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J Clin Invest. 1984;74(6):2249-2253. <https://doi.org/10.1172/JCI111651>.

Research Article

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Mechanism of Enhanced Fibroblast Arachidonic Acid Metabolism by Mononuclear Cell Factor

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Abstract. Chronic inflammation is associated with an infiltration of mononuclear cells, fibroblast proliferation, and elevated levels of prostaglandin (PG) E₂. Mononuclear cell conditioned factor (MNCF) medium (5%) stimulated a 100-fold increase in basal human dermal fibroblast PGE₂ release over 48 h as compared with fibroblasts that were incubated with control medium (conditioned medium prepared without cells). The MNCF-induced PGE₂ production was suppressed by protein synthesis inhibitors. Fibroblasts pretreated with control medium released PGE₂ only modestly in response to 1 nM bradykinin for 1 h (basal, 50±7 pg PGE₂/μg protein; stimulated, 104±12 pg PGE₂/μg protein), whereas cells that had been pretreated with MNCF showed a greatly facilitated bradykinin-induced release of PGE₂. (basal, 297±59 pg PGE₂/μg protein; stimulated, 866±85 pg PGE₂/μg protein). The exaggerated agonist response is not specific for bradykinin because platelet-derived growth factor elicits a similar response. Exogenous arachidonic acid conversion to PGE₂ was also facilitated (two- to threefold) by MNCF pretreatment as compared with control. Both the enhanced agonist-stimulated and exogenous arachidonic acid-induced PGE₂ release from the MNCF pretreated cells were inhibited by actinomycin D or cycloheximide. A kinetic study of microsomal cyclooxygenase prepared from fibroblasts pretreated with MNCF showed a threefold increase in the maximum velocity (V_{max}) but the same Michaelis constant (K_m) as control-treated cells. This augmented arachidonic acid metabolism and subsequent enhanced PGE₂ production may play an important role

in macrophage-fibroblast interactions at sites of inflammation.

Introduction

Chronic tissue inflammation is generally associated with an infiltration of mononuclear cells and a fibrogenesis. Monocytes, when stimulated, release a whole spectrum of products, including arachidonic acid (AA)¹ metabolites (1), complement components (2), and monokines (3), which elicit a large number of responses on local vasculature, immune processes, and various target cell functions. Endogenous mediators of inflammation, such as bradykinin and prostaglandins (PG), are derived from the injured tissue. Bradykinin is formed by cleavage of kininogen by kallikrein (4), and during the inflammatory reaction acts as a vasodilator (5) and increases vascular permeability (6), thereby contributing to the edema. Bradykinin also elicits PG release from various intact organ systems (7) as well as different cell types such as fibroblasts (8) and endothelial cells (9). Chronic inflammation has been associated with elevated levels of PGE₂ at the site of injury. PGE₂ is a vasodilator (10), enhances vascular permeability (11), and plays a role in the regulation of the immune response by modulating T lymphocyte proliferation, macrophage cytotoxicity, and lymphokine production (12).

Macrophages seem to actively modulate fibroblast proliferation and PG production (13). Human peripheral blood mononuclear cells in vitro release a variety of factors over a 72-h period into the culture medium. The supernatant medium from these cultures contains a factor that results in a marked (50–200-fold) stimulation of PGE₂ biosynthesis from dermal fibroblasts (13), gingival fibroblasts (14), synovial cells (15), and lung fibroblasts (16). In some models, such as pulmonary fibrosis, the monocyte-conditioned medium suppresses fibroblast proliferation by stimulation of PGE₂ production (16). The rabbit hydronephrotic kidney (HNK), a model of renal injury, exhibits histological evidence of a macrophage invasion

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Received for publication 25 May 1984 and in revised form 20 August 1984.

