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Activin/EDF as an inhibitor of neural differentiation

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Activin/EDF, a stimulator of the secretion of follicle stimulating hormone (FSH) from pituitary gland and an inducer of erythroid differentiation for Friend leukemia cells, has since been implicated in a variety of biological roles. Here, we show some novel effects of activin on murine embryonal carcinoma cells (EC cells). First, activin acts as a growth factor on undifferentiated P19 cells, a well characterized EC cell line for the study of mammalian development. Second, activin inhibits the retinoic acid (RA) induced differentiation of P19 cells to neurons and glial cells. The inhibitory effect of activin on neural differentiation, which has yet to be proved in other physiological peptides, is confirmed also on the differentiation of various neuroblastoma cell lines. Our results suggest a possible role of activin as a negative regulator of neural differentiation in mammalian development.

Activin, a member of the TGF-β family(1), was originally purified from ovarian fluids as a stimulator of the secretion of FSH from anterior pituitary gland(2,3). Independently, erythroid differentiation factor (EDF) was isolated from a human monocytic leukemia cell line as a peptide that causes differentiation of Friend erythroleukemia cells(4). Molecular cloning of cDNA revealed that activin and EDF were identical. Furthermore, they share 35% homology in amino acid sequence with TGF-β1(5). In addition to the activities shown above, other diverse

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biological effects of activin have also been disclosed. For example, activin modulates secretion of growth hormone and prolactin from pituitary gland(6) and stimulates insulin secretion from pancreatic islets(7). Recently, activin was also shown to be a mesoderm-inducing factor in Xenopus laevis(8,9).

We have previously carried out binding assays using ^{125}I-activin for a number of cultured cell lines. A relatively high number of activin receptors were expressed in EC cells. P19 cells and PCC3 cells expressed high affinity receptors (P19 cells; 5200 sites/cell, Kd=0.4nM., PCC3 cells; 6500 sites/cell, Kd=0.3nM) found in Friend erythroleukemia cells (3500 sites/cell, Kd=0.15nM), while F9 cells demonstrated only low affinity receptors (3500 sites/cell, Kd=1.5nM) (10). In the case of Friend erythroleukemia cells, activin is capable of inducing them to differentiate into haemoglobin-synthesizing cells by specifically binding to this high affinity receptor.

We suspected that the high numbers of activin receptors on EC cells implied some significant physiological roles of activin in the differentiation of these cells and perhaps in the embryo. EC cells represent an early blastula stage of the embryo which can be induced to differentiate by treating with RA(11). To investigate the effect of activin on EC cells, we examined P19 cells, the differentiation of which was widely used as a model of early development(11).

Materials and Methods

Cell culture; EC cells (P19 cells and F9 cells) were obtained from Dr. M. W. McBurney (University of Ottawa). Neuroblastoma cells (IMR-32 cells, GOTO cells and NB-1 cells) were obtained from Japanese Cancer Research Resources Bank (JCRB). Unless otherwise indicated, cells were incubated in α-minimal essential medium (GIBCO, USA) supplemented with 10% fetal bovine serum (FBS) (Cell Culture Laboratories, USA) and 10μg/ml gentamycin sulfate at 37° C in a humidified atmosphere of 5% CO2.

^{3}H-tyhmidine incorporation; Cells were grown under 3% FBS for 24 h in advance and after trypsinization, transferred to 12 wells plate dish (Corning, USA) containing 0.5% FBS (5X10^{3} cells/well). They were treated with 1nM activin alone or 0.3μM RA, either with or without addition of 1nM activin. At indicated times, medium was replaced to serum-free one and incubation was continued in the presence of ^{3}H-thymidine (1μCi/ml) for 1h. ^{3}H-thymidine incorporation was measured as described(12). Data are the means of duplicate experiments. At the same time, phase-contrast photomicrograph of each sample was taken at 72 hrs. Activin was produced in Chinese hamster ovary cells as previously described(5).

