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Activin A-Induced Differentiation in K562 Cells Is Associated With a Transient Hypophosphorylation of RB Protein and the Concomitant Block of Cell Cycle at G_1 Phase

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Abstract The human erythroleukemic cell line, K562, can be induced to differentiate by the addition of activin A, a newly purified protein belonging to the TGF-β_1 family. The present studies used flow cytometric cell cycle analysis, indirect immunofluorescence staining of the proliferating cell nuclear antigen (PCNA), and thymidine incorporation assay of cell proliferation to study the effects of activin A on the cell cycle during differentiation in K562 cells. Activin A-treated K562 cells were found to undergo a transient block in cell cycle, temporarily halting progression from G_1 to S phase. The latter can be observed after approximately 24 hr of incubation with activin A and then disappears after this early stage of induction of differentiation. Cell cycle kinetics analysis using synchronized K562 cells also confirms that in the presence of activin A, K562 cells progress normally through various phases of cell cycle, except that there is prolongation of the G_1 phase between 10 to 24 hr of culture. Furthermore, this transient arrest in G_1 is correlated with dephosphorylation of a nucleoprotein, the RB gene product, which occurs within 9–24 hr of incubation with activin A; and phosphorylation of RB protein then develops afterward. In addition, these cell cycle-related events are observed to occur earlier than the accumulation of hemoglobins in K562 cells. It is concluded that transient dephosphorylation of RB protein and prolongation of G_1 phase of cell cycle precede and accompany erythroid differentiation caused by activin A and chemical inducers, thus constituting part of the mechanism for induction of differentiation in the erythroleukemia cells.

Key words: prolongation of G_1, activin A, RB protein, cell cycle, differentiation

INTRODUCTION

Activins are a family of newly purified protein dimers consisting of two β-subunits (14,000 daltons per subunit) [Vale et al., 1986; Ling et al., 1986]. There are two distinct β-subunits, β_A and β_B, with 116 and 115 amino acid residues, respectively. The complete amino acid sequences of the precursors of these subunits (β_A and β_B) have been deduced from cDNA sequences [Mason et al., 1986; Mayo et al., 1986]. The most abundant form is activin A, (i.e., $\beta_A\beta_A$); the other two are $\beta_B\beta_B$ and $\beta_A\beta_B$. Activin was first purified to homogeneity from gonadal fluids and recognized as gonadal protein, which stimulated the secretion of pituitary follicle-stimulating hormone [Vale et al., 1986,, 1990; Ling et al., 1986]. Later, it was found that activin A increases globin transcripts and hemoglobins in developing human erythroid cells [Shao et al., 1992], as well as the human erythroleukemia cell line, K562 [Frigon et al., 1992]. Activin A also causes induction of mouse Friend cells to become positive, when stained with benzidine reaction [Eto et al., 1987]. It was also found that activin A could modulate the colony formation of BFU-E and CFU-E in human bone marrow culture [Yu et al., 1987; Broxmeyer et al., 1988; Yu et al., 1989; Shao et al., 1992]. Furthermore, at least the β_A-subunit of this protein was found to be expressed at the RNA level in the bone marrow of rats [Meunier et al., 1988], and activin A was reported to be produced locally in human bone marrow [Shao et al., 1991; Yu et al., 1991;

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Yamashita et al., 1992]. These data all suggest a possible role of activin A in the regulatory control of erythropoiesis [Yu et al., 1987].

In addition to these functions, activins have also been found to elicit many different tissue-specific responses [Vale et al., 1990]. They stimulate hormone production in ovarian and placental tissues [Vale et al., 1986, 1990; Ling et al., 1986], promote neural cell survival [Schubert et al., 1990], and induce axial mesoderm and anterior structures in explants from *Xenopus* and chick blastulae [Smith et al., 1990; Van den Eijnden-van Raaij et al., 1990; Thomsen et al., 1990; Green and Smith, 1990; Green et al., 1990; Sokol and Melton, 1991]. Activin-binding sites have been identified on a number of activin-*responsive* cells [Campen and Vale, 1988]; recently, a repertoire of activin receptor isoforms belonging to the protein serine/threonine kinase family have also been cloned [Mathews and Vale, 1991; Attisano et al., 1992]. However, the intracellular signalings of activin A are unknown. In the present studies, erythroleukemia cell line, K562, was used as a model for human erythroid differentiation. In addition, K562 is a transformed cell line; and a logical function for the product of a tumor suppressor gene such as retinoblastoma (RB) might be to modulate proliferation and promote cellular differentiation in this cell line. The RB protein is believed to suppress cell growth and appears to be regulated by cell cycle-specific phosphorylation [Weinberg, 1989]. This protein is underphosphorylated when cells are quiescent and hyperphosphorylated in S, G_2, and early M phase [Chen et al., 1989; Ludlow et al., 1990; DeCaprio et al., 1989]. Current data suggest that the underphosphorylated form of RB may act as a growth suppressor by blocking exit from the G_0 or G_1 phase into S phase, and phosphorylation of RB protein inhibits the growth suppression function of the underphosphorylated RB, allowing the cell to enter S phase [Chen et al., 1989; Ludlow et al., 1990; DeCaprio et al., 1989]. In the present studies, the effects of activin A on the cell cycle and the relationship between activin A-induced differentiation and phosphorylation state of RB protein were examined.

