Rapid Disappearance of Statin, a Nonproliferating and Senescent Cell-specific Protein, upon Reentering the Process of Cell Cycling

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ABSTRACT   Statin, a 57,000-D protein characteristically found in nonreplicating cells, was identified by a monoclonal antibody produced by hybridomas established from mice injected with extracts of in vitro aged human fibroblasts (Wang, E., 1985, *J. Cell Biol.,* 100:545–551). Fluorescence staining with the antibody shows that the expression of statin disappears upon reinitiation of the process for cell replication. The rapid de-expression is observed in fibroblasts involved in the in vitro wound-healing process, as well as in cells that have been subcultured after trypsinization and replated from a confluent culture. Kinetic analysis shows that 50% of the cell population lose their statin expression at 12 h after replating, before the actual events of mitosis. Immunogold labeling with highly purified antibodies localizes the protein at the nuclear envelope in nonreplicating cells, but not in their replicating counterparts. Immunoblotting analysis confirms the disappearance of statin in cells that have reentered the cycling process. Using the technique of flow cytometry to examine the large number of nonreplicating fibroblasts in confluent cultures, we have found that statin is mostly expressed in those cells showing the least amount of DNA content, whose growth is blocked at the G_0/G_1 stage of the cell cycle. This close correlation is rapidly altered once the cells are released from the confluent state. These results suggest that the expression of statin may be regulated by a fine mechanism controlling the transition from the nonreplicating to the replicating state, and that the protein is structurally associated with the nuclear envelope.

Studies to distinguish proliferating cells from their nonproliferating counterparts were limited to a description of phenotypic characteristics until the recent development of powerful probes, such as cDNA clones and monoclonal antibodies. Lately, a growing number of new proteins have been identified in cells at particular stages of development or differentiation by the use of unique monoclonal antibodies (3, 7, 8). With a similar approach, several novel proteins found only in proliferating or in nonproliferating cells have been localized either on the cell surface or in the nucleus (2, 6, 11).

We reported earlier that a protein of 57,000 D is present in the nucleus of nonproliferating cells from several lines of in vitro aged human fibroblasts, but not in their proliferating counterparts (11). However, the presence of this nuclear antigen was not restricted to senescent fibroblasts; young fibroblasts can be induced to express it upon the arrest of proliferation such as culturing to confluency. The nuclear presence of this protein also is characteristic of many tissue sites

composed of nonproliferating cells (12). The absence of this protein is noted in tissues, such as epithelium of the gastrointestinal tract and kidney and the basal layer of the epidermis, where cells are replicating.

We now report that the expression of statin can be rapidly turned off once the restriction of cell replication at G_0/G_1 is removed. The deactivation of statin expression is heterogeneous among different cells but, in general, occurs before the actual event of mitosis. Therefore, the expression of statin seems to be incompatible with the mechanism that promotes cell cycling events.

MATERIALS AND METHODS
Cultures of Human Fibroblasts

Two cell strains of human fibroblasts were obtained from the Human Genetic Cell Repository (Camden, NJ). These cell strains were initiated by

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[1] *Abbreviation used in this paper:* PDLs, population doubling levels.

culturing skin biopsies derived from two different donors. Reference number 0011 was designated for the fibroblast cultures derived from a donor of 8 fetal wk, and 3529 for cultures derived from another donor 66-yr-old. The complete in vitro life spans of these two cell strains have been established in our laboratory and reported (11). Cultures of varied life spans as represented by population doubling levels (PDLs)[1] were acquired by serial passage as described (4, 11). Eagle's minimum essential medium supplemented with 10% fetal calf serum and 1% nonessential amino acid was used for growth and maintenance of cultures at 37°C with humidified air and 5% CO_2.

Antibodies

A mouse hybridoma clone S-30 was identified to produce an antibody that detects a protein of 57,000 D present in abundance only in nonproliferating cells. This hybridoma clone was established by injecting a mouse with insoluble cellular materials derived from the senescent fibroblasts of the 3529 cell strain. A detailed description of the antigenic activity was reported previously (11). The quality of monoclonal antibody was improved by generating a serum-free supernatant of clone S-30 cultures. After precipitation with ammonium sulfate, the secreted immunoglobulin (IgG) was concentrated 100-fold and used as the stock solution for various immunoassays of the present study.