J. Clin. Invest.

© The American Society for Clinical Investigation, Inc.
0021-9738/84/12/2249/05 \$1.00

Volume 74, December 1984, 2249–2253

1. Abbreviations used in this paper: αMO, minimal essential medium alpha; AA, arachidonic acid; CLK, contralateral kidney; HNK, hydronephrotic kidney; MNCF, mononuclear cell factor; PDGF, platelet-derived growth factor; PG, prostaglandin.

and fibroblast proliferation, and is associated with a greatly exaggerated renal AA metabolism in response to stimulation with bradykinin (17). An *in vitro* cell culture derived from HNK cortical explants contained macrophages and fibroblasts and elicited a profound PGE₂ synthesis in response to bradykinin and exogenous AA (18). Since mononuclear-cell conditioned medium stimulates basal fibroblast PGE₂ production, we reasoned that the conditioned medium might also alter agonist-stimulated fibroblast PGE₂ production. This paper addresses the mechanism by which mononuclear cell factor alters both basal- and agonist-stimulated fibroblast AA metabolism.

Methods

Cell cultures. Human dermal fibroblasts were obtained from the American Type Cell Culture Collection, Rockville, MD (CRL 1445). Cells were maintained in 150 cm² tissue culture flasks (Corning Glassworks, Corning, NY) in minimal essential medium alpha (Basic Cancer Center, Washington University, St. Louis, MO), 15 mM Hepes buffer, L-glutamine (2 mM), penicillin (100 U/ml), and streptomycin (100 µg/ml) (termed α MO) supplemented with 10% fetal calf serum (KC Biological Inc., Lenexa, KS). Cells were fed twice weekly, passaged at confluence, and used for experiments between the 5th and 20th subpassage in culture.

Supernatant preparation. Human peripheral blood mononuclear cells from heparinized venous blood of normal volunteers were prepared by the method of Böyum (19). Basically, the mononuclear cells were isolated on a Ficoll-Hypaque density centrifugation gradient (Histopaque; Sigma Chemical Co., St. Louis, MO). The mononuclear cell-enriched interface fraction was then washed three times with α MO. For production of supernatant, mononuclear cells (5×10^5 /ml) were cultured for 72 h at 37°C in a 5% CO₂/95% air humidified atmosphere in α MO supplemented with 10–20% fetal calf serum. To terminate cultures, tubes of the conditioned medium were centrifuged for 10 min at 1,000 rpm. The supernatant was retained and passed through a 2-µm filter (Millipore Corp., Bedford, MA). Cell-free supernatant, which is termed mononuclear cell factor (MNCF), was stored at –20°C until used. Control medium was prepared in the same way as MNCF but without any mononuclear cells.

Assay for MNCF-induced PGE₂ production. Fibroblast cultures were harvested by brief exposure to trypsin (0.25%), which was washed off the cells by centrifugation. The cells were resuspended at 10⁵ cells/ml in α MO. Equal 100-µl volumes of the fibroblast suspension and of diluted conditioned medium (10%) were added to quadruplicate wells of 96-well flat-bottomed tissue culture plates (Costar, Cambridge, MA) and cultured for 48 h at 37°C in a 5% CO₂/95% air humidified atmosphere. At the termination of the culture, the medium was removed and stored at –20°C until assayed for PGE₂ by radioimmunoassay (RIA).

Assay for agonist-induced PGE₂ production. Fibroblasts were pretreated with conditioned medium for 48 h. Then the medium was removed, cells were washed three times with α MO, and the appropriate agonist in α MO was added back to the cultures for an additional hour. To terminate the incubation, the medium was removed and stored at –20°C until assayed for PGE₂ by RIA. Data shown are of representative experiments and are expressed as mean \pm SEM of quadruplicate wells. Experiments were repeated at least three times.

Protein determination. Cells from all experiments were washed three times with phosphate-buffered saline (50 mM potassium phosphate). 200 µl of 0.62 N NaOH was added to each well to solubilize the protein. The protein concentration of the cells was determined fluorometrically with fluorescamine using bovine serum albumin as a standard. Proteins generally ranged from 2 to 4 µg protein/well.

