Vol. 188, No. 1, 1992 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
October 15, 1992 Pages 310-317

TUMOR NECROSIS FACTOR AND INTERLEUKIN-1 INDUCE ACTIVIN A GENE EXPRESSION IN A HUMAN BONE MARROW STROMAL CELL LINE

Shunji Takahashi, Kaoru Uchimaru, Ken-ichi Harigaya *, Shigetaka Asano and Takayuki Yamashita **

Fourth Department of Internal Medicine, Faculty of Medicine, University of Tokyo, Bunkyo-ku, Tokyo 112, Japan

* First Department of Pathology, Chiba University School of Medicine, Chuou-ku, Chiba 260, Japan

Received September 1, 1992

SUMMARY: Activin A, a homodimer of the βA chain, regulates hematopoiesis. In a human bone marrow-derived stromal cell line, KM-102, phorbol myristate acetate, tumor necrosis factor-α and interleukin -1β induced great increases in βA chain mRNA levels and production of activin A activities. The phorbol ester-induced βA chain gene expression was inhibited by cycloheximide and down regulation of protein kinase C, whereas the cytokine-induced expression was little affected by these treatments. These results indicate that the inflammatory cytokines directly stimulate βA chain gene expression via protein kinase C-independent pathways.

Activins are dimeric proteins highly homologous to transforming growth factor β. There are three activins (A, AB and B), comprising different combinations of two closely related β chains (βA βA, βA βB and βB βB, respectively). The β chains can form other dimer proteins, inhibins with α chains (For review, see Ref 1, 2). Since accumulating evidence indicates that activins regulate growth and

** To whom correspondence should be addressed at Fourth Department of Internal Medicine, University of Tokyo, Faculty of Medicine, 3-28-6 Mejirodai, Bunkyo-ku, Tokyo 112, Japan.

Abbreviations: TNF, tumor necrosis factor; IL, interleukin; PMA, phorbol myristate acetate; PDBu, phorbol 12,13-dibutyrate; rh, recombinant human; CM, conditioned medium.

0006-291X/92 $4.00

differentiation in various cell systems (1, 2), the expression of these proteins is expected to be strictly regulated in many tissues. However, a little is known about the regulation, partly because there are a few experimental systems suitable for studying this issue.

Several lines of evidence strongly suggests that activin A regulates hematopoiesis. It induces hemoglobin synthesis in murine and human leukemic cell lines (3, 4). Recently, we (5) and others (6) reported that activin A also induces megakaryocytic differentiation in murine leukemic cells. In normal hematopoietic cells, it stimulates the growth of normal erythroid and multipotential precursor cells via accesory cells (7-9) and directly enhances the differentiation of late-stage erythroid precursors (10). Two groups reported that in vivo administration to rodents stimulates erythropoiesis (11, 12). The physiological role of this factor is supported by detection of its mRNA in bone marrow. (13).

To clarify the regulation of activin A production in hematopoietic tissue, we explored the expression of this factor in bone marrow stromal cells, which are known to play a central role in the regulation of hematopoiesis (for review, see Ref. 14). Recently, we reported that murine bone marrow stromal cells produce activin A (15). In the present study, we found that tumor necrosis factor (TNF)-α and interleukin (IL)-1 β as well as phorbol myristate acetate (PMA) induced rapid expression of βA chain mRNA and activin A production in a human bone marrow stromal cell line KM-102 and that the signalling pathways probably differ between PMA and the cytokines.

METHODS

Materials PMA and phorbol 12,13-dibutyrate (PDBu) were obtained from Sigma. Follistatin, purified from porcine ovary (16), was a kind gift from Dr. Y. Eto (Ajinomoto Co., Kawasaki, Japan) with permission of Dr. H. Sugino (Institute of Physical and Chemical Research, Saitama, Japan). Recombinant human (rh) activin A, IL-1 β, and TNF-α were kindly provided by Ajinomoto Co., Ohtsuka Pharmaceutical Co.(Tokushima, Japan), and Suntory Co.(Tokyo, Japan), respectively.

Cell culture KM-102 cells were established by transfection of SV-40 containing plasmid to primary culture of human bone marrow stromal cells (17). The cells were cultured in MEM Iscove (Boehringer Mannheim, Germany) supplemented with 5% fetal calf serum in a humidified atmosphere of 5% CO2 and passaged every four days at a split ratio of 1:4, using 0.25% trypsin containing 0.02% EDTA. The murine erythroleukemic cells F5 (18) were cultured in Ham's F-12 supplemented with 10% fetal calf serum and passaged every 3-4 days at a cell density of about 5 X 10 cells/ml.

Assay of activin A activities in conditioned medium (CM) The cells were seeded at $5X10^4$ cells per well in 1 ml of medium in 24 well plates, and cultured for 4-5 days to confluent states, then media were replaced by 0.5 ml of the fresh media containing various agents. CM was collected at 72 h after stimulation and used for determination of activin A activity, as described elsewhere (15). In brief, activin A-responsive F5 cells were cultured with 25 % of CM at $2X10^3$ cells per well in 100 μl of medium in 96 well plates and the percentages of hemoglobin-positive cells were scored after a 5-day culture.