Reinitiation of Proliferating Activity

IN VITRO WOUNDING EXPERIMENTS: Young fibroblasts with high replicating activity at an early stage of their life span were grown into confluency to halt the activity of proliferation. Growing cultures of both 3529 and 0011 cell strains at PDLs less than 15 were used. Cultures were initiated at a density of 3,550 cells/cm², and confluency was reached as cells grew to ten times this density ~7 d after initial plating. These cultures were kept in this confluent state for another 3 d before use. The absence of proliferating activity was monitored by time-lapse cinematography showing little mitotic activity, and by quantitation of DNA content by flow cytometry (see below). Denudation of an area of ~2 × 4 cm² was introduced into these confluent cultures by sterile razor blades.

TRYPSINIZATION AND REPLATING: The same type of confluent culture as described above was used for reinitiation of cell replication by subculturing at lower growth density. Cells were dislodged from the confluent monolayer by treatment with trypsin at 0.5%, and replated into the growth medium at a density of 1,770 cells/cm². Mitosis was reinitiated and examined by kinetic studies at various time intervals after the seeding of the fibroblasts.

Time-Lapse Cinematography

The mitotic frequency in cultures of nonreplicating cells, as well as fibroblasts with reinitiated replicating activity, was investigated by cinematographic studies using a time-lapse video recording device. Cultures were filmed directly on a Nikon Diaphot inverted microscope with 6× phase optics (Nikon Inc., Garden City, NY). The temperature of the environment immediately surrounding the culture was maintained at 37°C by a Sage air incubator (Sage Instruments Div., Cambridge, MA). Cell locomotion as well as the actual events of cell division were recorded on Beta-max videotape by a National Electrostatics Corp. (Middleton, WI) time-lapse videotape recorder. The recorded tape was then analyzed on a regular Audiotronics monitor (Audiotronic, Inc., North Hollywood, CA). Experiments for cinematographic studies were designed in parallel to those cell specimens used for various immunoassays. These parallel experiments allow us to compare the expression of statin in various cell cultures with mitotic frequency.

Immunofluorescence and Immunoelectron Microscopy

Presence of statin was identified at the light microscopic level by labeling the formaldehyde-fixed and acetone-extracted cell specimens with the mouse monoclonal antibody, S-30. Fluoresceinated secondary antibody, goat anti-mouse immunoglobulin, was used to localize the binding sites of S-30 IgGs. The details of the labeling procedure have been reported (11). To identify the ultrastructural location of S-30 binding sites, we have used the technique of immunogold labeling (5). In brief, cell specimens were prepared by formaldehyde fixation, followed by extraction with 0.5% Triton in phosphate-buffered saline (PBS), at pH 7.2. These specimens were then labeled with the antibody to statin, followed by labeling with protein A conjugated with 5-nm colloidal gold particles as described (14). Afterwards, the samples were processed for routine electron microscopy (13).

Immunoblotting Analysis

Fibroblasts of confluent cultures of the 0011 strain as described above were processed to obtain cellular materials of nonreplicating cells. Cells were harvested in PBS and pelleted by low speed centrifugation (1,000 rpm for 10 min). The cell pellets were then homogenized and lysed with a dounce homogenizer in lysis buffer containing 20 mM Tris, 10 mM KCl, 5 mM $MgCl_2$, 0.1 mM phenylmethylsulfonyl fluoride, 4 mM EDTA, and 2 mM EGTA. The cell lysate was treated with deoxyribonuclease I (DNase I) for 30 min at 4°C to remove the DNA, followed by tip-sonication to dissociate the insoluble cytoskeletal materials. Cellular proteins were then separated electrophoretically on 10% polyacrylamide gels. Replicating fibroblasts of a sparse culture, with only 2% of cells showing staining activity for statin, were processed in the same fashion for gel electrophoresis. The same amount of protein specimens for both nonreplicating and replicating fibroblasts were used for gel electrophoresis. Proteins were then transferred to nitrocellulose paper in a Hoefer Trans-blot chamber (Hoefer Scientific Instruments, San Francisco, CA), as described (9). Normal goat serum at 0.05 μg/ml was used to remove nonspecific activity before incubation overnight with the primary S-30 antibody at 1:500 dilution in PBS containing 0.03% Tween-20. The second antibody was peroxidase-conjugated goat anti-mouse IgG (HyClone Laboratories, Logan, UT) at 0.04 μg/ml for incubation at room temperature for 1 h. The detailed procedure has been reported (10).