MATERIALS AND METHODS

Cell Preparations

K562 cells were grown in RPMI 1640 medium supplemented with 50 IU/ml of penicillin, 50 μg/ml of streptomycin and 10% fetal calf serum (FCS) (Hyclone Lab., Logan, UT), as previously described [Singh and Yu, 1984]. Actively growing cells were then seeded in culture with or without activin A at 25 ng/ml to an initial density of 0.5–2.0 × 10^5/ml. The final cell density at 3–5 days of culture was ≤ 1 × 10^6/ml.

Incorporation of Radioactive Thymidine

A microtiter plate containing 200 μl/well of the suspension of K562 cells at a density of 1.2—5 × 10^5 cells/ml with or without activin A was incubated for different periods of time. Afterward, to each well was added 1 μCi of ^{3}H-methyl-thymidine; incubation was continued for 2 hr, and the cells were collected and washed in a multiple automatic sample harvester [Matsumoto et al., 1982]. The incorporation of radioactivity into nucleic acids was measured by the collection of these cells on a glass fiber filter strip and washings of the filters with water. After drying, the filter was analyzed for radioactive thymidine incorporation by liquid scintillation counting.

Cell Cycle Analysis

K562 cells were washed twice in ice-cold PBS, resuspended at 1–2 × 10^6 cells/ml, aliquoted into 1 ml samples, and subsequently kept on ice. To each 1 ml sample, 2 ml of ice cold methanol was added dropwise, while gently mixing. Samples were then incubated on ice for at least 30 min. At this point, samples were either stained immediately with propidium iodide or stored at 4°C for less than 5 days before staining. For staining, samples were centrifuged at 300*g* for 5 min, and the supernatant was discarded. Then, 500 μl of 0.1 mg/ml propidium iodide staining solution containing 0.1% Triton X-100, 0.1 mM EDTA in PBS, was added to each cell pellet and the cells were resuspended. Subsequently, 500 μl of RNAse A (Sigma, St. Louis, MO) was added to a final concentration of 100 U/ml. Samples were again vortexed and incubated in the dark at room temperature for 30 min. Samples were then stored on ice in the dark and analyzed within 1 h. Finally, samples were analyzed using the FL2 parameter of a Becton Dickinson FACScan System fitted with a milliwatt argon laser set at an excitation wavelength of 488 nm. Approximately 20,000 cells were analyzed per sample. The fraction of cells in different phases of cell cycle (G_1, S, or G_2/M) was determined by mathematical modeling, using the Becton Dickinson Cellfit Software SOBR Model. Data were percentages of total cells analyzed; each experi-

ment was repeated for a minimum of three times in each case.

Synchronization of K562 Cells

K562 cells were seeded at a density of 10^5 cells/ml in RPMI 1640 medium supplemented with 10% FCS, as described. A presynchronization control aliquot was removed and processed for cell cycle analysis and RB protein study. Remaining cells were synchronized by incubating for 18 h in the presence of aphidicolin at 1 μg/ml. Subsequently cells were washed three times in fresh culture medium and resuspended again at 4–5 × 10^5 cells/ml in two groups of flasks for control and activin A treatment. Aliquots were removed and pooled at 1 h postwashing, after which time activin A was added to the experimental group of flasks at 25 ng/ml. Cells were incubated at 37°C and 5.0% CO_2; further aliquots were removed from both groups at 4, 7, 12, 18, and 24 h postwashing of aphidicolin-synchronized cells.

Indirect Immunofluorescence for PCNA

K562 cells were deposited to slides by cytospin centrifugation (500 *g*, 5 min) and fixed with methanol for 5 min at −20°C followed by acetone for 2 min at −20°C. After a brief rinse with PBS and air drying, cells were incubated with 40 μl of human anti-PCNA serum (a generous gift from the W.M. Keck Autoimmune Disease Center at the Scripps Research Institute) diluted 1:100 in PBS for 30 min at room temperature in a humid chamber. After two 10-min washes with PBS, cells were incubated with 40 μl of fluorescein-labeled polyvalent goat antihuman IgG (Caltag Laboratories, San Francisco, CA) at a dilution of 1:100 in PBS. After 30 min of incubation at 37°C, slides were washed with PBS and mounted with cover glass in mounting buffer containing 66% glycerol, 60 mM glycine pH 8.6, and 100 mM NaCl. Stained cells were observed with Zeiss Axioskop fluorescence microscope fitted with a ×100 oil immersion objective.

