Activin A/Erythroid Differentiation Factor Induces Thromboxane A2 Synthetic Activity in Murine Erythroleukemia Cells*

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Activin A, a protein homologous to transforming growth factor β, was shown to induce hemoglobin synthesis in murine erythroleukemia (MEL) cells and was also termed erythroid differentiation factor (EDF) (Eto, Y., Tsuji, T., Takezawa, M., Takano, S., Yokogawa, Y., and Shibai, H. (1987) Biochem. Biophys. Res. Commun. 142, 1095–1103). We found that activin A/EDF also induced thromboxane (TX) A2 synthetic activity in these cells. Synthesis of TXA2 from arachidonic acid is catalyzed by cyclooxygenase and TX synthase. Activin A/EDF induced the latter TX synthase activity, whereas the cyclooxygenase activity was constitutively expressed. The induction of this enzyme activity was inhibited by cycloheximide, suggesting that activin A/EDF induced de novo protein synthesis of TX synthase. Furthermore, we studied the relationship between the induction of TXA2 synthetic activity and erythroid differentiation in MEL cells, since the former is not an erythroid phenotype. We found 1) that the two responses to activin A/EDF were distinctly affected by the initial cell density; 2) that the dose-response curves for activin A/EDF were similar (ED50 = ~100 pt), whereas the time course of induction of TXA2 synthetic activity was much faster; and 3) that other erythroid differentiation inducers of MEL cells, namely dimethyl sulfoxide and hexamethylene bis-acetamide, had little or no effect on TXA2 synthesis. These results indicate that activin A/EDF induces TXA2 synthetic activity independently of erythroid differentiation.

Thromboxanes (TX), prostaglandins (PG), leukotrienes, and lipoxins, collectively termed eicosanoids, are metabolites of arachidonic acid (AA) and regulate diverse cellular functions as autacoids (for review, see Refs. 1 and 2). Two major enzymatic pathways have been defined. The cyclooxygenase pathway produces PGs and TX (1), and lipoxygenases synthesize leukotrienes and lipoxins (for review, see Refs. 2 and 3). It has been widely accepted that each cell type possesses characteristic metabolic pathways and produces a specific pattern of eicosanoids, once AA is released from membrane phospholipids by various stimuli (1). Increasing attention is focused on the regulation of expression of the enzymes in these pathways; a number of growth or differentiation factors and cytokines, such as platelet-derived growth factor, epidermal growth factor, transforming growth factor β, interleukin-1, and interleukin-2, alter the production of eicosanoids by regulating the expression of the enzymes at various steps (4–9).

TX synthase is an enzyme synthesizing TXA2 from PGH2, which is produced from AA by cyclooxygenase (1). TXA2 is a labile but biologically active compound in smooth muscle cell constriction and platelet aggregation (for review, see Ref. 10). TX synthase was purified from human platelets and porcine lung and was found to be a cytochrome P-450 protein with a Mr of 53,000–58,800 (11, 12). Immunoblot analysis revealed that this protein is abundant in platelets and in the colon, duodenum, lung, kidney, and stomach (13). However, little is known of the regulation of expression of this enzyme, although cytochrome P-450 proteins are a well-known inducible protein family (for review, see Ref. 14). Induction of TX synthase activity has been observed in human promyelocytic HL-60 cells when they differentiate into monocyte/macrophage-like cells in the presence of a tumor-promoting phorbol ester, diacylglycerols, or 1,25-dihydroxyvitamin D3 (15, 16). These results are consistent with the observation that monocytes/macrophages produce TXA2 (17).