Simultaneous Examination of Cell Cycle Analysis and Statin Expression

The newly developed technique of flow cytometry was used to examine statin expression via antibody staining and the distribution of cell cycle phases by DNA content (1). The detailed procedure of simultaneous measurements of DNA quantity and the fluorescent intensity for antibody labeling of cellular antigens was recently described (15). In brief, the DNA content was measured by staining intensity with propidium iodide, which can be detected by a band of 600–650-nm wavelength for red fluorescence. The statin expression is measured by the band of 515–575-nm wavelength to detect the green fluorescence of fluorescein isothiocyanate–labeled secondary antibody. A third parameter, red pulse width determined by nuclear diameter, was used to discriminate single cells from cell aggregates. Cells were initially prepared for indirect immunofluorescence microscopy, and after treatment with RNase were counterstained with propidium iodide for DNA content. The measurements of statin expression and cell cycle distribution were performed on the Cytofluorograf FC200 (Ortho Diagnostic Instruments, Westwood, MA) with the generous assistance of the laboratory of Dr. Andreeff of Memorial Sloan-Kettering Cancer Center, New York.

RESULTS

In vitro wounding was the first approach to reinitiate cell cycling activity in confluent cultures of nonreplicating fibroblasts. In general, a time span of 6 h was needed for the culture to recover from the mechanical shock incurred during the wounding. Subsequently, cells migrated into the wounded area by forming fan-shaped leading edges. Fig. 1A shows locomotion activity of migrating fibroblasts of the 0011 cell strain at 12 h after the initiation of the wound. A layer consisting of cells that have moved away from the dense region of the confluent monolayer sheet has already formed at the edge of the wound. Staining of this cell specimen with S-30 antibody shows that these cells are negative for the presence of statin, whereas those cells remaining in the confluent region show positive staining for the antibody. While most of these cells display rigorous motility and have broken away from contact with the neighboring cells, mitotic activity is not yet observed. Results of time-lapse cinematographic studies show that a high frequency of mitosis was not noticed until 24 h after the establishment of the wound. Figs. 1, C and D demonstrates a similar phenomenon of the loss of statin in the locomoting cells of the 3529 cell strain.

A second approach to reinitiate replicating activity involved passing the confluent cultures to lower cell density. The
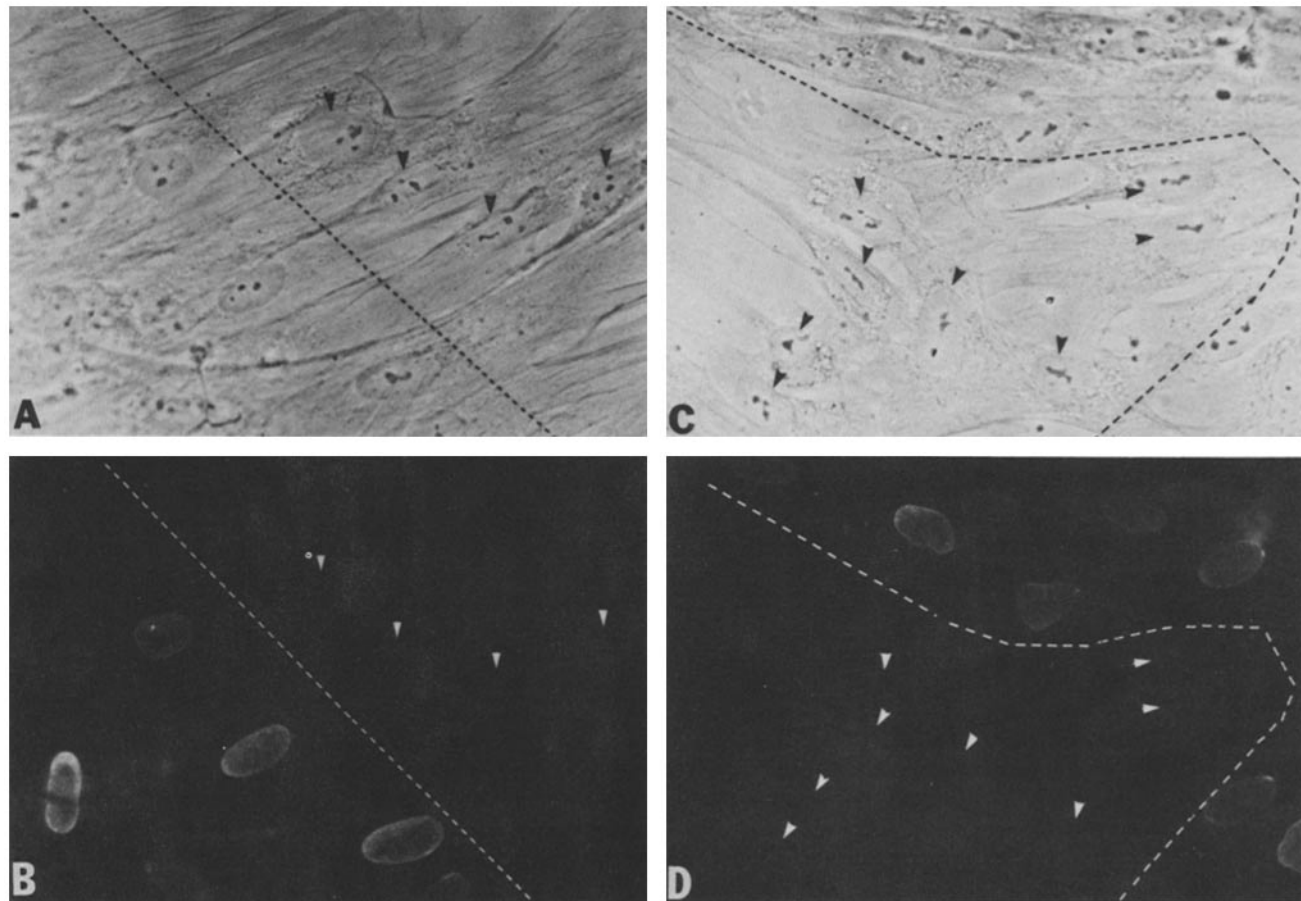
FIGURE 1 The disappearance of statin-associated nuclear staining in cells (arrowheads) that have migrated from dense cultures to wound regions. A denuded area was introduced by mechanical wounding at the area indicated by the dotted lines. 12 h after the establishment of the wound, fibroblasts had already migrated into the open area and lost their nuclear staining activity. However, the statin staining activity was still detectable in the nuclei of those cells that remained in the dense area, whose growth continued to be arrested by contact with the neighboring cells. (A and B) Cultures of human fibroblasts (0011 cell strain) derived from a donor of 8 fetal wk were maintained at high density (35,550 cells/cm^2) at early stages of life with PDLs of 12. (C and D) Cultures of human fibroblasts (3529 cell strain) derived from a donor 66-yr-old were maintained at the same density with PDLs of 14. Identical cell specimens were visualized by phase contrast (A and C) and fluorescence (B and D) optics to show relative positioning of each cell in the cultures. × 450.

