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Transforming Growth Factor-Beta1 Induces Expression of Statin During Differentiation of Human Promonocytic Leukemia Cells

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Abstract Transforming growth factor-Beta (TGF-β) is a potent growth inhibitor for several cell types including epithelial cells and hematopoietic progenitor cells. Using a human promonocytic leukemia cell line, THP-1, we have shown that TGF-β inhibits their proliferation and promotes differentiation into cells exhibiting macrophage-like properties. Therefore, a key question is whether TGF-β influences the expression of genes associated with proliferation and/or growth inhibition. TGF-β treatment of THP-1 cells results in downregulation of expression of *c-myc*. We also observe that TGF-β1–treated cells express reduced levels of the cell cycle regulated histone, H2B, but express elevated levels of an RNA splicing variant of this histone that has been observed to be upregulated in growth inhibited and terminally differentiated cells. In addition, a nuclear protein associated with senescence and withdrawal of cells from the cell cycle, statin, is also expressed by THP-1 cells in response to TGF-β1 treatment. These results suggest that TGF-β1 is capable of inducing expression of specific nuclear proteins associated with differentiation and/or cessation of proliferation that may result in changes in nuclear organization and altered gene expression. Such changes in nuclear organization may be incompatible with continued proliferation of the cells.

Key words: cell cycle, growth inhibitory genes, growth factors, growth arrest factors

Negative growth regulation is as important as a mechanism in the regulation of embryonic development, differentiation, and tumor formation as is positive regulation. TGF-β is one of the most potent endogenous inhibitors of cell proliferation known [Moses et al., 1990; Roberts et al., 1985]. TGF-β1, a 25 kDa homodimeric polypeptide, is the prototype of a family of factors that are intimately involved in embryogenesis, regulation of cell proliferation, and differentiation and phenotype expression. Members of this family have been identified in species ranging from insects to humans, further reinforcing their critical role throughout the evolutionary spectrum [reviewed in Massagué, 1990; Ignotz, 1991]. Included in this family are at least five distinct forms of TGF-β, Müllerian inhibiting substance, activin and inhibin, bone morphogenetic proteins, Vg1 and VGR-1, and the decapentaplegic product of Drosophila.

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TGF-β is abundant in platelets and has been implicated in wound healing responses. Thus, TGF-β is chemotactic for monocytes, stimulates the synthesis of several extracellular matrix components (fibronectin, collagen, proteoglycans, and others) and their cell surface receptors in vitro [Ignotz, 1991], and induces fibrosis, angiogenesis, and formation of granulation tissue in vivo [Sporn et al., 1983; Roberts et al., 1986]. TGF-β1 can induce proliferation of fibroblasts as a secondary effect to the induction of *c-sis* [Leof et al., 1986] but inhibits proliferation of endothelial [Takehara et al., 1987] and other epithelial cells [Knabbe et al., 1987; Daniel et al., 1989], hematopoetic progenitors [Otha et al., 1987], and lymphocytes [Kehrl et al., 1986]. TGF-β can also suppress immune cell functions [Rook et al., 1986; Wahl et al., 1989].

Despite the broad spectrum of cells, tissues, and responses affected by TGF-β, details of the mechanisms by which TGF-β accomplishes this diverse array of activities are poorly understood. TGF-β binds to several cell surface receptors, the molecular natures of which are only now being elucidated [Massagué, 1992]. The cell surface proteoglycan, betaglycan, that binds TGF-β

with high affinity has recently been purified [Andres et al., 1991] and the cDNA cloned [Lopez-Casillas et al., 1991; Wang et al., 1991]. It has been suggested that betaglycan may bind TGF-β and control its access to or presentation to signaling receptors similar to the involvement of heparan sulfate in FGF binding to its receptor [Rapraeger et al., 1991]. The cDNA for the Type II receptor for TGF-β has also been cloned recently and shown to possess a serine-threonine kinase domain [Lin et al., 1992] similar to that of the activin receptor [Mathews and Vale, 1991]. However, substrates for this receptor kinase have not yet been identified. Complementation analysis of TGF-β receptor mutants for restoration of TGF-β responsiveness has suggested that some interaction between the Type II and Type I receptors may occur to facilitate cellular responses [Laiho et al., 1991].