Activin A was first purified from porcine ovarian fluid as a protein that enhances the secretion of follicle-stimulating hormone from cultured pituitary cells (18, 19) (for review, see Ref. 20). This protein is a homodimer of the β A chain which shows about 40% homology to a subunit of transforming growth factor β-1 (18–20). Independently, Eto et al. (22) purified, from the culture supernatant of a human monocytic leukemia cell line THP-1 (21), a protein inducing erythroid differentiation of murine erythroleukemia (MEL) cells and designated this protein as erythroid differentiation factor (EDF). Subsequently, EDF has proven to be coded by the same gene as activin A (23). Several lines of recent evidence indicate that activin A/EDF functions as a physiological regulator of erythroid lineage cells. It induces hemoglobin synthesis in a human erythroleukemia cell line K562 (24) and enhances the growth of normal erythroid precursor cells, not only in culture systems (24–26) but also when given to rodents in vivo (27, 28). Furthermore, activin A/EDF was expressed in several myelomonocytic cell lines as well as in the normal bone marrow (29–31). In this paper, we report that activin A/EDF induces TX synthase activity in MEL cells and present evidence indicating that this phenotype is expressed inde-
pendently of erythroid differentiation and is possibly megakaryocytic.

**EXPERIMENTAL PROCEDURES**

*Materials*— Recombinant human activin A/EDF was a gift from Dr. T. Nakamura (Ajinomoto Co., Kawasaki, Japan). [1-14C]AA (52.8 mCi/mmol) was purchased from Du Pont-New England Nuclear. Authentic PGs, TXB2, 12-hydroxy-5,8,10-heptadecatrienoic acid (HHT), and OKY-046, a specific inhibitor of TX synthase (32), were provided from Ono Pharmaceutical Co. (Osaka, Japan). Hexamethylene bisacetamide (HMBA) and cycloheximide were purchased from Sigma. Dr. Y. Eto (Ajinomoto Co., Kawasaki, Japan). [l-"C]AA (52.8 mCi/mmol) was purchased from Du Pont-New England Nuclear. Authentic PGs, TXB2, 12-hydroxy-5,8,10-heptadecatrienoic acid (HHT), and OKY-046, a specific inhibitor of TX synthase (32), were provided from Ono Pharmaceutical Co. (Osaka, Japan). Hexamethylene bisacetamide (HMBA) and cycloheximide were purchased from Sigma.

*Cell Culture*— MEL cells (F5–5) used for experiments were originally established from the DDD mouse by Ikawa et al. (34) and subcloned by Eto et al. (22) as a clone highly sensitive to activin A/EDF in erythroid differentiation. Experiments were performed using cells at passages 10–30. The cells were cultured in a humidified atmosphere of 5% CO2 and 95% air in Ham’s F-12 medium supplemented with 10% fetal calf serum (GIBCO). The cells were maintained by dilution every 3 days at about 5 × 10^6 cells/ml in the fresh medium.

**Analyses of AA Metabolism**— Cells from the cultures were harvested and washed twice with Hanks’ solution buffered at pH 7.5 with the same solution. For assay of the overall TXA2 synthesis from AA, cells were incubated with 5 μM [1-14C]AA (0.2 μCi) and 2 μM A23187 for 4 min at 37 °C in a total volume of 0.5 ml. For assay of TX synthase activity, the cells were incubated with 10 μM [1-14C]PGH2 for 4 min at 24 °C in a total volume of 100 μl. The reactions were halted by adding 3 volumes of ethyl ether, methanol, 0.2 m citric acid (30:4:1, v/v/v) precooled at −20 °C. The cell suspension was immediately vortexed and centrifuged at 5,000 rpm for 10 min at 4 °C. The organic layer was evaporated to dryness under a stream of N2 and redissolved in 100 μl of ethyl acetate. The samples and authentic PGs (6-keto-PGF1α, PGF2α, PGF2β, and PGE2) and TXB2 were applied to silica gel thin layer chromatography (TLC) plates (Merck, F-254, 20 × 20 cm), which were developed using the following solvent systems: (i) A-1 system (benzene/dioxane/acetic acid, 30:20:1, v/v/v) or (ii) A-9 system (the organic phase of ethyl acetate/isooctane/acetic acid/water, 110:50:20:100, v/v/v/v). Because TXA2 is extremely unstable and nonenzymatically hydrolyzed to TXB2 (1, 30), TXA2 synthesis was monitored by the formation of TXB2. The radioactive bands were visualized by autoradiography. Thereafter, the locations of authentic PGs and TXB2 standards were visualized with iodine vapor, and the radioactive zones were defined. The silica gel was scraped from the glass plates, and the radioactive activity was determined in a liquid scintillation counter (Beckman LS 3801). TXA2 synthetic activity was determined by the conversion rate of [1-14C]AA into TXB2.