Analysis of RB Protein Phosphorylation

Cultured K562 or MOLT-4 (control) cells were washed twice in PBS and then lysed by freeze/thawing twice in lysis buffer (50 mM Tris–HCl, pH 7.4, 250 mM NaCl, 5 mM EDTA, 0.1% Nonidet P-40, 50 mM NaF, 1 mM PMSF, 0.05 mg/ml aprotinin, 0.05 mM leupeptin) at 10^7 cells/ml. Lysates were clarified by centrifugation (4°C, 20,000 *g*) for 10 min. Supernatants were quick frozen and stored at −70°C until further processing. For analysis, thawed lysates were kept on ice and incubated with pMG3-245 anti-fRB monoclonal antibody (generous gift of Dr. Wen-Hwa Lee at the University of California, San Diego, CA) for 6 h. Protein A–Sepharose swelled in lysis buffer was added, and the lysates were rotated at 4°C overnight. Immunoprecipitated complexes were washed in lysis buffer, solubilized in SDS–lysis buffer (62.5 mM Tris–HCl pH 6.8, 2% SDS, 10% glycerol, 5% β-mercaptoethanol, and 0.001% pyronin Y) and separated by 7.5% SDS–PAGE until heavy chains of immunoglobulins ran out of gels. Proteins were transferred to Imobilon-P membrane (Millipore, Bedford, MA), using a Transblot Cell (Biorad, Richmond, CA) at 60 V for 2.5 h. Membranes were incubated in blocking solution (25 mM Tris–HCl, pH 7.4, 125 mM NaCl, 0.1% Tween-20, 4% nonfat dry milk, 0.1% NaN_3) at 4°C on a tilting platform for 1 h. Subsequently, membranes were incubated in pMG3-245 diluted with blocking solution (1/200) rotating at room temperature for 6 h. Membranes were then washed in blocking solution before overnight incubation with phosphatase-conjugated goat antimouse IgG at 1:1,500 dilution (Promega, Madison, WI). Chromogenic substrate was added for color development according to the manufacturer's specifications.

RESULTS

K562 Cells Arrest Temporily in G_1 Phase With the Addition of Activin A

To analyze the effect of activin A on cell cycle, the distribution of DNA content in K562 cells was measured by propidium iodide staining and flow cytometric analysis. Figure 1 represents DNA histograms of actively growing K562 cells. In the control culture, approximately 29.7% of the cells were in G_1 with peak at channel number 356 (Fig. 1A). They are separated from cells in G_2/M phase at peak channel 670 (approximately 13.6%) by S phase cells, characterized by an intermediate amount of DNA with a mean distribution at channel number 513 (approximately 56.7%). It was shown that 3 h of incubation with activin A (Fig. 1B) did not incur significant changes in the proportion of cells in G_1, G_2/M and S phases (32.0, 12.1, and 55.1%, respectively) of the cell cycle as compared with control cells (Fig. 1A). However, by 24 h of culture with activin A, approximately 67.1% of

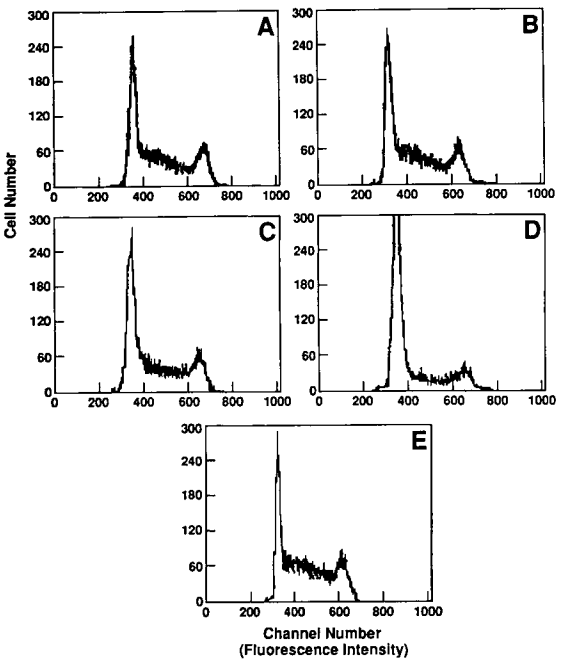

Fig. 1. Effect of activin A on the cell cycle of actively growing K562 cells. K562 cells growing actively in suspension at a density of 0.5–2 × 10^5/ml were incubated in the absence (**A,C**) and in the presence (**B,D,E**) of 25 ng/ml activin A. At 3 h (A,B), 24 h (C,D) and 5 days (E), aliquots were taken from the stock culture for DNA histograms, as described. In addition, DNA histogram of K562 cultures prior to experiment was analyzed and shown to be similar to that presented in Fig. 1A (picture not shown).

the cells were in G_1 phase in the activin A-treated cultures (Fig. 1D), compared with 35% in the untreated cultures (Fig. 1C). Meanwhile, in the activin A-treated cultures, a decrease in the proportion of cells entering S phase was also noted (about 24.8% of cells in the S phase in Fig. 1D). By comparison, 52.6% of the cells were in S-phase at 24 h in the control cultures (Fig. 1C). In other words, incubation with activin A for 24 h caused significant accumulation of cells at G_1 phase in K562. After 5 days in culture with activin A, the proportions of cells in various phases of cell cycle returned to normal (Fig. 1E). In other experiments the proportions of cells in G_1 and S phases of cell cycle in the control and activin A-treated K562 samples were analyzed over 3 h, 1, 3, 4, and 5 days (picture not shown). These results all suggest the increase of the cells at G_1 phase in the activin A-treated samples at the initial 24 h of incubation is transient and accompanied by a concurrent decrease in the proportion of cells at S phase.