Northern blot analysis Human βA chain cDNA (*PstI*/ *Hin*cII) (19) was kindly supplied from Dr. Y. Eto (Ajinomoto Co., Japan). cDNAs of human βB and α chains (20, 21) were kindly provided by Dr. K. Miyamoto (National Cardiovascular Center, Osaka, Japan). Total RNA was extracted from the cells by guanidium thiocyanate-cesium chloride method (22). Ten μg of total RNA was separated on 1 % formamide/ agarose gel by electrophoresis and transferred to nylon membrane filters (Pall Ultrafine Filtration Corp., Glen Cove, N.Y.). The filters were prehybridized for 12h at 42℃ in 5 X SSC (SSC:0.15M NaCl/ 15 mM sodium citrate, pH 7.0), 50 % formamide, 50 mM phosphate buffer at pH 8.0, 250 μg/ml denatured salmon sperm DNA and 5 X Denhardt's solution. Hybridization with ^{32}P-labeled cDNA probes was performed for 16h at 42 ℃ in 5 X SSC, 50 % formamide, 100 μg/ml denatured salmon sperm DNA, 5 X Denhardt's solution and 10% dextran sulfate. The filters were washed in 2 X SSC and 0.1% sodium dodecyl sulfate three times for 5 min at room temperature and then 0.1 X SSC and 0.1% sodium dodecyl sulfate three times for 30 min at 60℃ Autoradiography was performed with intensifying screen at -80 ℃ for 24 hours or longer.

RESULTS AND DISCUSSION

Fig. 1 shows CM taken from KM-102 cells treated with PMA, TNF-α, or IL-1 β induced erythroid differentiation of F5 cells, an activin-sensitive murine erythroleukemia cells (3). whereas CM of control cells had little such activity. Follistatin, an activin-binding protein (16), almost completely inhibited the erythroid differentiation induced by rh activin A and CM of the stromal cells but not that induced by hexamethylenebisacetamide, a chemical inducer

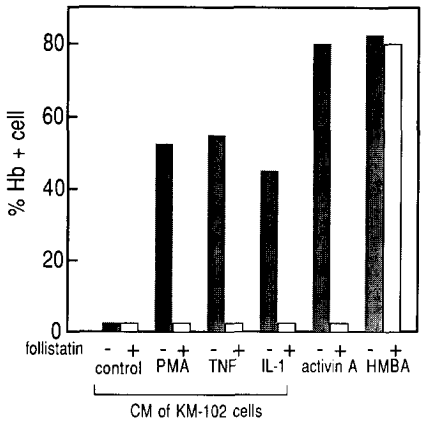

Fig.1. Production of activin A in stromal cells. CM was taken from control or PMA- (100 nM), TNF-α (10 ng/ml)-, or IL-1 β(100 U/ml)-treated KM-102 cells. F5 cells were cultured with 25 % of CM, rh activin A (1 nM), or hexamethylenebisacetamide (HMBA) (5 mM) in the absence (-) or presence (+) of follistatin (100 ng/ml) and hemoglobin-positive (Hb+) cells were scored after 5 days. Other details are described in " METHODS". Values are the percentages of Hb+ cells. The data are means of duplicate experiments.

(23). These results indicate that the stimulated stromal cells secrete activin into CM.

Fig. 2 shows that PMA, TNF-α and IL-1β induced rapid increases in βA chain mRNA levels in KM-102 cells. Neither βB nor α chain mRNA was detected in these samples (data not shown), thereby indicating that neither activin AB nor inhibin A was expressed. These cells expressed at least four βA chain mRNA species of about 6.0,

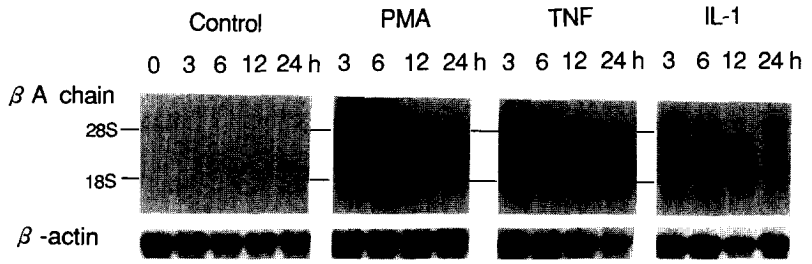

Fig.2. Expression of βA chain gene expression in stromal cells. Confluent cultures of KM-102 cells were treated for with PMA (100 nM), TNF-α (10 ng/ml), or IL-1β (100 U/ml) for indicated time. Ten μg of total RNA was isolated, electrophoresed and blotted onto nylon membrane filters, then the filters were hybridized with 32 P-labeled cDNA probes for βA chain and β-actin. Other details are described in "METHODS". The data are representative of three independent experiments.