At the biochemical and molecular levels, growth inhibition by TGF-β treatment results in downregulation of *c-myc* expression [Mulder and Brattain, 1988; Coffey et al., 1988] and inhibition of phosphorylation of pRb that normally occurs prior to entry into S-phase [Laiho et al., 1990]; it may affect G-protein mediated activities [Howe et al., 1989] and inhibits phosphorylation and activity of $p34^{cdc2}$ [Howe et al., 1991].

Several genes and proteins that appear to be associated with cessation of cell proliferation have recently been identified. A 57 kDa nuclear protein, statin, has been identified in senescent human fibroblasts, growth inhibited fibroblasts, and rat liver cells [Wang, 1985; Sester et al., 1990]. Statin has been localized to the nuclear periphery, possibly the nuclear envelope, but a function for statin has not been demonstrated. Complementary DNA clones for several other genes, growth arrest specific genes (gas 1, gas 2, gas 3) have been identified and reported [Schneider et al., 1988]. Expression of gas 1 and gas 2 mRNAs is induced by serum starvation of NIH 3T3 cells but not by *v-fos* or *v-src* transformed NIH 3T3 cells. Gas 2, with an apparent molecular weight of 36 kDa, appears to be a component of the microfilament network [Brancolini et al., 1992] while gas 3 is a component of myelin [Manfioletti et al., 1990]. Several other gene products that function in aspects of growth arrest are the tumor suppressor gene products pRb, p53, and DCC [Weinberg, 1991]. Loss of expression or function of these proteins often results in transformation and tumor formation.

The retinoblastoma susceptibility gene product, pRb, undergoes a change in phosphorylation late in G1 of the cell cycle. Treatment of epithelial cells in tissue culture with TGF-β1 inhibits proliferation and blocks the normal change in phosphorylation of pRb and presumably alters or prevents pRb function [Laiho et al., 1990]. In this report, we have begun to examine other proliferation inhibition associated factors. We observe that treatment of a human promonocytic leukemia cell line, THP-1, results in growth inhibition and terminal differentiation into macrophage-like cells. During this process, the cells downregulate expression of *c-myc* and the cell cycle histone H2B but begin to express an mRNA splicing variant of this histone and also express statin, a nuclear protein associated with withdrawal of cells from the cell cycle.

MATERIALS AND METHODS

The human promonocytic leukemia cell line THP-1 was obtained from the American Type Culture Collection. Cells were grown in a 5% CO_2 atmosphere in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS). Transforming growth factor-Beta1 (TGF-β1), isolated from porcine platelets, was purchased from R & D Systems, Inc. (Minneapolis, MN).

For Northern blot analysis, cells were treated with 200 pM TGF-β1 in RPMI-1640 containing 2% fetal bovine serum (FBS). After 3 days, cultures were split into cells that had become adherent to the culture plates and those that had remained in suspension. RNA was extracted with 4 M guanidine isothiocyanate and recovered by centrifugation through 5.7 M $CsCl_2$ according to Chirgwin et al. [1979]. Total cell RNA (25 μg per sample) was fractionated on 1% agarose gels containing 2.2 M formaldehyde, transferred to nylon membranes (Zetaprobe, Bio-Rad Laboratories), and hybridized to ^{32}P-labeled cDNA probes for *c-myc*, histone H2B, and the splicing variant H2B (GL105) [Collart et al., 1991]. Probes were labeled by the random-prime method using a kit from New England Nuclear, DuPont, Corp.