The decrease of cells at S phase in the activin A-treated samples was also manifested in the characteristic immunofluorescent staining pattern of a cell proliferation marker, PCNA, in the S phase nuclei [Bravo and Macdonald-Bravo, 1987]. Previous reports have shown that the characteristic distribution of PCNA in various cell lines has been correlated with the S phase of cell cycle [Bravo and Macdonald-Bravo, 1987]. Indirect immunofluorescence microscopy shows that early in S phase, granular nucleoplasmic immunofluorescence appears, then strengthens and becomes punctate within these nuclei. As shown in Figure 2, the percentage of PCNA-positive cells decreased significantly 24 h after the addition of activin A to the culture medium (from approximately 58.0% to 33.5%) (Fig. 2B). In parallel cultures without the addition of activin A, many PCNA-positive cells were observed (Fig. 2A). These experiments thus demonstrate a noticeable decline in cellular proliferation in the activin A-treated samples, although only qualitative differences between control and treated cultures were examined using this indirect immunofluorescence analysis for PCNA. In order to study DNA synthesis quantitatively, thymidine incorporation was also measured for K562 cells after incubation with activin A for different periods of time. As shown in Figure 3, K562 cells exhibited a substantial decrease in the incorporation of thymidine after incubation with activin A for 24 h; then, 3–4 days thymidine incorporation of the activin A-treated cells recovered and, at day 5, exceeded that for the control. These experiments confirm that there is a transient decline in DNA synthesis activity associated with the treatment of activin A at the early stage of incubation.

Activin A Does Not Affect the Progression of Cell Cycle, Except for G_1 Phase

It was reported that aphidicolin halts cells at the G_1/S border thus allowing a simple and rapid synchronization of suspension cells. After 18 hr incubation with aphidicolin, cell cycle profiles of K562 cells were altered and K562 cells were largely halted at G_1 phase of the cycle (Fig. 4A). The number of cells in G_1 phase (51.2%) increased significantly with treatment of aphidicolin. This inhibition of entry into S phase appears to be due to the selective inhibition of the replicative DNA polymerase α by aphidicolin [Pedrali-Noy et al., 1980]. These aphidicolin-synchronized K562 cells were released from synchronization by washings and reculturing in

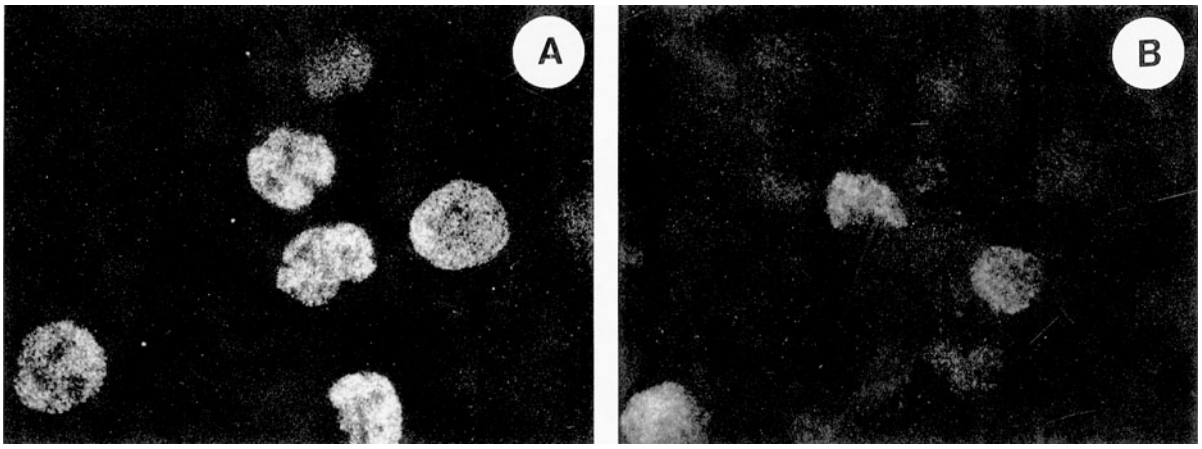

Fig. 2. Changes in the staining pattern of PCNA following addition of activin A. K562 cells were incubated in the absence (**A**) and presence of activin A (**B**), as described in Fig. 1. After 24 h of incubation, cells were prepared for indirect immunofluorescence microscopy using anti-PCNA antibodies as described under Materials and Methods and examined using a ×100 oil immersion Neofluor lens fitted to a Zeiss Axioskop fluorescence microscope. The exposure times for these two photographs are identical.

