Membrane-associated inhibitor of DNA synthesis in senescent human diploid fibroblasts: Characterization and comparison to quiescent cell inhibitor

(aging/serum stimulation/cell cycle/IMR-90/simian virus 40-transformed human cells)

GRETCHEN H. STEIN AND LAURA ATKINS

Department of Molecular, Cellular and Developmental Biology, University of Colorado, Boulder, CO 80309-0347

Communicated by David M. Prescott, August 18, 1986

ABSTRACT Cell membranes prepared from senescent human diploid fibroblasts (HDF) inhibited entry into S phase by 35% when added to the medium of replicating young HDF. This membrane-associated inhibitory activity was (i) sensitive to trypsin, heat, and periodate, which suggests that the inhibitor is a glycoprotein, and (ii) not to inhibit DNA synthesis in simian virus 40-transformed HDF, which indicates that not all types of cells are sensitive to this inhibitor. Quiescent young HDF also have a surface membrane-associated inhibitor of DNA synthesis. A comparison of the senescent HDF and quiescent HDF inhibitory activities indicates that they may have the same chemical and physical nature and the same specific activity, but their regulation is different. The inhibitory activity of quiescent young HDF is abolished within 20 hr after refeeding with fresh serum-containing medium, whereas that of senescent HDF remains unchanged. Quiescent old HDF (two or three population doublings remaining) exhibit an intermediate response to serum with approximately two-thirds of the inhibitory activity abolished. The fraction of cells in S phase at 20–24 hr post-stimulation (37% in young HDF, 24% in old HDF, and 0% in senescent HDF) is inversely proportional to inhibitor levels. This suggests that inability to neutralize the inhibitory activity in response to serum stimulation could be involved in the inability of senescent HDF to enter S phase. Disappearance of the inhibitory activity from quiescent young HDF occurs late in G1 phase. Thus, the inhibitor may play a role in determining the length of the G0 to S phase transition in these cells.

Human diploid fibroblasts (HDF) have a finite proliferative life-span, at the end of which they are in a viable G1-arrested senescent state (1, 2). Senescent HDF cannot be induced to synthesize DNA by subcultivation or by refeeding with fresh serum-containing medium. Previous studies of the behavior of heterokaryons formed by fusion of senescent HDF with replicating young HDF suggested that senescent HDF contain an inhibitor of entry into S phase (3, 4). Further characterization of the senescent HDF inhibitory activity in heterokaryons and cybrids showed that (i) it does not inhibit cells transformed by DNA viruses, such as simian virus 40 (SV40)-transformed HDF (5, 6); (ii) it does inhibit carcinogen-transformed HDF (6); (iii) it is present in enucleated senescent cytoplasmic (7, 8); and (iv) it depends on a protein, or proteins (9, 10), which probably includes a cell-surface protein because trypsin-treated senescent cytoplasts lose their inhibitory activity (11).

Quiescence in young HDF that are crowded or serum-deprived is similar to senescence in old HDF in many ways. The key difference is that quiescence is reversible by subcultivating or by refeeding with fresh serum-containing medium, whereas senescence is not. The similarities are that (i) quiescent HDF are in a viable G1-arrested state (12, 13), and (ii) they behave like senescent HDF in heterokaryons and cybrids, with one exception (11, 14–16). Cycloheximide treatment of serum-deprived quiescent HDF did not abolish their inhibitory activity in heterokaryons and cybrids as it did the inhibitory activity of senescent HDF (11, 16). It is not known whether this difference in response to cycloheximide reflects an inherent difference between the senescent HDF inhibitor and the quiescent HDF inhibitor or a difference in rates of protein degradation in high serum-containing medium versus low serum-containing medium. Recent studies have shown that both serum-deprived quiescent HDF and density-inhibited quiescent HDF have DNA synthesis inhibitory activity in a plasma membrane-enriched fraction of the cells (11, 17–19).