For immunofluorescent detection of statin, THP-1 cells were placed into Lab-Tek tissue culture slide chambers and treated with or without TGF-β1 in RPMI-1640 containing 2% FBS. After three days, the unattached cells were collected and spotted onto microscope slides by

Cytospin centrifugation. The adherent cells were washed once in phosphate-buffered saline (PBS). The cells were incubated for 10 min at room temperature in PBS containing 0.5% Triton X-100 and then fixed with methanol:acetone (1:1) at −20°C for 30 min. The slides were rinsed once in PBS followed by incubation in PBS containing 0.5% bovine serum albumin (BSA) for 1 h at 4°C. Samples were next incubated with anti-statin antibody (S-44 monoclonal antibody was generously provided by Dr. Eugenia Wang, McGill University) diluted in PBS with 0.5% BSA for 2 h at room temperature. Slides were washed three times followed by a 1 h incubation with a FITC-labeled secondary antibody and counterstained with DAPI. Cells were examined under fluorescence optics.

Western blotting for statin was accomplished by harvesting cells following various treatments, adjusting each sample to contain 1–2 × 10^6 cells and pelleting cells at 13,000*g* for 2 min. Cell pellets were directly solubilized in 200 μL SDS-PAGE sample buffer [Laemmli, 1970], sonicated to shear the DNA, boiled for 2 min, and electrophoresed into 9% polyacrylamide gels. Proteins were transferred to Immobilon P membranes (Millipore) and incubated in blocking buffer (5% dried milk, 0.5% Tween 20, 150 mM NaCl, 25 mM Tris-HCl, pH 7.4), followed by incubation overnight with the anti-statin antibody diluted in the blocking buffer. The blots were washed and statin visualized by chemiluminescent detection using an Amersham ECL kit.

Growth responses of THP-1 cells were determined by ^{3}H-thymidine labeling of various cultures for 12 h with 0.5 μCi/ml of ^{3}H-methylthymidine (6.7 Ci/mmol; DuPont) followed by harvesting of the cells and spotting onto microscope slides via Cytospin centrifugation. Slides were dipped into Kodak NTB nuclear track emulsion for autoradiographic analysis. After development of the slides, cells were counted and the percent of labeled cells determined.

RESULTS AND DISCUSSION

Rapid Decline in Labeling Index Accompanies TGF-β1 Induced Cell Adhesion

We have previously reported that TGF-β1 treatment of THP-1 promonocytic leukemia cells are growth inhibited and that a portion of the cells differentiate into cells expressing a macrophage phenotype [Bombara and Ignotz, 1992]. One aspect of the differentiation response is an elevation in the expression of certain integrins,