mitogenic effect via trypsinization and replating into a less crowded environment allows the cells to reenter the process of cycling. Figs. 2, A and B demonstrates that most cells still exhibit the statin-positive phenotype during the initial 4–6 h after subculturing. Not until 12 h after replating is the statin-negative phenotype observed in recognizable frequency (Figs. 2, C and D). This heterogeneous expression of statin continues in culture for another 12 h, with an increasing number of cells showing the negative phenotype (Figs. 2, E and F). At 24 h after replating, close to 90% of the cell monolayer shows negative staining activity for antibody to statin (Figs. 2, G and H). Time-lapse cinematographic studies show that the culture at this point begins to show noticeable mitotic activity.

Quantitation of the statin loss in post-trypsinized cultures was made by direct examination of fluorescent staining activity, counting ~500 cells of the specimens; the results are shown in Fig. 3. For the initial 4 h after release from the contact arrest of growth, >80% of the cells still retain statin expression. At 12 h, the loss of statin expression reaches to 50% of the cell cultures. At 24 h, the loss of nuclear staining reaches its peak as ~80% of the fibroblasts show negative antigenic reaction to statin.

Immunogold labeling with high-titred antibody showed that statin is primarily located at the nuclear envelope in nonproliferating fibroblasts of confluent cultures (Fig. 4 a). Statin is distributed along the entire nuclear envelope, as indicated by the positioning of gold particles. A few gold particles were seen associated with the nucleoplasm as well as adjacent intermediate filaments on the cytoplasmic side. This is possibly due to nonspecific background staining, since the same type of gold labeling was observed in control specimens when the primary antibody was replaced with a monoclonal antibody to neurofilaments (which are not found in human fibroblasts). As shown in Fig. 4b, the nuclear envelope-specific labeling was not observed when anti–statin IgGs were incubated with replicating fibroblasts, when the restriction of growth by confluency was removed.