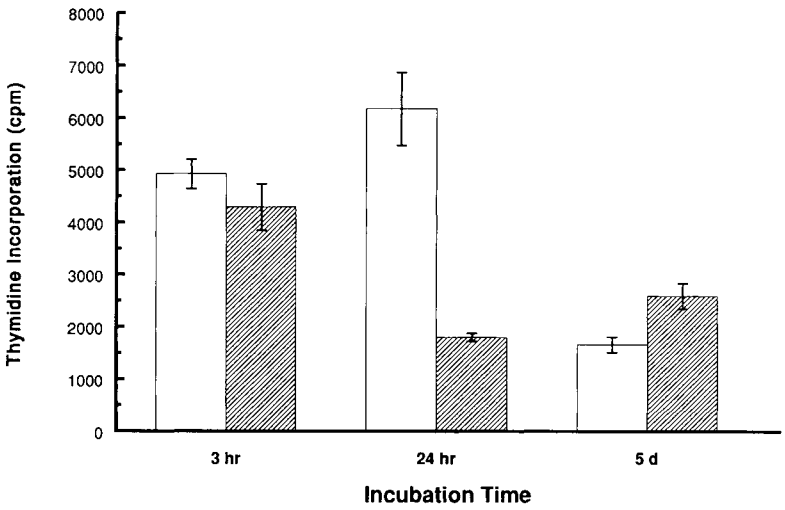

Fig. 3. Effect of activin A on thymidine incorporation in K562 cells. K562 cells were incubated in the presence or in the absence of activin A for different periods of time, as indicated in Fig. 1. Then, aliquots of cells were taken and uptake of radioactive thymidine was analyzed as described.

fresh medium. Progression through the cell cycle in the presence and the absence of activin A is given by the DNA histograms in Figure 4B–K; 7 h after release from aphidicolin, the cells have reached halfway through S phase of cell cycle (Fig. 4C). Progression through S phase was nearly completed within 12 h of resuspension in fresh medium (Fig. 4D). In addition, following release from aphidicolin, the number of cells remained relatively constant as it maintained at 4–5 × 10^5/ml within the first 12 h. Shortly thereafter, the cells divided in synchrony and doubled between 18 to 24 h, indicating that they had traversed through G_2/M phase (Fig. 4E). By 24 h, there was a significant increase of control cells entering S phase (65.5%) (Fig. 4F). This analysis of the synchronized cells agrees with previous estimates of the generation time of K562 in cultures to be approximately 20–24 h. Thus, exposure to aphidicolin did not affect the capacity of cells to traverse through the cell cycle upon removal of the drug. In the same experiments, activin A was added 1 h after release of K562 cells from synchronization. As shown in Figure 4G–K, exposure to activin A did not affect the capacity of cells to traverse from

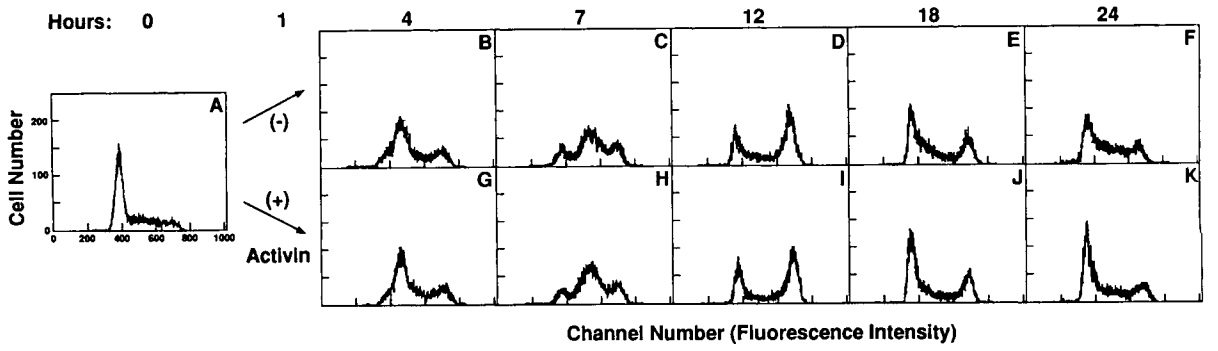

Fig. 4. Cell cycle kinetics analysis of synchronized K562 cells cultured in the absence and presence of activin A. K562 cells maintained 18 h in the presence of 1 μg/ml of aphidicolin, were washed three times and allowed to resume growth in fresh prewarmed culture medium; 1 h after release from synchronization, samples were separated into two portions, which were then cultured in the absence (B–F) and in the presence of 25 ng/ml activin A (G–K). DNA histograms were made immediately after release from aphidicolin (**A**), at 4 h (**B,G**), 7 h (**C,H**), 12 h (**D,I**), 18 h (**E,J**) and 24 h (**F,K**) after reversal.