Preparation of microsomes. Cells (5×10^6 cells/150 cm² T-flask) were pretreated with either control or MNCF medium for 48 h. The cells were then washed, scraped from the flask, and centrifuged in α MO for 10 min at 1,000 rpm. The cells were resuspended in 1 ml of 50 mM Tris buffer (pH 7.8) containing 1% bovine serum albumin and 10 mM EGTA. The suspension was sonicated for 30 s in a Cell Disruptor 350 (Sonifier) at pulsed 40% duty cycle, three output control. Microscopic examination revealed that the cells were completely broken apart. The disrupted cells were centrifuged at 8,000 g for 15 min, and the resulting supernatant was centrifuged at 100,000 g for 60 min. The pellets obtained were washed with 50 mM Tris buffer (pH 7.8) and 1 mM EGTA. The pellet was then resuspended in a glass homogenizer with 500 µl of the 50 mM Tris buffer (pH 7.8), 1 mM EGTA.

Kinetic analysis of microsomes. The time course for microsomal conversion of AA to PGE₂ (as detected by RIA) indicated the conversion to be linear for 30 min. The microsomes were incubated with various concentrations of AA (0–30 µM) for 10 min at 37°C in the presence of 1.2 mM epinephrine and 1.0 mM reduced glutathione. The reaction was terminated on ice and then stored at –20°C until assayed for PGE₂. For control purposes, some microsomes were preincubated with indomethacin (5 µg/ml) for 20 min on ice.

RIA of PGE₂. PGE₂ was analyzed with rabbit antiserum as previously described (20). The cross-reactivity at 50% displacement of other PGs with the antiserum is as follows: 6-keto-PGF₁ α , 0.39%; PGF₂ α , 0.04%; TxB₂, 0.009%.

Results

Effect of MNCF on basal fibroblast PGE₂ accumulation. When fibroblasts (10⁴ cells/well) were incubated with 5% MNCF (determined to be an optimal dose; data not shown) or 5% control medium for 48 h (Table I), there was approximately a 100-fold stimulation of PGE₂ from MNCF-treated cells. The MNCF-induced PGE₂ production was completely abolished by ibuprofen, a cyclooxygenase inhibitor. To determine if the exaggerated MNCF-induced fibroblast PGE₂ release was dependent upon protein synthesis, the fibroblasts were incubated simultaneously with the conditioned medium and protein synthesis inhibitors (Table I). Both actinomycin D and cycloheximide at least partially (50–70%) abolished the MNCF-induced fibroblast PGE₂ release. The effect of the protein synthesis inhibitors was not due to a loss of cell viability, since there was no change or perhaps a slight enhancement of PGE₂ in the control treated cells, and the adherent fibroblasts excluded trypan blue. Higher doses of actinomycin D and cycloheximide nonspecifically inhibited cyclooxygenase, as determined by exogenous AA conversion, and in some instances were toxic to the cells. Therefore, even though higher doses of the inhibitors provided a more complete inhibition of the response,

Table I. Effect of MNCF on Basal Fibroblast PGE₂ Production

	5% Control	5% MNCF
	pg PGE ₂ /μg protein	
No treatment	14±2 (9)	1,694±93 (14)
Ibuprofen (10 μM)	9±1 (6)	69±6 (5)
Actinomycin (40 nM)	74±5 (3)	519±18 (3)
Cycloheximide (36 nM)	16±2 (3)	798±150 (3)

Fibroblasts (10⁴/well) were treated for 48 h and assayed for PGE₂ as described in Methods. Data are expressed as the mean±SEM of the number of determinations from separate experiments indicated in parentheses.

we chose the lowest dose of inhibitor, in which there were no deleterious effects to the cells.