To determine that the absence of nuclear staining seen by fluorescence microscopy is indeed the absence of statin, we have subjected the insoluble protein extracts from the non-replicating fibroblasts of the confluent cultures to gel electrophoresis, followed by transfer to nitrocellulose paper. When the paper was incubated with S-30 monoclonal antibody, the 57,000-D protein and the accompanying high molecular
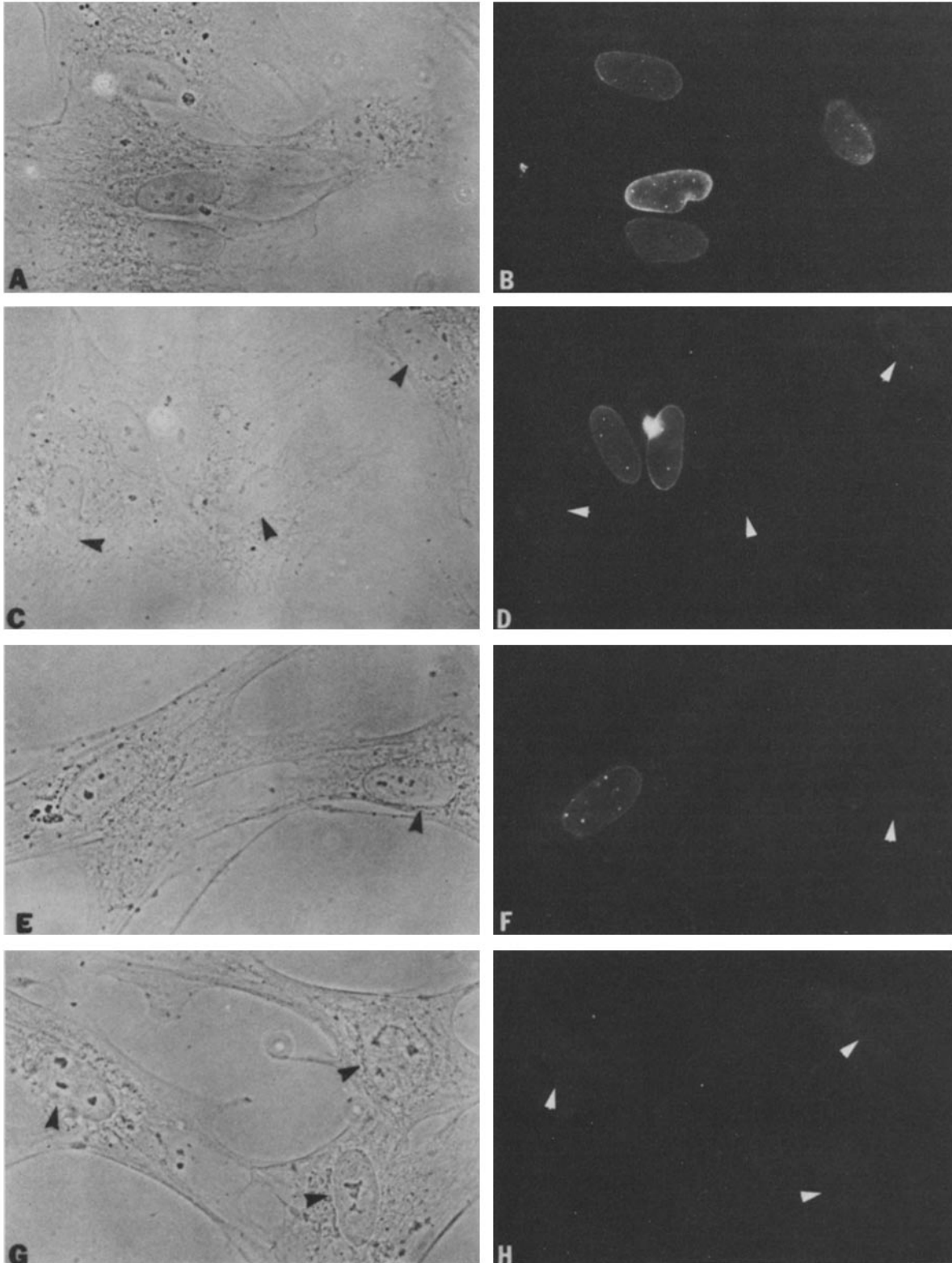
FIGURE 2 Rapid disappearance of statin expression in cells whose replicating activity was reinitiated by trypsinization and replating as described in Materials and Methods. Kinetic studies using immunofluorescence microscopy showed the heterogeneous loss of statin in cultures at 4 h (A and B), 12 h (C and D), 16 h (E and F), and 24 h (G and H) after trypsinization and replating. During the initial hours, most cells remained statin-positive (A and B). More than 50% of cells displayed the negative phenotype at 12 h after subculturing (C and D). By 24 h, >85% of cells lost their statin expression (G and H). Phase contrast (A, C, E, and G) and fluorescence (B, D, F, and H) images of the same cell specimens are presented to show the statin-negative (arrowheads) and the statin-positive cells. Confluent cultures of 0011 cell strain as described for Fig. 1, A and B PDLs of 12 were used for trypsinization and subculturing. × 550.
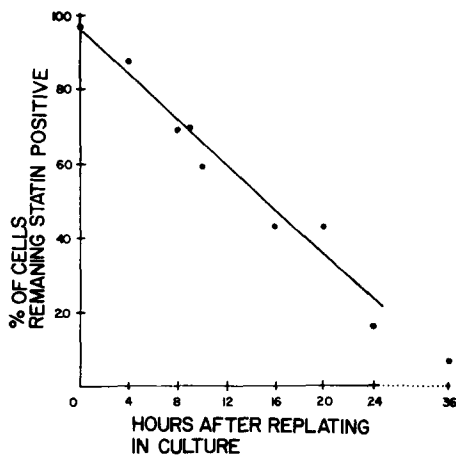
FIGURE 3 Kinetic studies of statin disappearance in cells after reinitiation of cell cycling activity by trypsinization and replating as described for Fig. 2. Quantitation was performed by direct visualization of the nuclear staining activity for statin in 500 cells at various time points after replating as indicated by the closed circles. As indicated here, ~50% of the cell culture had lost their statin expression after the removal of replication-restriction by subculturing from the high density of 35,550 cells/cm² to a low density of 1,770 cells/cm².