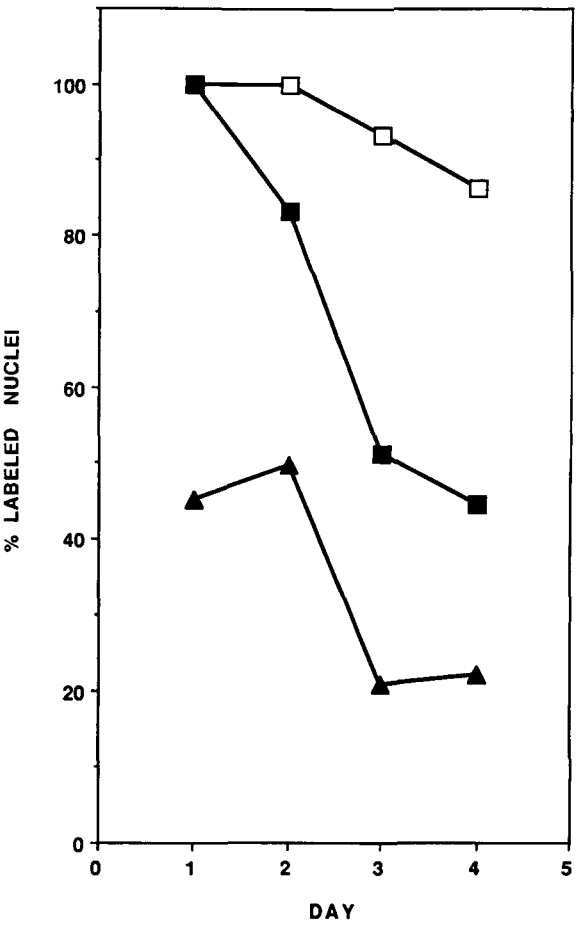

Fig. 1. TGF-β inhibits replication of THP-1 cells. THP-1 cells were transferred to Lab-Tek microscope chambers in RPMI-1640 containing 2.5% fetal bovine serum (FBS). Some cultures were supplemented with 200 pM TGF-β1. At 24 h intervals, cultures were given 0.5 μCi ^{3}H-thymidine and incubated an additional 12 h. Cells were then harvested, washed once in PBS, and spotted onto microscope slides. TGF-β treated cells were split into two groups, those that had differentiated and attached to the culture slides and those that had remained in suspension. Slides were processed for autoradiography. The percent of labeled nuclei was determined by counting at least 500 cells for each point. Untreated cells, □; TGF-β1 treated (suspension) ■; TGF-β1 treated (adherent and differentiated) ▲.

in particular the fibronectin receptor (α5β1) and enhanced cell adhesion. To determine whether there is a difference in the growth of the THP-1 cells that adhere and differentiate as compared to those that do not attach to the culture surface, we subjected cells to ^{3}H-thymidine labeling and autoradiography. After the labeling period, the TGF-β1 treated cultures were divided into cells that had attached to the culture slides, an indication of differentiation, and those cells that remained unattached. Autoradiographic analysis indicates that the cells that become attached

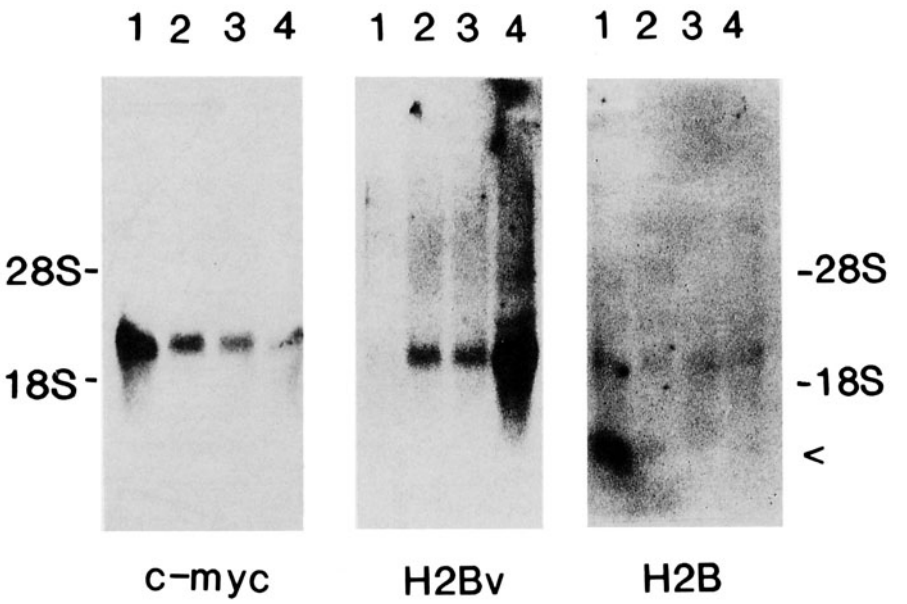

Fig. 2. TGF-β1 downregulates expression of *c-myc* and histone H2B mRNAs. THP-1 cells were transferred to RPMI-1640 containing 2% FBS. Some cultures received either 200 pM TGF-β1 of 50 ng/ml phorbol myristate acetate (PMA). After 4 days, the cells were harvested, RNA extracted, and subjected to Northern blot analysis for *c-myc*, histone H2B, or the histone mRNA splicing variant H2Bv (GL105). The H2B blot is the identical blot as the H2Bv blot after being stripped and reprobed which accounts for the higher background. The arrow indicates the position of the H2B mRNA at about 0.9 kb. The H2Bv mRNA is approximately 2.3 kb while the *c-myc* mRNA was about 2.5 kb. **Lanes 1:** Untreated cells. **Lanes 2:** TGF-β1 treated (suspension) cells. **Lanes 3:** TGF-β1 treated adherent cells (differentiated). **Lanes 4:** PMA treated cells.

have a lower labeling index than the unattached cells (Fig. 1). One day after addition of TGF-β1 essentially 100% of the unattached cells incorporated ^{3}H-thymidine and showed heavily labeled nuclei, whereas only about 45% of the attached cells contained labeled nuclei. The percent of labeled nuclei declined for both cell populations throughout the course of the experiment. Labeling of untreated cultures remained elevated (greater that 80%) throughout the 4 days of the experiment. The slight decline in labeling of the untreated cells is likely due to depletion of nutrients from the medium as the cultures did not receive fresh medium during the experiment. Thus, it appears that THP-1 cells that become attached to the culture slides following TGF-β1 treatment withdraw from the cell cycle at a faster rate than cells that remain in suspension.