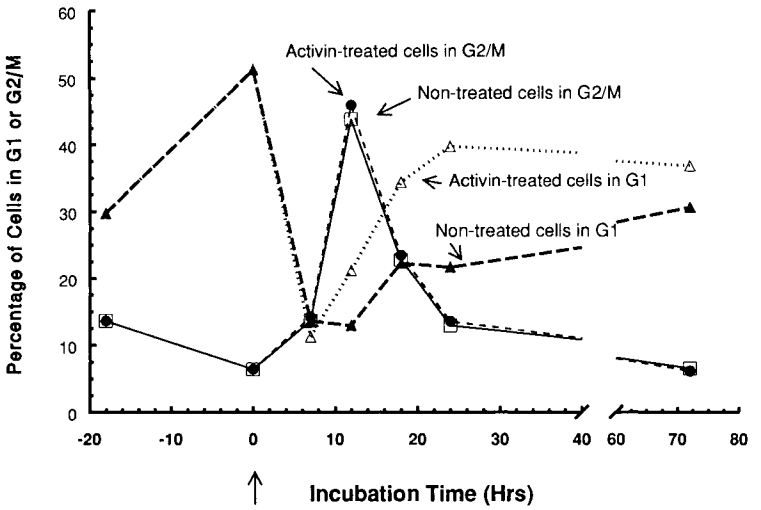

Fig. 5. Effect of activin A on the percentage of cells in G_1 and G_2/M phases of synchronized K562 cells. K562 cells were synchronized after incubation for 18 h with 1 μg/ml of aphidicolin (the time of release from aphidicolin is indicated with arrow mark); 1 h after release from synchronization, samples were separated into two portions and cultured with or without activin, as shown in Fig. 4. At specific time of incubation, aliquots were taken for DNA histograms and the fractions of cells in G_1 or G_2/M phases of cell cycle were determined as described under Materials and Methods. The percentages of control cells in G_1 and G_2/M are represented as --▲--▲-- and ——□—□——; the percentages of cells incubated with the addition of activin A 1 h after release from synchronization are shown as ...△..△... and --●--●-- respectively.

G_1 to S, then G_2/M and finally G_1 phase. However, between 18 and 24 h of incubation, a significant percentage of activin A-treated K562 cells were still maintained at G_1 phase. These activin A-treated samples accumulated 39.7% of cells at G_1 (Fig. 4K), approximately twice as many as the proportion of cells at G_1 in the untreated control (i.e., 21.6%) (Fig. 4F). The differences in percentages of cells in G_1 or G_2/M phases of cell cycles from synchronized K562 in the presence or absence of activin A are summarized in Figure 5. It was obvious that the presence of 25 ng/ml of activin A did not affect the progression of synchronized K562 cells through G_2/M phase, reaching a peak at 12 h after release from synchronization and rapidly declining (Fig. 5). Furthermore, as compared to control samples, activin A-treated K562 cells did not show difference in proportion of cells at G_1 phase within the first 7 h of incubation. However, after 18–24 h of incubation, there were twice as many cells at the G_1 phase in the activin

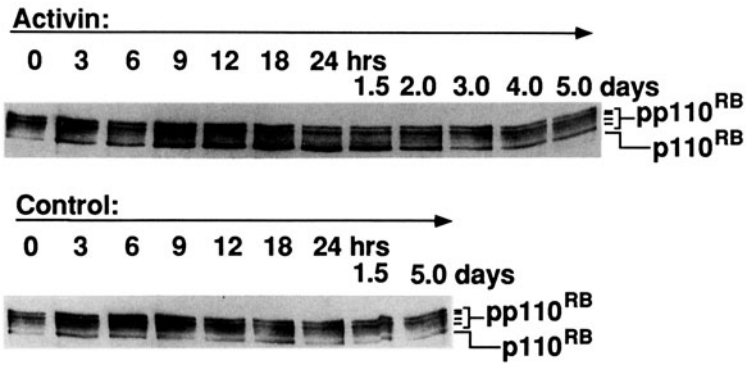

Fig. 6. Changes of phosphorylation of RB protein during induction of cell differentiation by activin A. K562 cells were incubated in the presence and absence of 25 ng/ml activin A. At 0, 3, 6, 9, 12, 18, and 24 h and at 1.5, 2.0, 3.0, 4.0 and 5.0 days, aliquots were taken from the stock cultures. Analysis of RB protein by Western blotting was performed as described. The $pp110^{RB}$ represents the highly phosphorylated forms of RB protein, whereas the $p110^{RB}$ refers to the less phosphorylated form [Chen et al., 1989; DeCaprio et al., 1989].

A-treated samples as compared to control samples (Fig. 5). These cell cycle analyses using synchronized K562 cells were repeated four times. In addition, it was found that the increase in the percentage of cells remaining at G_1 phase of cell cycle at 18–24 h of incubation of synchronized K562 cells was transient because there was no significant difference between control and activin A-treated samples at 72 h of incubation.