**Assay of Erythroid Differentiation**— After treating the cells with activin A/EDF for 4 days, the percentages of hemoglobin-positive cells were scored by benzidine staining according to Orkin et al. (35). At least 500 cells were counted in each assay.

**Acetylcholinesterase and α-Naphthyl Butyrate Esterase Staining**— After the cells were treated with activin A/EDF for 4 days, about 100,000 cells were smeared on a slide glass and stained according to Karnovsky and Roots (36) for acetylcholinesterase staining and to Li et al. (37) for α-naphthyl butyrate esterase staining.

**RESULTS**

To clarify the effect of activin A/EDF on the exogenous AA metabolism of MEL cells, we incubated untreated MEL cells and activin A/EDF-treated MEL cells with 1-14C-labeled AA and analyzed the products with TLC. Fig. 1 shows the autoradiogram of the TLC plates developed using two different solvent systems, A-9 and A-1. In both systems, the results were essentially the same. The control cells converted exogenous AA into materials that comigrated with authentic PGF2α, PGE2, PGD2, and HHT, whereas activin A/EDF-treated cells also converted AA into a material that comigrated with TXB2 (a stable metabolite of TXA2). For further identification, we examined the effect of indomethacin, a cyclooxygenase inhibitor, and OKY-046, a specific TX synthase inhibitor (32). Indomethacin completely blocked the conversion of AA into products that comigrated with PGs, TXB2, and HHT (data not shown), and OKY-046 blocked specifically the conversion of AA into a product that comigrated with TXB2 in the activin A/EDF-treated cells. These results demonstrate that activin A/EDF induced TXA2 synthetic activity in MEL cells.

Next, we questioned whether the effect of activin A on TXA2 synthetic activity was due to an increase in cyclooxygenase activity (AA → PGH2) or in TX synthase activity (PGH2 → TXA2). Since the synthetic activities of other PGs in the activin A/EDF-treated cells were similar to or less than those in the control cells (Fig. 1), an increase in TX synthase activity was presumably responsible. To verify this presumption, we analyzed the metabolism of exogenous PGH2 in the control and activin A/EDF-treated cells. Activin A/EDF-treated cells converted PGH2 into a material that comigrated with authentic TXB2, while the control cells were deficient in this activity (Fig. 2). This reaction did not occur in the presence of 1 μM OKY-046, a specific inhibitor of TX synthase (32). All these results indicate that TX synthase is responsible for the increase in TXA2 formation.

We examined the effect of cycloheximide on the activin A/EDF-induced TXA2 synthetic activity to see whether new protein synthesis is needed for the action of activin A/EDF. In case of the simultaneous presence of 1 μM cycloheximide, the induction of TXA2 synthetic activity was completely blocked, whereas the synthetic activities of other PGs were affected to a much lesser extent (Fig. 3).

As we described in the previous paper (38), activin A/EDF-induced erythroid differentiation was markedly affected by the cell density in the initial inoculum. At the initial density of 1 × 10^6 cells/ml, the percentage of hemoglobin-positive cells was 80–90%, whereas it was reduced to 10–20% when the cells were seeded at 1 × 10^5 cells/ml (Table I). The cell density had an inverse effect on the induction of TXA2.
Actinin A-induced Thromboxane A₂ Synthesis in MEL Cells

**Fig. 2.** TLC autoradiogram of products after incubation of activin A/EDF-treated MEL cells with [1-¹⁴C]PGH₂. MEL cells were seeded at 1 × 10⁵ cells/ml in medium with or without 1 nM activin A/EDF and then cultured for 48 h. Metabolism of exogenous [1-¹⁴C]PGH₂ by these cells was analyzed as described under “Experimental Procedures.” The TLC plates were developed in an A-9 system. Pretreatment with OKY-046 was as described for Fig. 1. Lane 1, control cells; lane 2, activin A/EDF-treated cells; lane 3, cells treated by activin A/EDF and incubated with OKY-046. Arrows indicate locations of authentic AA, PGs, and TXB₂. The data are representative of three experiments.