Regulation of Proliferation Related Genes by TGF-β

Examination of steady-state mRNA levels by Northern blot analysis shows that *c-myc* expression is downregulated in THP-1 cells during growth inhibition mediated by TGF-β1. Both the adherent population of cells and THP-1 cells that remain unattached to the culture plates express much reduced levels of *c-myc* mRNA as compared to untreated cultures (Fig. 2). Downregulation of *c-myc* mRNA expression has also been reported for several epithelial cell types [Mulder and Brattain, 1988; Coffey et al., 1988; Moses et al., 1990]. Phorbol myristate acetate (PMA) also induces differentiation of THP-1 cells and reduces *c-myc* mRNA levels very dramatically.

Histone H2B mRNA is expressed by cells in S-phase [Stein et al., 1984]. Actively growing cultures of THP-1 cells express high levels of the 0.9 kb mRNA for H2B. Messenger RNA for histone H2B was undetectable in both populations (adherent and suspension) of TGF-β1 treated as well as PMA treated promonocytes. In contrast, an mRNA splicing variant of histone H2B mRNA (GL105) of 2.3 kb identified in and expressed during terminal differentiation of HL-60 cells [Collart et al., 1991] becomes expressed in both TGF-β1 and PMA induced THP-1 cells. The appearance of this non-replication dependent histone has also been observed in HeLa cells where it appears to be constitutively expressed at low levels throughout the cell cycle [Collart et al., 1992]. Thus, two mRNAs associated with an active proliferative state ap-

pear to be inhibited by TGF-β1 while a third mRNA associated with terminal differentiation of HL-60 cells is elevated following TGF-β1 treatment of THP-1 promonocytic leukemia cells.

TGF-β1 Induces Statin Expression

The expression of the nuclear protein, statin, which was originally identified in senescent human fibroblasts and growth inhibited fibroblasts [Wang, 1985] was also examined. Detection of statin was accomplished by immunofluorescent staining of cells and by Western blotting of whole cell extracts. Figure 3 shows a typical immunofluorescent analysis for statin. Untreated, actively proliferating THP-1 cells do not contain detectable levels of statin nor do TGF-β1 treated cells that have not attached to the culture surface. However, THP-1 cells that become attached to the culture surface in response to TGF-β1 treatment do express easily detectable statin. Statin remains detectable in this population of cells even after removal of TGF-β1 and refeeding of the cultures with fresh medium containing 5% fetal bovine serum. Statin could be detected in TGF-β1 treated adherent cells within 24 h (not shown); however, the number of cells in the population that had attached to the culture slides in one day was quite small. Thus, the nuclear appearance of statin correlates with the terminal differentiation of these cells.

To rule out the possibility that statin was present in all populations of THP-1 cells but in a confirmation or subcellular compartment not detectable by immunofluorescence until after adhesion, Western blot detection assays were performed. As before, THP-1 cells were treated with or without TGF-β1 to initiate the differentiation program. Alternatively, some cultures received PMA to induce differentiation. After three days, cells were harvested, extracted, and subjected to Western blot analysis. Figure 4 shows two examples from separate experiments. Statin was not detectable in extracts from untreated THP-1 cells. Cells that had been induced to differentiate with phorbol esters express high levels of two immunoreactive polypeptides of approximately 55 kDa and 60 kDa. The TGF-β1 treated cells that had attached to the culture surface also expressed these two polypeptides but to a lesser extent than the phorbol ester treated cells. In some assays, the 60 kDa species was more prominent than the 55 kDa species. The cells that did not attach following TGF-β1