Activin A Causes Transient Hypophosphorylation of RB Protein

To investigate whether phosphorylation of RB protein is modulated following activin A treatment, we examined phosphorylation of RB protein in K562 cells during activin A-induced differentiation. Since the more highly phosphorylated forms of RB protein ($pp110^{RB}$) have slower mobilities upon SDS–PAGE than the less phosphorylated form ($p110^{RB}$), immunoblotting can be employed to gauge the phosphorylation status of RB protein (Fig. 6). In control K562 cells, only the phosphorylated RB protein was detected (Fig. 6, day 0). Between 9 and 24 h after induction with activin A, cells began to accumulate more underphosphorylated RB protein, as compared with the hyperphosphorylated RB forms in the same samples, as was correlated with the appearance of more rapidly migrating form in the SDS–PAGE (Fig. 6). In addition, the proportion of underphosphorylated RB protein was largely decreased after 2 or 3 days of treatment with activin A. These suggest that the inhibition of phosphorylation of RB protein caused by activin A is also a transient event. Besides, the amount of RB protein relative to total cellular proteins did not vary significantly between resting and induced cells.

In earlier studies, it was found that the addition of activin A to K562 cells for a period of 3–5 days induced these cells to become positive when stained with benzidine [Yu et al., 1987]. Based on immunocytochemical analysis using antihuman hemoglobin antibodies and S_1 nuclease analysis using specific globin DNAs as probes, it was also confirmed that activin A induced the accumulation of hemoglobins and globin RNA transcripts in K562 cells [Yu et al., 1991; Frigon et al., 1991, 1992]. In the present studies, the time course of the effect of activin A on induction of differentiation and RB dephosphorylation in K562 cells was examined. It was found that incubation of cells with activin A for 12–18 h was not sufficient to render the cells to become benzidine positive; incubation for 1–2 days started to induce hemoglobin accumulation, while incubation for greater than 3 days caused nearly complete induction and cells became hemoglobin-containing cells (Fig. 7). On the other hand, a large proportion of RB protein was found in the hypophosphorylated form by 9–24 h (Fig. 6), at which time K562 cells showed little morphological evidence of differentiation. These experiments demonstrate that RB dephosphorylation precedes and accompanies erythroid differentiation in K562 cells.

DISCUSSION

Activins, which are related to the TGF-β family, are broadly distributed and have actions on multiple tissues [Vale et al., 1990]. They play a

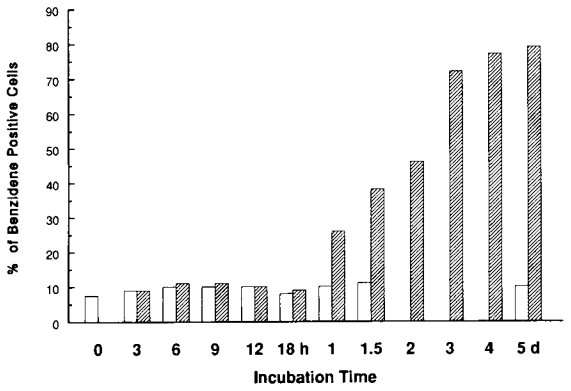

Fig. 7. Time course for the effect of activin A on the accumulation of hemoglobins and the proportion of dephosphorylated RB protein in K562 cells. K562 cells were incubated in the presence (▨) or in the absence (□) of 25 ng/ml of activin A. At different periods of time, aliquots were taken and examined for hemoglobinized cells with benzidine staining reaction as described under Materials and Methods and for RB protein analysis, *presented in* Fig. 6.

variety of types of roles as hormonal, paracrine, and autocrine regulators of cullular function and proliferation [Vale et al., 1990]. It has been shown that activin A increases the proliferative state of erythroid progenitors from both bone marrow and peripheral blood [Yu et al., 1989], only through the mediation of monocytes and T lymphocytes [Yu et al., 1989; Broxmeyer et al., 1988]. In addition, activin A has more direct effects on erythroid cells in driving the immature erythroid precursors to form hemoglobinized cells [Yu et al., 1987]. This was supported by the findings that activin A induced K562 and mouse Friend cells to become benzidine-positive cells [Yu et al., 1987; Eto et al., 1987] and enhanced the levels of globin mRNAs and hemoglobins in developing human erythroid cells [Frigon et al., 1992], and K562 [Shao et al., 1992]. In addition, the actions of activin A are presumably exerted through interaction with high affinity receptors in these cells [Frigon et al., 1992]. It will be of particular interest to find out how activin A modulates erythroid expression intracellularly and what mechanisms of signal transduction activin A uses after reacting with receptors on the erythroid cells.