Effect of MNCF on agonist-induced fibroblast PGE₂ release. Since MNCF had such a profound effect on basal fibroblast PGE₂ release, we wished to determine if MNCF could also enhance agonist-induced fibroblast PGE₂ release. Cells were pretreated with either 5% control medium or 5% MNCF for 48 h, washed three times as described in Methods, and then incubated with the appropriate agonist for 1 h. In a 1-h incubation without any agonist, conditioned medium, or serum, there is more fibroblast PGE₂ production from MNCF-pretreated fibroblasts than from control-treated fibroblasts (Fig. 1, A and B). A bradykinin dose-response curve is shown in Fig. 1 A. Cells that had been pretreated with control medium showed only a modest response to bradykinin, whereas MNCF-pretreated cells showed a greatly enhanced response to bradykinin with a dose as low as 1 nM bradykinin. To determine if this facilitated response was specific for bradykinin or if in fact

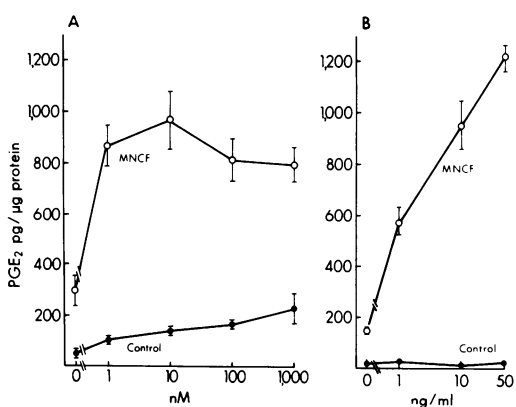


Figure 1. Effect of MNCF on hormonally induced fibroblast PGE₂ release. Fibroblasts pretreated with 5% control medium (●) or 5% MNCF (○) were incubated with varying doses of bradykinin (A) or PDGF (B), and PGE₂ release was assayed by RIA. All MNCF-treated cell values are statistically different ($P \leq 0.01$) from control treated cells.

other agonists were also affected, we incubated the cells with platelet-derived growth factor (PDGF). PDGF has been shown for some fibroblasts (21) to elicit a PGE₂ response, although our control pretreated cells did not respond. However, MNCF-pretreated cells showed a rather substantial release of PGE₂ in response to PDGF that was dose dependent (Fig. 1 B).

Since this altered peptide-induced fibroblast PGE₂ release was quite dramatic, we explored the mechanism by which this might occur. Exogenous AA at varying doses was incubated with cells (Fig. 2) that had been pretreated with conditioned medium in the same manner as with bradykinin and PDGF. Fibroblasts pretreated with control medium were able to convert AA to PGE₂ quite readily. However, for a fixed dose of AA, MNCF-pretreated cultures converted AA to significantly more PGE₂, indicative of more cyclooxygenase activity.

Effect of MNCF on fibroblast microsomal cyclooxygenase activity. In whole cells, exogenous AA is readily incorporated to the phospholipid membrane, and therefore, assessment of cyclooxygenase activity is difficult to quantitate. To specifically address whether MNCF was altering fibroblast cyclooxygenase activity, we prepared fibroblast microsomes after a 48-h conditioned medium pretreatment. Fibroblast cyclooxygenase activity was quantitatively determined by RIA of the PGE₂ produced from the conversion of AA by microsomes. The reaction rates of microsomes prepared with AA as substrate were linear for 30 min of incubation at 37°C. Thus, the initial rate comparisons were studied at 10 min. Although the Michaelis constants (K_m 's) for the fibroblasts' cyclooxygenase under both conditions were similar (Table II), the maximum velocity (V_{max}) for MNCF-pretreated fibroblasts' cyclooxygenase was about threefold greater than the V_{max} for control treated cells.