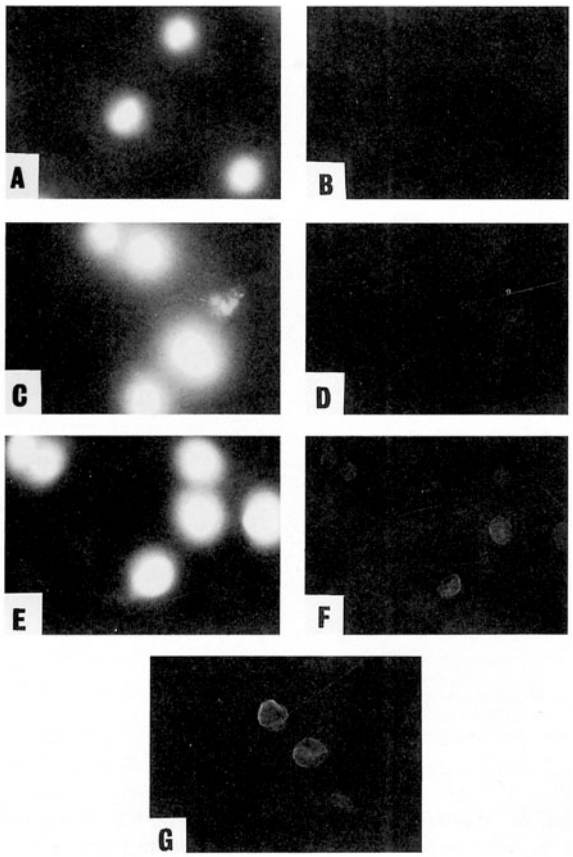

Fig. 3. TGF-β1 induces statin expression in THP-1 cells. THP-1 cells were treated with or without 200 pM TGF-β1 in RPMI-1640 supplemented with 2% FBS. After 3 days, the cells were harvested and subjected to immunoflourescent staining for statin with the S-44 monoclonal antibody. **A,C,E:** DAPI staining to show positions of cells. **B,D,F:** Immunofluorescent images of fields A, C, and E, respectively. A,B: Untreated cells. C,D: TGF-β1 treated (suspension) cells. E,F: TGF-β1 treated adherent (differentiated) cells. **G:** TGF-β1 treated adherent cells 2 days after removal of TGF-β1 and refeeding with medium containing 5% FBS. (×600.)

treatment expressed a very low level of the 60 kDa polypeptide that was detectable only after prolonged exposures. This low level of statin may be from contamination of the non-adherent cell population with some of the adherent cells that may have detached during harvesting of the cultures. It appears from these observations that only cells that attached to the culture surface are capable of expressing readily detectable amounts of both immunoreactive forms of statin.

Statin was originally described as a 57 kDa polypeptide; however, more recent studies have shown that two immunoreactive species of approximately 57 kDa and 53 kDa can be detected in rat hepatocytes [Sandig et al., 1991] similar in