weight doublets were recognized (Fig. 5, lane B). In contrast, the antibody did not react with the protein extracts derived from the replicating fibroblasts of the same specimens used for the experiments of Fig. 2, G and H (Fig. 5, lane C).

Fig. 6 demonstrates the cell cycle distribution and intensity of statin expression in nonreplicating fibroblasts of confluent cultures, and in the replicating fibroblasts that have been released from the restraint of density arrest by subculturing. As shown in A, the majority of cells among the nonreplicating fibroblasts, showing positive staining for statin, are the same group of cells whose growth is arrested at the G_0/G_1 phase of the cell cycle. This close correlation was lost rapidly when the cells were released from the confluent state. The number of cells exhibiting positive staining reaction, as well as the fluorescent intensity of those positive cells, are significantly reduced (Fig. 6B). By the analysis of DNA content, fibroblasts are distributed in all stages of the cell cycle, including G_0/G_1, S, and G_2 phases. Simultaneous examination of the activity of antibody staining shows that regardless of which stage of the cell cycle the cells are at, the majority of these replicating fibroblasts of post-trypsinized cultures contain very little statin expression.

DISCUSSION

Statin, a protein of 57,000 D, was initially described to be present only in nonreplicating and senescent fibroblasts (11). Here, I report that the expression of statin in young fibroblasts of confluent culture can disappear rapidly once the cells are released from the environment of density-dependent arrest of growth. In cells reentering the cycling process, the disappearance of a statin-positive phenotype usually precedes the actual mitosis. The timing for the loss of statin is heterogeneous among different cells. Our preliminary results of serum-starved nonreplicating cells show that statin is present in them too, and upon restoration of serum to the normal growth strength (10%), the expression of statin disappears in the same time scale as described for wounded and post-trypsinized
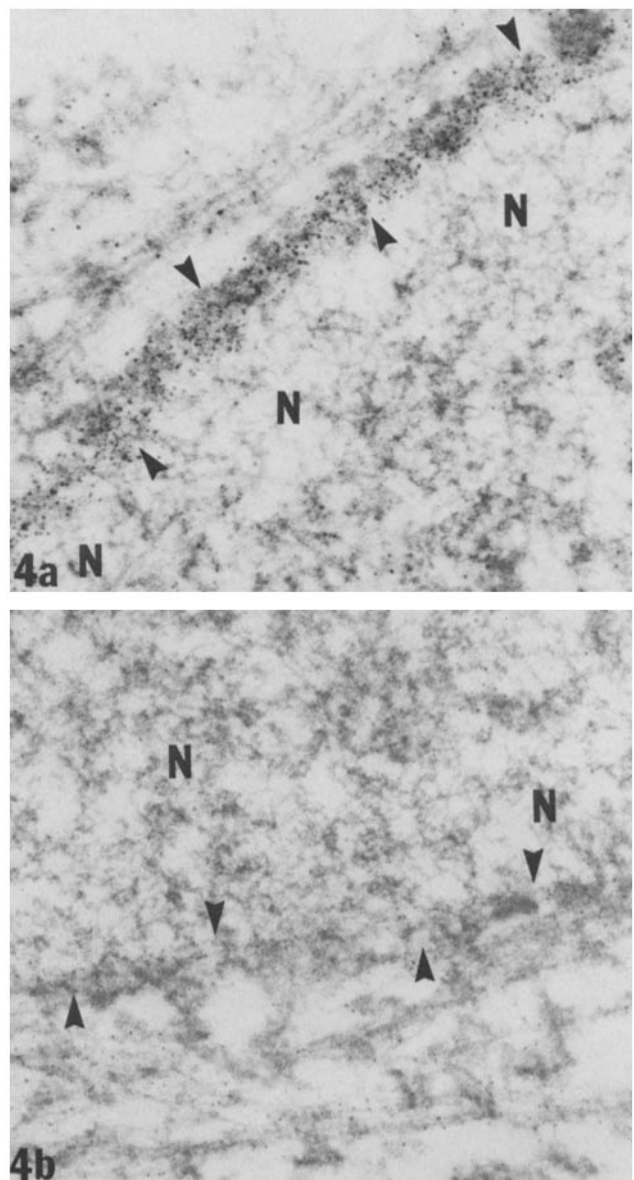