Effect of protein synthesis inhibitors on agonist-induced PGE₂ release. To determine if the facilitated agonist-induced PGE₂ release was protein synthesis dependent, we performed the following experiment. Fibroblasts were grown in the presence of 5% control medium or 5% MNCF with and without protein synthesis inhibitors (Table I). The medium was then

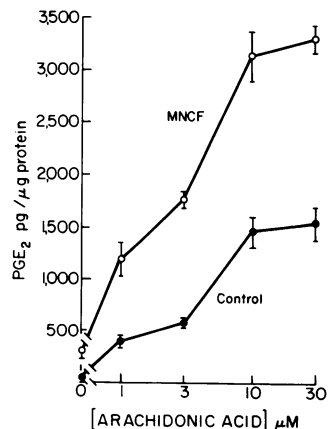


Figure 2. Effect of MNCF on exogenous AA conversion by fibroblasts. Fibroblasts pretreated with 5% control medium (●) or 5% MNCF (○) were incubated with varying doses of AA, and PGE₂ release was measured by RIA. All MNCF-treated cell values are statistically different ($P \leq 0.01$) from control treated cells.

Table II. Effect of MNCF on Microsomal Cyclooxygenase Activity

	V_{max}	K_m	V_{max}
	fmol PGE ₂ /μg/min	μM	MNCF/control
Experiment 1			
Control	7.5	1.3	2.6
MNCF	19.3	1	
Experiment 2			
Control	4.0	.9	3.1
MNCF	12.5	1.6	

Effect of MNCF on fibroblast cyclooxygenase activity. Microsomes were prepared from fibroblasts (5×10^6 cells/flask) cultured for 48 h with 5% control medium or 5% MNCF as described in Methods. Varying concentrations of AA were incubated with 40–50 μg of microsomal protein for 10 min at 37°C. In some incubations, microsomes were preincubated with indomethacin before incubation with AA, which completely abolished the response.

removed and various agonists were added for 1 h (Fig. 3). Actinomycin D completely abolished the enhanced agonist-induced PGE₂ release from MNCF-pretreated fibroblasts. A

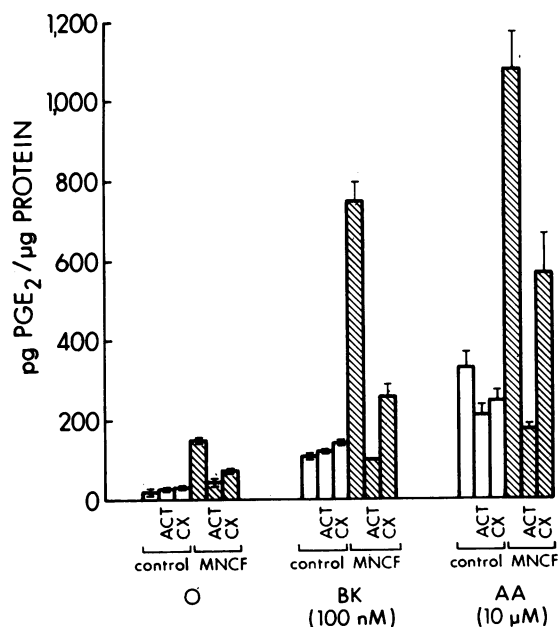


Figure 3. Effect of protein synthesis inhibitors on agonist-induced PGE₂ release. Fibroblasts pretreated with 5% control medium (□) or 5% MNCF (▨) with and without actinomycin D (ACT; 40 nM) or cycloheximide (CX; 36 nM) were incubated with bradykinin (100 nM) or AA (10 μM), and PGE₂ production was assayed by RIA. All MNCF-treated values are statistically different ($P \leq 0.01$) from both control treated cultures and MNCF plus protein synthesis inhibitor treated cultures. There is no statistical difference between control or control plus protein synthesis inhibitor cultures.

similar result was seen with cycloheximide, although the inhibition was not as complete. Clearly the inhibitors were not acting as nonspecific cyclooxygenase inhibitors, since the control-treated cells had unchanged responses. The inhibition of the facilitated bradykinin and AA-induced fibroblast PGE₂ response indicates that these responses were in fact due to new protein synthesis.

Since PGE₂ has been shown in some systems to modulate DNA synthesis as determined by thymidine incorporation (22), we wished to determine if the elevated levels of PGE₂ induced by MNCF were modulating the enhanced bradykinin and AA responses. The MNCF-induced PGE₂ release was inhibited with ibuprofen (Table I), a readily reversible cyclooxygenase inhibitor. After 48 h, the ibuprofen was washed off the cells, which were then stimulated with various agonists. There was no difference in response to bradykinin or AA for fibroblasts pretreated with MNCF with and without ibuprofen (data not shown).