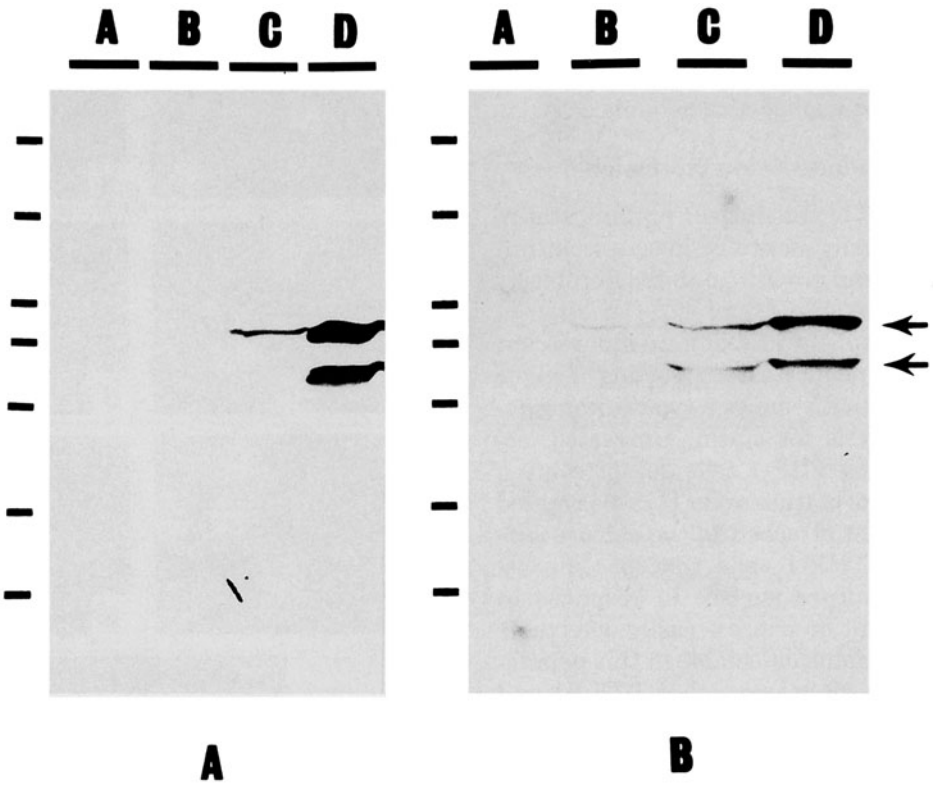

Fig. 4. Western blot detection of statin in TGF-β1 treated cells. THP-1 cells were treated as described for Fig. 2. After 3 days of treatment with either TGF-β1 or PMA, cells were collected, samples adjusted to contain equal numbers of cells (2×10^6), extracted, and subjected to Western blot analysis for the presence of statin as described under Materials and Methods. **Lanes A:** Untreated cells. **Lanes B:** TGF-β1 treated (suspension) cells. **Lanes C:** TGF-β1 treated adherent cells. **Lanes D:** PMA-treated cells. Arrows indicate the positions of 2 immunoreactive polypeptides detected with the anti-statin antibody. Prestained molecular weight markers from Sigma Chemical Co. are indicated to the left as 205 kD, 115 kD, 84 kD, 58 kD, 48.5 kD, 36.5 kD, and 26.6 kD.

molecular weight to the two immunoreactive polypeptides reported here. Sandig et al. have also suggested that the 57 kDa species may arise as the result of phosphorylation of the 53 kDa isoform. It is interesting to speculate that changes in phosphorylation of statin may be regulated in a cell cycle dependent manner although this remains to be demonstrated.

CONCLUSIONS

We have reported that TGF-β treatment of THP-1 promonocytes induces these cells to differentiate into macrophage-like cells [Bombara and Ignotz, 1992]. Differentiation of THP-1 cells is accompanied by cessation of proliferation and reduced expression of *c-myc* and the cell cycle regulated histone H2B. At the same time, a splicing variant of H2B mRNA (GL105) that is not cell cycle regulated is expressed and a nuclear protein, statin, associated with senescence and withdrawal of cells from the cell cycle appears in the differentiated cells. Thus, a reciprocal relationship exists between the expression of *c-myc* and H2B mRNAs and the expression of statin and the H2B variant mRNA. This change in gene expression also correlates with the decline in cell proliferation and enhanced cell adhesion.

Cell adhesion may provide a necessary three-dimensional architecture within the cell which is permissible or required for expression of statin and possibly other genes associated with terminal differentiation or growth arrest of THP-1 cells. Werb et al. [1989] have suggested the possibility of signal transduction through the fibronectin receptor and regulation of expression of collagenase and stromelysin genes. Giancotti and Ruoslahti [1990] have demonstrated that elevated expression of fibronectin receptors by Chinese hamster ovary cells can partially suppress their transformed properties. TGF-β1 elevates expression of the fibronectin receptor in

many cell types including THP-1 cells. Reorganization of cellular architecture through elevated integrin expression, altered cytoskeletal arrangement, and expression of statin and other nuclear components in response to TGF-β1 treatment may render cells incapable of continued proliferation and allow terminal differentiation of the cells. Further studies will be required to fully address this possibility.

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