FIGURE 4 Localization of statin at the region of the nuclear envelope in nonproliferating fibroblasts of confluent cultures of 0011 cell strain (a). The absence of statin, as seen by the lack of cell-bound gold particles, was found in cells whose proliferating activity was reinitiated (b). The exact procedure for the immunogold labeling technique was described in Materials and Methods. As shown in a, most of the gold particles, reflecting the locations of statin, are localized at the nuclear envelope region as indicated by the arrowheads. A small proportion of gold particles is found in the nuclear matrix region (N). The association of gold particles with the intermediate filaments on the cytoplasmic side is regarded as nonspecific, as the same degree of binding was observed with monoclonal antibody to neurofilaments (data not shown). The absence of statin, as reflected by the lack of gold particles at the nuclear envelope region, is apparent in the replicating fibroblasts (b). Again, a small number of gold particles associated with the cytoskeleton is observed in this specimen (b). × 100,000.

cultures (data not shown).

Analysis by flow cytometry shows that those nonreplicating cells showing high statin expression in confluent cultures are indeed at the G_0/G_1 stage of the cell cycle. Once cell replication is reinitiated, the rapid disappearance of statin is associ-

ated with the shift of cells from the G_0/G_1 block to other phases of the cell cycle. The advantage of using the technique of flow cytometry was appreciated not only for the possibility

FIGURE 5 Biochemical analysis of the disappearance of statin after the reinitiation of cell proliferation. Lane A shows the electrophoretic mobility of protein standards at 150, 92, and 58 kD. Lane B shows the presence of a protein at 57,000 D and two high molecular weight bands by immunoblotting technique when the statin antibody is incubated with specimens derived from the nonreplicating fibroblasts as in Fig. 3 a. The absence of recognizable protein bands is noticed in lane C when the same antibody at the identical IgG concentration is incubated with cell extracts from replicating fibroblasts as in Fig. 3 b. The same protein concentration was used for gel electrophoresis for both lanes B and C. This was performed by applying the same optical density units at 280 to the two gel lanes. The efficiency of protein transfer to nitrocellulose paper from the two polyacrylamide gel lanes was controlled by blotting simultaneously in the same run at 150 mA for 12 h at room temperature.

of simultaneous examination of DNA content and antibody staining for statin, but also for the ability to perform the survey with large cell samples.

In post-trypsinized cultures such as those of Fig. 2, G and H, one can usually observe 5–10% of cells displaying the fluorescence positive staining for statin. However, we cannot detect the same degree of protein presence with the immunoblotting technique. This difference may result from the fact that specimens used for protein identification are pooled materials, whereas the microscopic visualization indicates the staining activity in single cells. Another possibility could result from the difference of sensitivity between immunoperoxidase, used for the blotting procedure, and fluorescence used for the microscopic studies.

In the nonreplicating fibroblasts, statin is primarily located at the region of the nuclear envelope. This is different from our previous report demonstrating that the S-30 antigenic activity is present in the entire nuclear region including the nuclear envelope (11). The difference of immunogold localization may indeed reflect the quantitative difference of statin expression between contact-inhibited nonreplicating young fibroblasts and nonproliferating senescent fibroblasts. We have noticed repeatedly that former cells show less fluorescence staining activity than do senescent fibroblasts. This quantitative variation is being studied by the technique of cytofluorometry, where a large number of cells (>5,000) from different cultures can be readily analyzed.

Another difference between the nonreplicating young and senescent fibroblasts is the mere fact of reversibility for cell cycling events in the former but not in the latter (except by strenuous means of in vitro cell transformation). We do not know whether, assuming the protein is functionally related to the traverse of the cell cycle, there is a threshold of statin expression determining the irreversibility of replicating ability for fibroblasts. Do the cells at the terminal stage of their life span contain too much statin to return to the replicative pattern?