**Table I**
The distinct effects of cell density on activin A/EDF-induced TXA₂ synthetic activity and erythroid differentiation

<table>
<thead>
<tr>
<th>Initial cell density</th>
<th>Hemoglobin-positive cells (%)</th>
<th>TXA₂ synthetic activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 × 10⁴ cells/ml</td>
<td>81 ± 5</td>
<td>0.8 ± 0.1</td>
</tr>
<tr>
<td>1 × 10⁵ cells/ml</td>
<td>14 ± 2</td>
<td>2.6 ± 0.3</td>
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**Fig. 3.** Effect of cycloheximide on TXA₂ synthesis in activin A/EDF-treated MEL cells. MEL cells were seeded at 1 × 10⁵ cells/ml in medium containing 1 nM activin A/EDF and 1 μM cycloheximide. AA by these cells was analyzed by a TLC autoradiogram, as described under “Experimental Procedures.” The TLC plates were developed in an A-1 system. Arrows indicate locations of authentic AA, PGs, TXB₂, and HHT.

synthetic activity; at 1 × 10⁴ cells/ml, TXA₂ activity was about 0.8%, whereas at 1 × 10⁵ cells/ml, it increased to about 2.6%.

The kinetics of the two responses of MEL cells to activin A/EDF (TXA₂ synthetic activity and erythroid differentiation) were compared. MEL cells were seeded at 1 × 10⁵ cells/ml for the induction of TXA₂ synthetic activity and 1 × 10⁴ cells/ml for erythroid differentiation. Both responses were time- and dose-dependent. Fig. 4 shows the time courses. TXA₂ synthetic activity was detected 12 h after addition of this factor and reached the maximum at 36-48 h. This time course was faster than that of the induction of hemoglobin-positive cells in the same cell line. Fig. 5 shows that the dose-response curves of both reactions for activin A/EDF were

**Fig. 4.** Time courses of the induction of the TXA₂ synthetic activity and erythroid differentiation. MEL cells were seeded at 1 × 10⁵ cells/ml for the induction of the TXA₂ synthetic activity and at 1 × 10⁴ cells/ml for erythroid differentiation in medium containing 1 nM activin A/EDF, and then cultured for the indicated periods of time. The TXA₂ synthetic activity (O) and the percentages of hemoglobin-positive cells (O) were determined as described under “Experimental Procedures.” All values represent the mean and standard error of triplicate determinations.

**Fig. 5.** Dose-response curves of the TXA₂ synthetic activity and erythroid differentiation for activin A/EDF. MEL cells were seeded at 1 × 10⁵ cells/ml for the induction of the TXA₂ synthetic activity and at 1 × 10⁴ cells/ml for erythroid differentiation in medium containing various concentrations of activin A/EDF, then cultured for 48 h. The TXA₂ synthetic activity (O) and the percentages of hemoglobin-positive cells (O) were determined as described under “Experimental Procedures.” All values represent the mean and standard error of triplicate determinations.
similar. The effects of activin A/EDF were evident at 20–50 pm and maximum at 1 nM. The ED₅₀ values were about 100 pm, consistent with that reported previously for erythroid differentiation (22, 38).

The effects of other erythroid inducers, HMBA and di-methyl sulfoxide (Me₂SO) (for review, see Ref. 39), on TXA₂ synthetic activity were examined. As shown in Fig. 6, HMBA showed no potential to increase TXA₂ synthetic activity, whereas the Me₂SO-treated cells showed slight TXA₂ synthetic activity. Activin A/EDF functioned as a much more potent inducer of TXA₂ synthetic activity.

Finally, to search for evidence that activin A/EDF induces non-erythroid differentiation, we performed cytochemical analyses. Since TXA₂ synthetic activity is expressed in monocytic (17) or megakaryocytic cells (40, 41), we examined α-naphthyl butyrate esterase staining for a monocytic marker (37) and acetylcholinesterase staining for a megakaryocytic marker (42, 43). α-Naphthyl butyrate esterase staining was positive in the control cells, and no change was caused by activin A/EDF (data not shown). Acetylcholinesterase-positive cells were not seen in the control cells, whereas a small but significant portion (0.02–0.05%) of positive cells was induced by activin A/EDF (Fig. 7).