Immunofluloresence and immnoblotting; P19 cells were differentiated according to the method of Rudnicki and McBurney(11) with minor modifications. Cells were aggregated and exposed to 0.3μM RA with or without treatment of 1nM activin for 4 days. At day 4, aggregates were transferred to tissue culture grade dish (CORNING, USA) and their morphological changes were followed. Photographs of phase contrast micrography and immunofluorescence using monoclonal anti-neurofilament

160(13) antibody (BioMarker,Israel) at day 7 were taken. Immunoblot analyses using the same antibody as above and monoclonal anti-glial fibrillary acidic protein (GFAP), clone G-A-5 (Sigma, USA) were performed using the same materials according to the method of Towbin et al(14). At day 6 and day 10 respectively, cells were lysed directly with SDS solbilization buffer; 2% SDS, 5% 2-mercaptoethanol, 15% glycerol, 125mM Tris-HCl (pH6.8). Cells without treatment of RA had overgrown to death before day10. As a positive control, a sample from mouse spinal cord was also prepared. Proteins resolved by SDS-PAGE were transferred to nitrocellulose sheets (PH 79, Schleicher & Schuell, Inc. 0.1μm) using the semi dry blot system. The blots were washed with Tris buffered saline (TBS), 0.15 M NaCl, 20mM pH 7.8, and blocked for 1h with 10% skim milk in TBS. After incubation with the first antibody for 2h at room temperature, the blots were washed with TBS, incubated with goat affinity-purified antibody to mouse IgG conjugated with alkaline phosphatase (Sigma) at a 1:1000 dilution for 2h at room temperature, and washed with 0.05% Tween 20 in TBS for 30 min and then with TBS. Alkaline phosphatase bound to proteins were developed with the bromochloroindolyl phosphate / nitro blue tetrazolium substrates.

Differentiation of neuroblastoma cells; IMR-32 cells(15) were treated with 1uM RA in the presence or absence of 1nM activin in the medium. GOTO cells(16) were subjected to serum starvation with or without addition of 1nM activin. Medium was changed every 3 days and their morphological changes were followed. Phase-contrast photomicrographs were taken at day 8.

Results and Discussion

First, we have found that activin acts as a growth factor on undifferentiated P19 cells. Cells were grown in the medium containing 0.5% FBS in the presence or absence of RA and their growth rates were evaluated by ^{3}H-thymidine incorporation. Under these low serum conditions, the growth rate of P19 cells slows down within 48 h and ceases within 72 h (Fig1.a). Cells given 1nM activin in the medium continued to grow without changing growth rate during this period (Fig1.a). Microscopic examination showed that the former cells aggregated themselves into a somewhat rounded shape (Fig1.d), while the latter cells grew spread in an undifferentiated state (Fig1.c). When cells were treated with RA under low serum conditions, the appearance of aggregated cells or dead cells became even more remarkable (Fig1.f). By contrast, addition of activin to this medium made some populations of cells remain in an undifferentiated state (Fig1.e). The mitogenic effect of activin was dose-dependent and its maximal effect was obtained by the concentration of 1nM activin (data not shown). On F9 cells having only low affinity receptors, activin had no effects (Fig1.b). These observations were also confirmed on soft agar colony formation assay (data not shown).

Next, the effect of activin on the differentiation of P19 cells was examined. In the presence of a high concentration (0.3μM) of RA, P19 cells can be differentiated into neurons and glial cells under normal serum conditions. Interestingly, the addition of activin at 1nM inhibited