4.0, 2.8 and 1.7 kb. Tanimoto et al. suggested that cell type-specific alternative polyadenylation generates five species of βA chain mRNA in a human fibrosarcoma cell line (24). Probably, a similar mechanism explains the multiplicity of βA chain mRNA species in KM-102 cells. PMA, TNF-α, and IL-1β induced the expression of the mRNA species in an essentially similar pattern, whereas relative amounts of each species considerably varied in repeated experiments. The larger two species (6.0 and 4.0 kb) seemed to be labile and that of 2.8 kb was most stable.

We performed two sets of experiments to characterize the actions of TNF-α and IL-1β in comparison with that of PMA. First, we examined the effects of actinomycin D and cycloheximide. Actinomycin D almost completely inhibited the βA chain mRNA increases by any of these agents (data not shown). Cycloheximide had little effects on the cytokine-induced βA chain mRNA expression, whereas it markedly inhibited the response to PMA (Fig. 3A). Second, to examine whether protein kinase C is involved in the actions of the cytokines, we tested the effects of prolonged treatment with PDBu, which is known to down regulate protein kinase C in many cell types (25). This treatment abolished the PMA-induced βA chain mRNA increase, thereby confirming that protein kinase C mediates the action of PMA (26), but not the responses to TNF-α and IL-1β (Fig. 3B). Therefore, we tentatively conclude that these inflammatory cytokines directly stimulate βA chain gene expression independently of protein kinase C, whereas the action of PMA requires de novo protein synthesis.

In the present study, we demonstrate that PMA, TNF-α, and IL-β induced βA chain mRNA expression and activin A production in KM-102 cells. We obtained essentially similar results in KM-101, 104, and 105, other clones derived from human bone marrow stromal cell cultures (17) (data not shown). The mode of activin A expression in these cells differs in some ways from that seen in the murine cells (15). First,

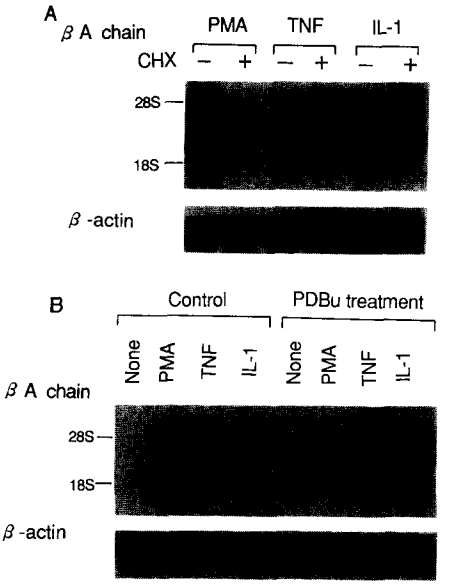

Fig.3. Effects of the presence of cycloheximide and prolonged PDBu pretreatment on the βA chain mRNA expression. Confluent cultures of KM-102 were treated for 3h with PMA (100 nM), TNF-α (10 ng/ml), or IL-1β (100 U/ml) in the absence or presence of cycloheximide (CHX) (10 μM) (A). The cells were cultured for 24h in the absence or presence of PDBu (100 nM), washed three times with medium, and treated for 3h with PMA (100 nM), TNF-α (10 ng/ml), or IL-1 β (100 U/ml) (B). The levels of βA chain mRNA were measured as described in the legend to Fig. 2. The data are representative of three independent experiments.

in the murine cells, activin A production induced by TNF- α by itself or synergistically with IL- β was very slow in kinetics (15) and not accompanied with elevation of βA chain mRNA levels (unpublished observation). Second, PMA induced a rapid increase in βA chain mRNA levels also in the murine cells but this increase was not inhibited by cycloheximide (unpublished observation). These differences might be due to the species difference or that KM-102 cells are transformed by SV-40 (17).

The present findings, together with the observation that a human fibrosarcoma cell line HT1080 produces activin A (24), raise the possibility that mesenchymal cells are sources of this protein. Tanimoto et al. reported that 8-bromo-cAMP elevated βA chain mRNA levels possibly by post-transcriptional mechanism, since they could not identify cAMP-responsive elements in the 5'-flanking region of the

human βA chain gene (24). Our results suggest that TNF-α and IL-1 β directly stimulate transcription of this gene in KM-102 cells. Since a putative AP-1 binding site is identified in the 3'-flanking region of the human βA chain gene (27) and TNF-α was reported to activate AP-1 (28), it is possible that TNF- α stimulates βA chain gene transcription by this pathway. Thus, KM-102 cells provide a good model system for studying regulation of βA chain gene expression in human mesenchymal cells.

ACKNOWLEDGMENTS

This investigation was supported in part by research grants from the Ministry of Education, Science and Culture of Japan (1991, 1992). We are grateful to Dr. Y. Eto for support.

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