Recently, a few reports have described the presence of a protein of 36,000 D in replicating cells but not in nonreplicating counterparts (3, 7, 8, 16, 17). The protein, originally identified by the sera of some patients with systemic lupus erythematosus, detects a nuclear antigen of cells with high
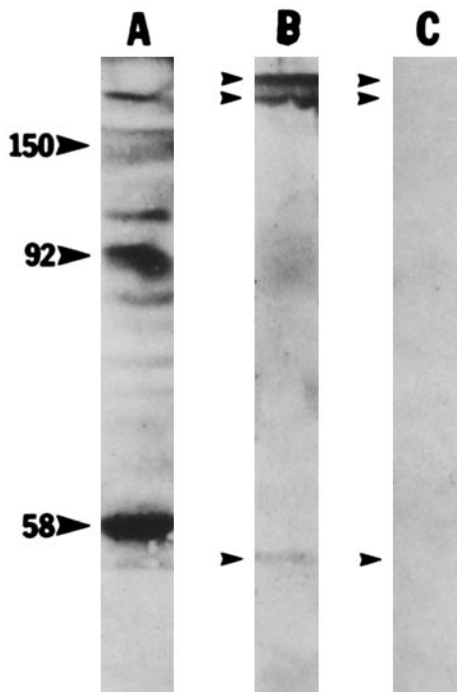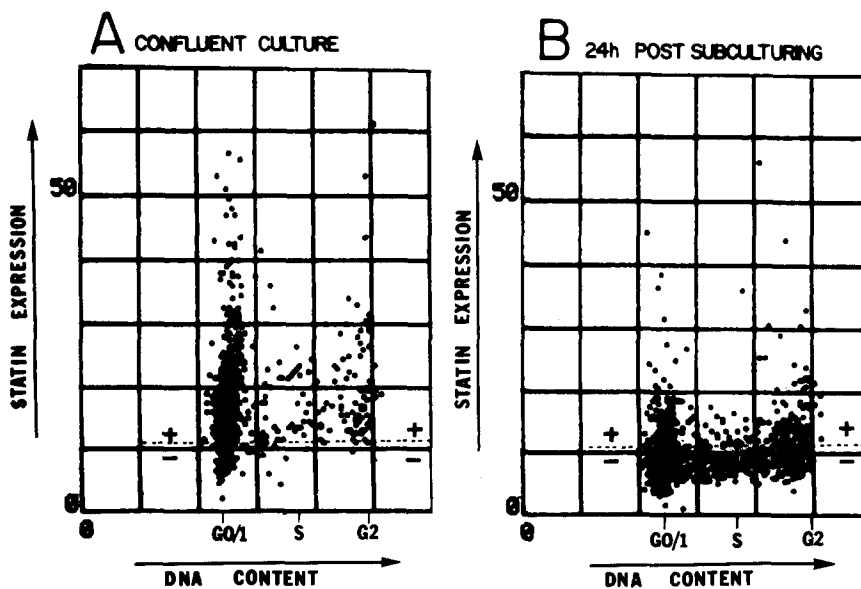

FIGURE 6 Simultaneous analysis of cell cycle distribution and statin expression by flow cytometric studies. (A) Nonreplicating fibroblasts of confluent cultures of 0011 cell strain at early stage of their life span (cumulative PDL < 12). (B) Replicating fibroblasts of culture with sparse density (3,550/cm²), taken at 24 h after trypsinization and replating. The detailed protocols for specimen preparation and manipulation of flow cytometry are described in Materials and Methods. The x-axis represents the content of DNA, and the y-axis shows the fluorescent staining intensity for statin. Each dot represents an individual cell measured. The measurements below the dashed line were considered negative, as derived from the background staining intensity of control experiments when the primary antibody to statin was replaced by PBS.

replicating activity. Since then a wide variety of cell types, including many permanent cultures of transformed lines, have been verified to contain this protein, termed either as proliferating cell nuclear antigen or cyclin. It is interesting to note that, during the cell cycle, the expression of statin is inversely related to that of cyclin. We have found through preliminary observations that the two proteins, statin and cyclin, are alternatively expressed during the transition from nonreplicating to replicating state or vice versa. Most probably, statin represents a novel group of proteins present only in cells that are not proliferating, whereas cyclin is a type of protein expressed only in proliferating cells.

The most important task now is to understand whether the expression of statin is the cause or the effect associated with G_0/G_1 arrest of cell replication. We are attempting in vivo studies such as microinjection into nonreplicating or replicating cells with powerful probes such as purified statin, and ultimately the corresponding genes and antibody. Examination of various phenotypes such as mitotic events and DNA synthesis in the injected cells may provide promising leads in the various functional investigations.

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