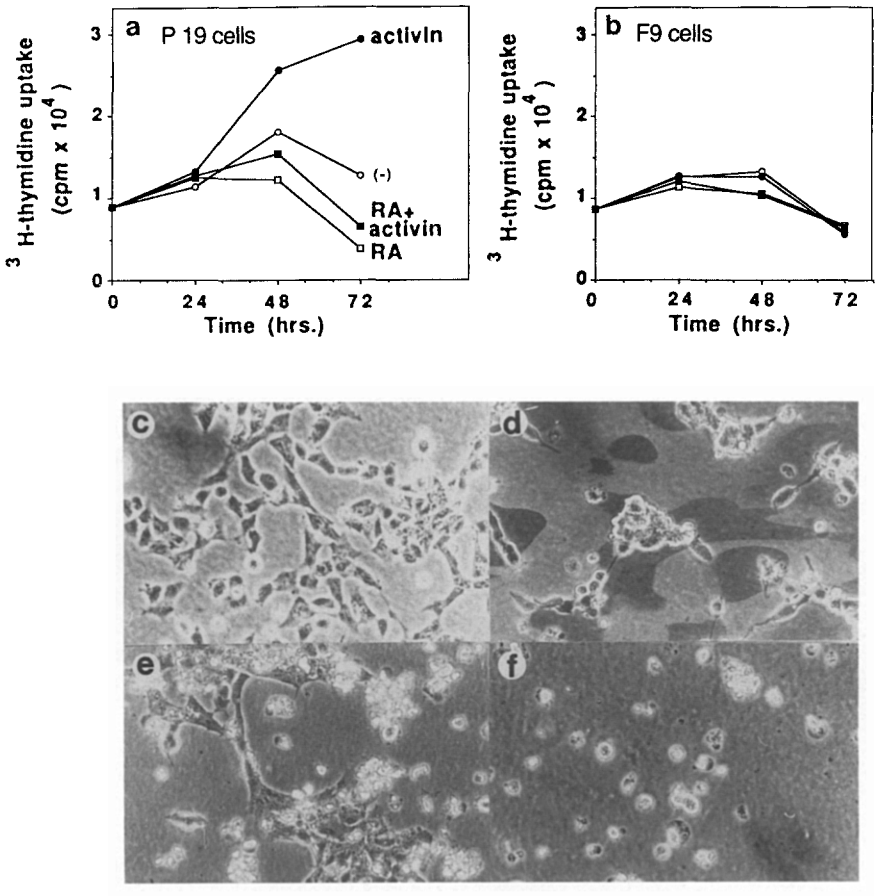

Fig.1 Effects of activin on the growth of P19 cells and F9 cells a and b, growth curves of undifferentiated and 0.3μM RA-treated cells (a; P19 cells, b; F9 cells) in the presence or absence of 1nM activin. c-f, morphology of P19 cells grown for 3 days under 0.5% serum conditions in the presence of 1nM activin alone (c) or 0.3uM RA, either with (e) or without (f) 1nM activin or without either (d).

this neural differentiation almost completely (Fig2.a,b). To confirm this observation, we investigated whether characteristic neural cell markers appear under these conditions. Indirect immunofluorecence using a monoclonal antibody against 160 kD neurofilament revealed that this filament was not present in cells treated with both RA and activin (Fig2.c,d). Immunoblotting experiments further confirmed this biochemically (Fig2.e). In addition, the synthesis of another neural differentiation marker, the 45 kD glial fibrillary acidic protein, was also found to be inhibited (Fig2.f). This inhibitory effect reached its plateau also at the concentration of 1nM activin (data not shown). When a lower concentration of RA was added to P19 cells, the cells differentiated into mesodermal tissues, including cardiac muscle cells, smooth muscle cells and skeletal muscle cells(11). We suspected that activin might also retard the differentiation to mesodermal tissues. However, the majority of cells continued to proliferate gradually,

which made it difficult to demonstrate the effect of activin clearly. Thus, we were able to prove the inhibitory effect of activin unequivocally only on the differentiation of P19 cells to ectoderm.