In order to understand the mechanism of induction by activin A, the present study examines the relationship among cell cycle, the dephosphorylation of RB protein and the induction of differentiation by activin A in K562 cells. The proportion of cells in S phase, the pattern of PCNA staining and the rate of DNA synthesis have been measured and compared between K562 cells cultured in the presence and absence of activin A. By these assays, it was found that the addition of activin A transiently inhibits the entry of K562 cells into S phase of the cell cycle. This inhibition is detected in nonsynchronous cultures about 24 h after beginning of cultures. Cell cycle kinetics analysis using synchronized cells also confirms that in the presence of activin A, K562 cells progress normally through various phases of cell cycle except that there is prolongation of G_1 phase between 18 and 24 h of cultures. Furthermore, this transient arrest of K562 cells in G_1 phase is found to be associated with dephosphorylation of RB protein, which occurs between 9 and 24 h of incubation with activin A. This action of activin A inhibiting exit from G_1 phase into S phase in erythroid cells seems consistent with the observations that activin A inhibits cells growth in BALB/C 3T3 [Kojima and Ogata, 1989], gonadal cell lines [Gonzalez-Manchon and Vale, 1989], and rat thymocytes [Hedger et al., 1989]. In fact, when added in "competent" cells, activin A was suggested to halt progression of 3T3 cells through the cell cycle, as induced by platelet-poor plasma [Kojima and Ogata, 1989].

There are several means of assessing cellular proliferation. Antibodies to the PCNA are a convenient marker for cells committed to proliferation [Bravo and Macdonald-Bravo, 1987]. The staining patterns of the antibodies against PCNA, observed in several cell lines, were reported to begin with weak nuclear staining in late G_1; granular nucleoplasmic immunofluorescence then appears early in S phase, strengthens and becomes punctated. At the time of peak DNA synthesis, the staining moves to the nucleoli before becoming punctate again and subsequently diminishing [Celis and Celis, 1985]. The response patterns of PCNA in K562 cells to activin A were similarly observed, in the present studies, to indicate that there was a decrease in the proportion of cells in S phase after the treatment with activin A. Furthermore, other reports have shown that phosphorylation of the RB product is modulated during cell cycle and cellular differentiation [DeCaprio et al., 1989; Chen et al., 1989]. This RB gene product is a 105- to 110-kd nuclear phosphoprotein expressed ubiquitously and throughout the cell cycle. But, this protein exists in multiple phosphorylated forms: the highly phosphorylated forms ($pp110^{RB}$) are found during S and G_2/M,

whereas the underphosphorylated RB forms ($p110^{RB}$) are the primary species seen in G_1 and in the growth-arrested state [Chen et al., 1989; DeCaprio et al., 1989]. Furthermore, the underphosphorylated $p110^{RB}$ seems to be the form with suppressive activity on the cell cycle [DeCaprio et al., 1989]. TGF-β, which is related to activins, is also linked to suppression of RB protein phosphorylation in its target cells [Laiho et al., 1990]. In addition, induction of differentiation in several human leukemia cell lines by treatment with retinoic acid and other chemical factors leads to dephosphorylation of RB protein [Chen et al., 1989]. Our results indicate that RB dephosphorylation precedes and accompanies erythroid differentiation, thus constituting part of the early events for induction by activin A. The fact that all chemical agents studied in erythroleukemia cells to date [Marks et al., 1978] are similar to the protein effector, activin A, in the ability to cause a transient prolongation of G_1 phase suggests possible roles of cell cycle-related events in the mechanism of induction of differentiation. The precise mechanism for the prolongation of G_1 and its relationship to the process of differentiation, however, remains to be resolved. It can be speculated that prolongation of G_1 phase in cells may permit accumulation (or degradation) of regulatory factor(s) to a concentration that can affect an alteration in the pattern of nucleoproteins, thus leading to the progression of the differentiation program for gene transcription. While activin A does not appear to increase the amount of RB protein in K562 cells, it was reported that induction of three mouse cell lines, including erythroleukemia, muscle and pre-B cells into differentiation, leads to increased expression of RB mRNA [Coppola et al., 1990].

It is interesting to observe that the effects of activin A on inhibiting phosphorylation of RB proteins and the exit of K562 cells from G_1 phase into S phase are both transient events. Since the phosphorylation of RB protein is believed to be required for the progression of cells from G_1 to S phase, our observations of an increase in RB phosphorylation after the early stage of induction in K562 cells agree well with the data on cell cycle and restoration of DNA synthesis. It also agrees with evidence suggesting that at least one round of DNA synthesis in culture with inducers occurs prior to expression of differentiation function in induced Friend cells [Marks et al., 1978]. It is also interesting to note that the nuclear oncoproteins of c-myc, c-myb, and c-fos are involved in a complex pattern of changes in expression [Marks et al., 1987; Richon et al., 1989]. During the early period of induction, there is a pronounced decrease in c-myc and c-myb mRNA and an increase in c-fos mRNA. However, the initial low level of c-myc RNA is also transient; in fact, c-myc mRNA increases, returning to levels found in uninduced cells afterward. It is intriguing to observe that, in the present studies, the levels of RB protein phosphorylation also show a biphasic change, and the blockage of cells to enter S phase is also transient. These observations agree with the suggestion that after initial G_1 arrest, K562 cells progress normally from G_1 into S phase of cell cycle. Taken together, these data, all inducing agents for erythroleukemic cells including activin A, may exert effects on a transient suppression of proliferation-related proteins, such as c-myc in the nuclei, and expression of antiproliferative activities of proteins, such as RB hypophosphorylation leading to the temporary accumulation of cells in G_1 phase of the cell cycle.

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