Discussion

Our experiments were designed to address the mechanism by which monocyte factors elicit a large basal increase in fibroblast PGE₂ release. One of the most striking findings in this study was that MNCF could alter fibroblast response to agonist-stimulated PGE₂ synthesis. Bradykinin, an autocoid generated at sites of inflammation, induced considerably more PGE₂ production from intact cultured fibroblasts pretreated with MNCF than from control-treated fibroblasts. This increase in bradykinin-induced AA metabolism was due to new protein synthesis, and was not affected by the elevated levels of PGE₂ during the 48-h pretreatment. MNCF-treated fibroblasts exhibited a protein synthesis-dependent increase, which at least in part accounts for the total 48-h PGE₂ accumulation. It is still unclear whether the entire enhanced basal production of PGE₂ was due to *de novo* protein synthesis in cyclooxygenase activity. Although this paper only addresses the mechanism of an augmented cyclooxygenase activity, it is quite likely that MNCF may also enhance PGE₂ production by other mechanisms such as (a) an increase in phospholipase activity, (b) a decrease in AA reacylation, or (c) a decrease in PG metabolic activity. The cyclooxygenase activity in MNCF-treated cells is about three times the activity in control treated cells, whereas the magnitude of the bradykinin response in MNCF-treated cells is 10 times that of the control-treated cells. Thus, whether the facilitated bradykinin response is due to just an increase in cyclooxygenase activity, or if in fact the phospholipase activity and/or bradykinin receptor are also altered by MNCF, remains to be established. The MNCF-induced facilitation of the agonist-stimulated PGE₂ release from fibroblasts is not specific for bradykinin, since the PDGF response is also enhanced. Platelets are also present at inflammatory or injury sites (23), and could readily interact with mononuclear cells and fibroblasts.

These experiments may help clarify possible interactions of monocytes and fibroblasts at sites of the tissue inflammation.

The basal increase in PGE₂ in fibroblast-like cells in gingivitis (16) or rheumatoid arthritis (15) may be due to the induction of more cyclooxygenase activity by monocytes. The HNK is another model of tissue inflammation characterized by the presence of fibroblasts and macrophages, and has an exaggerated arachidonate metabolic capacity (17). Microsomal studies of cyclooxygenase activity in the cortex of an HNK demonstrated that the V_{max} increased approximately fourfold with no change in the K_m as compared with the unobstructed contralateral kidney (CLK) (24). Thus, the exaggerated cyclooxygenase activity observed in the cortical microsomes of the HNK may in part reflect the mononuclear cell factor induction of fibroblast cyclooxygenase. Similarly, the facilitated bradykinin-induced PGE₂ in the isolated ex-vivo perfused HNK may reflect monocytes altering the bradykinin response. The heterogeneous HNK cell culture, derived from cortical explants, consisting of macrophages and fibroblasts, had an enhanced release of PGE₂ in response to bradykinin or exogenous AA compared with CLK cultures, which only contained fibroblasts (18). Passaged HNK cultures, which only contained fibroblasts, produced much less PGE₂ in response to bradykinin and AA, whereas CLK fibroblast responses remained unchanged with passage. Therefore, the mechanism invoked here is that macrophage in the primary HNK co-culture system can modulate the fibroblast cyclooxygenase and bradykinin response, consistent with our current data.

PGE₂ is thought to have many functions at a site of inflammation. This study demonstrates the possibility of an amplification scheme to greatly enhance local concentrations of PGE₂. Thus, not only mononuclear cells can chronically stimulate fibroblast PGE₂, but agonists such as bradykinin, present at the inflammation, can also have a profound impact on fibroblast PGE₂ release.

Acknowledgments

This manuscript was supported by National Institutes of Health training grants GM-07805, HL-14397, and HL-20787.

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