**DISCUSSION**

In this study, we found a novel action of activin A/EDF, induction of TXA₂ synthetic activity in MEL cells (Fig. 1), and clarified its enzymatic basis. Although activin A/EDF induces erythroid differentiation in these cells, TXA₂ synthetic activity has been observed in megakaryocytes/platelets (40, 41) and monocytes/macrophages (17) but not in erythroid cells. We therefore approached the relation between the expression of this enzyme activity and erythroid differentiation in the latter part of this study.

The enzyme responsible for the increase in TXA₂ synthetic activity is presumed to be TX synthase. This presumption is based on the observations that the control cells could synthesize other PGs (implying the constitutive presence of cyclooxygenase) and that TX synthase activity (conversion of PGH₂ to TXA₂) was detected only in the activin A/EDF-treated cells (Fig. 2). Cycloheximide selectively inhibited TXA₂ synthesis (Fig. 3), with little effect on the synthesis of other PGs, in the activin A/EDF-treated cells. These results suggest that activin A/EDF induced de novo protein synthesis of TX synthase. TX synthase produces TXA₂ and HHT in equimolar amounts (11–13, 47). However, HHT is also synthesized from PGH₂ under various nonenzymatic conditions (47). The finding that OKY-046 did not abolish HHT formation (Fig. 1) suggests that a large portion of HHT was formed through the TX synthase-independent pathway.

Although activin A/EDF also induces erythroid differentiation in MEL cells, TXA₂ synthetic activity is not a typical feature of erythroid cells. Thus, we analyzed in three experiments the relationship between the induction of TXA₂ synthetic activity and erythroid differentiation. First, we found that the cell density had inverse effects on activin A/EDF-induced erythroid differentiation and TXA₂ synthetic activity (Table I), suggesting that the expression of the activin A/EDF-induced two phenotypes is distinctly controlled, probably by cell-cell interactions. Second, we compared the kinetics of these actions of activin A/EDF. While the dose-response curves were similar (Fig. 5), the time course of the induction of TXA₂ synthetic activity was faster than that of erythroid differentiation (Fig. 4). Last, we examined the effects of other erythroid differentiation inducers, HMBA and Me₂SO, on TXA₂ synthetic activity. These inducers had little or no ability to induce this activity (Fig. 6). Taken together, these results indicate that the expression of TXA₂ synthetic activity is independent of erythroid differentiation.

Our findings raise the possibility that activin A/EDF induced a monocytic or megakaryocytic differentiation in MEL cells, since TXA₂ synthetic activity is known to be expressed in these non-erythroid cells (17, 40, 41). Our preliminary study revealed that activin A/EDF induced acetylcholinesterase activity, a widely accepted megakaryocytic marker in murine hematopoietic study (42, 43). Taken together, our observations suggest that activin A/EDF exerts the bipotential action to induce erythroid and non-erythroid, possibly megakaryocytic, differentiation in MEL cells. Broxmeyer et al. (26) reported that activin A/EDF increased not only erythroid colonies but also colonies containing multilineage cells,
granulocytes, erythroid cells, macrophages, and megakaryocytes in human bone marrow culture studies. Our system may be useful for studying such an action of activin A/EDF on multipotential progenitor cells.

The molecular basis of the bipotential action of activin A/EDF is unknown but may be explained by the existence of subtypes of receptors. The receptors for activin A/EDF expressed on MEL cells were studied by two groups of workers (48, 49). Their results showed that MEL cells had 3,200–3,500 of a single class of binding sites per cell, with a $K_d$ of 150–310 pm. Affinity cross-linking experiments with $^{125}$I-labeled activin A/EDF revealed three radiolabeled components with $M_r$ of 140,000, 76,000, and 67,000 in these cells (48). It is possible that different biological responses to activin A/EDF are mediated by different receptor molecules, since such mechanisms were documented with transforming growth factor $\beta$, a protein highly homologous to activin A/EDF (50–53). However, more study is necessary to substantiate this view on the mode of action of activin A/EDF.

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REFERENCES