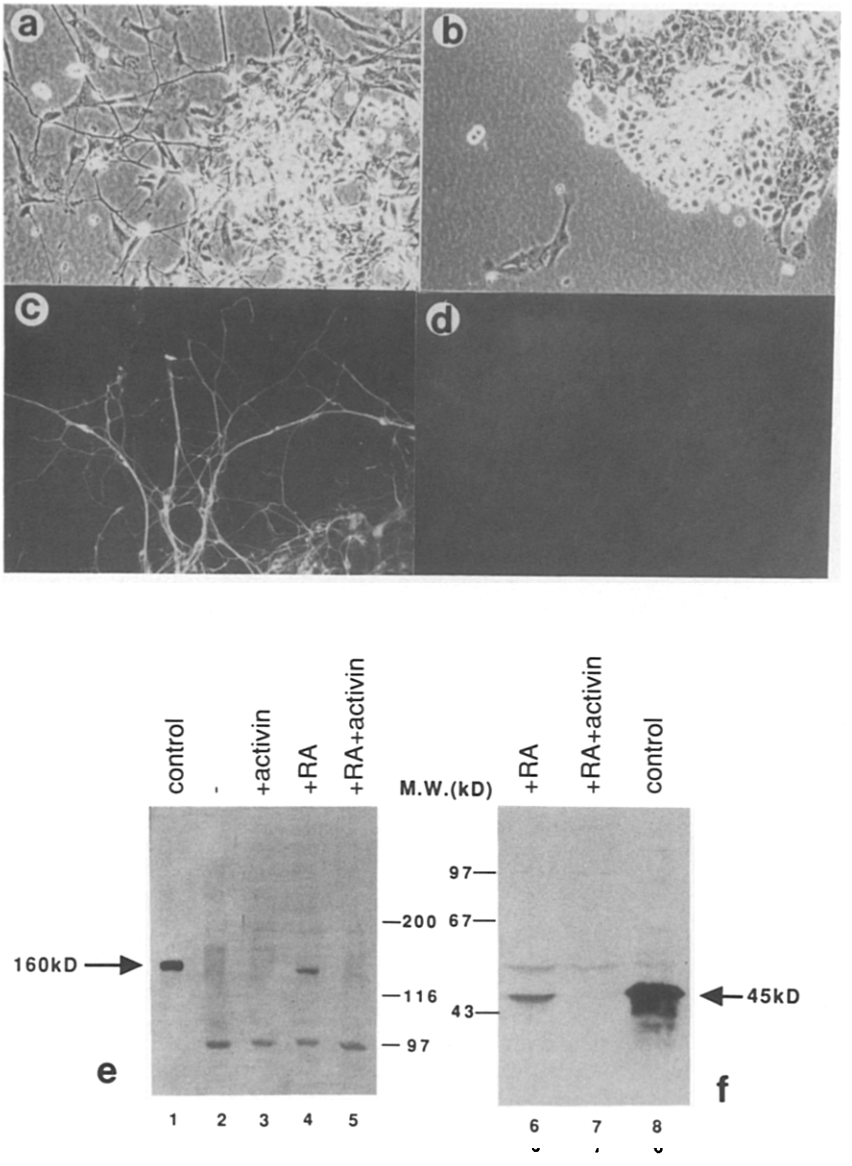

Fig.2 Suppressive effect of activin on RA-induced neural differentiation of P19 cells; Cells were treated as described in Materials and Methods. Morphology and immunoflorescence analysis of 160kD neurofilament in 0.3uM RA-treated P19 cells with (a,c) or without (b,d) treatment of 1nM activin are shown. Immunoblot analysis of 160kD neurofilament (a) and glial fibrillary acidic protein (GFAP) (b). Samples were prepared as described in Materials and Methods (lane 1 and 8; mouse spinal cord, lane 2; no treatment, lane 3; activin, lane 4 and 6; RA, lane 5 and 7; RA+activin) and run on a 6% (a) or 12% (b) SDS-polyacrylamide gel. Arrowheads denote the positions of the molecular weight (M.W.) marker (kD; kilo-dalton).

To see if activin could inhibit neural differentiation also in other cell systems, we examined neuroblastoma cell lines. Neuroblastoma cell lines are known to be induced to differentiate by chemical agents such as RA or by serum starvation. RA-treatment of IMR-32 cells, a human neuroblastoma cell line(15), results in cellular enlargement and neurites formation in 8 days with concomitant cessation of cell growth (Fig3.a). The addition of 1nM activin enabled them to continue to grow, although they changed into large, flattened and adherent cells (Fig3.a). GOTO cells, another human neuroblastoma cell line(16), are also induced to form neurites by a treatment of serum starvation for 8 days (Fig3.b). The addition of 1nM activin inhibited this process and kept the cells to grow in a flat and assembled shape (Fig3.b). Mitogenic effect of activin was also observed for another neuroblastoma cell line, NB-1 cells(17) (data not shown). In this way, the inhibitory effect of activin on neural differentiation was proved not only on pluripotent P19 cells but also on various neuroblastoma cell lines which were already committed to the neural pathway.

Recently, Schubert et al.(18) reported that activin can replace RA for the survival of P19 cells and concluded that activin is a nerve cell survival molecule. Our observations suggest that the effect of activin is somewhat different from that of RA. The increased survival of undifferentiated P19 cells were attained only by activin as a mitogen. In the differentiation of P19 cells and various neuroblastoma cells, the effect of activin was counteracting to the neural differentiation inducing activity of RA.

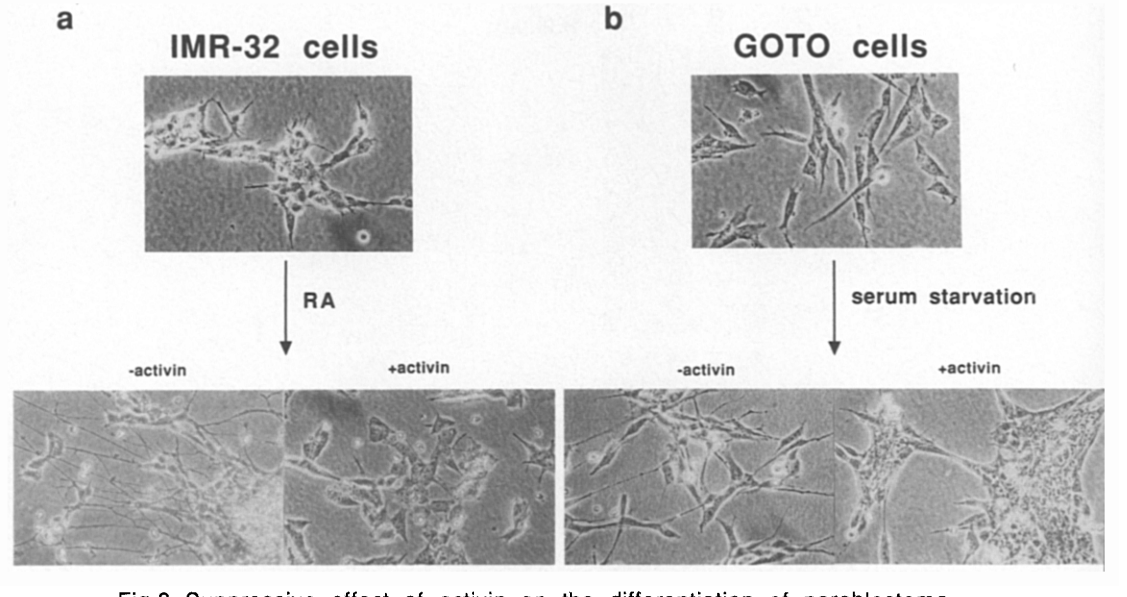

Fig.3 Suppressive effect of activin on the differentiation of neroblastoma cells; a; IMR-32 cells treated with 1uM RA in the presence or absence of 1nM activin. b; GOTO cells subjected to serum starvation with or without addition of 1nM activin. Phase-contrast photomicrographs are taken at day 8.

To date, only leukemia inhibitory factor (LIF), which appears to be engaged in many aspects of cell differentiation(19), has been identified as a peptide which has an inhibitory effect on the differentiation of EC cells(20). However, the inhibitory effects of activin and LIF appear different from each other in view of their target cell spectra. LIF inhibits the differentiation of F9 cells and NG-2 cells to endoderm-like cells(21), but has no effect on P19 cells (our unpublished data). On the contrary, activin has a strong effect on P19 cells but no effect on F9 cells. It is also noteworthy that TGF-β-1, a protein in the same family, acts as an inhibitor of myogenic differentiation(22). The maintenance of certain undifferentiated or growing states in various developing tissues will be crucial for the regulation of early development and this may be controlled by various cytokines at specific sites. Further investigations, including the molecular cloning of the activin receptor, are required for the understanding of the exact roles and mechanisms of activin in mammalian development.

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