The similarities between senescent HDF and quiescent HDF suggest that they may contain the same inhibitor of entry into S phase. We have proposed the following mechanism to explain how this is compatible with the reversibility of quiescence and the irreversibility of senescence (6, 15). This hypothesis states that (i) whenever HDF experience poor growth conditions, such as serum deprivation or cell crowding, they produce an inhibitor protein, which is degraded or inactivated when the cells are again given good growth conditions; and (ii) as HDF age, they gradually become less and less able to respond to serum or other mitogenic growth factors so that they become functionally mitogen-deprived, which triggers production of the same inhibitor protein. In the latter case, the inhibition is not reversible because the mitogen-deprived state is a consequence of a change in the cells rather than a change in the culture medium. The idea of an age-related loss of responsiveness to serum growth factors stems from Ohno’s (20) finding that as HDF age, they require rapidly increasing amounts of serum to maintain a constant growth rate.

The data presented in this paper show that a plasma membrane-enriched fraction of senescent HDF has DNA synthesis inhibitory activity. Characterization of this membrane-associated inhibitor suggests that (i) it is a glycoprotein, (ii) it may be the same molecule as a membrane-associated inhibitor present in quiescent HDF, and (iii) it is resistant to elimination following serum stimulation of senescent HDF. These data are consistent with the hypothesis described above.

MATERIALS AND METHODS

Cell Lines and Culture. IMR-90 human fetal lung fibroblasts (HDF) and 90VA-V1 (SV40-transformed IMR-90, AG2804) were obtained from the Coriell Institute for Medical Research.

Abbreviations: HDF, human diploid fibroblasts; PD, population doublings; SV40, simian virus 40.

9030
(Camden, NJ). Young HDF were used at 20–28 population doublings (PD) and senescence was achieved at 45–50 PD. Cells were routinely grown at 37°C in EF medium [a 1:1 mixture of Eagle’s minimal essential medium and Ham’s F-12 (17)] plus 10% fetal bovine serum.

Density-inhibited quiescent young HDF were prepared by subcultivating IMR-90 (28 PD) at a 1:8 split ratio in EF medium/10% fetal bovine serum, refeeding them 1 week later, and incubating them for 10 more days. Density-inhibited quiescent old HDF were prepared in the same manner from old IMR-90 (3 PD remaining) that were subcultivated at a 1:4 split ratio. Replicating old HDF were IMR-90 (3 PD remaining) that were subcultivated as described above and harvested 4 days later. Serum-deprived HDF were prepared by subcultivating young IMR-90 (27 PD) at a 1:16 split ratio into EF medium/1% fetal bovine serum, adding an equal volume of EF medium 3 days later to make the final concentration EF medium/0.5% fetal bovine serum, and incubating the cells for 10 more days. Senescent HDF were prepared by subculturing young IMR-90 until they could no longer achieve a 1 PD in at least 2 weeks, with weekly refeddings. Senescent HDF were used in experiments either 14 days after subcultivation (i.e., 7 days after one refeeding) or 16 days after subcultivation (i.e., 2 days after a second refeeding). There was no difference in the inhibitory activity present in membranes of senescent HDF, regardless of when they were refed.

Membrane Preparation. Membranes from density-inhibited, serum-deprived, replicating, and senescent HDF were prepared by the method of Raben et al. (21). Briefly, the cells were harvested by scraping, broken in a Dounce homogenizer, and fractionated on a Ficoll step gradient. This procedure yields a fraction enriched primarily in plasma membranes (21, 22). The number of cells harvested for each preparation ranged from 2.5 x 10^7 to 1.2 x 10^6 cells, and the protein content of each membrane fraction was determined by using the Bio-Rad protein assay. Membranes were stored in small aliquots at ~70°C in Tris-buffered saline (21). Prior to use, the membranes were thawed, diluted in EF medium/10% fetal bovine serum to obtain the necessary concentrations, and sterilized for 15 min under germicidal ultraviolet light.

DNA Synthesis Assays. In each experiment, one of the Petri dishes of cells prepared for membrane isolation was used for [3H]thymidine labeling to measure the replicative state of the cells. In various experiments, the cells were labeled for 12 min, 120 min, or 24 hr with [3H]thymidine (2.5 μCi/ml; 1 Ci = 37 GBq), then trypsinized, replated on slides with a cytocentrifuge, fixed, and processed for autoradiography (23).

DNA Synthesis Inhibition Assays. Young replicating HDF (20–28 PD) were seeded at 1.25–5 x 10^3 cells per cm^2 in 1 ml of EF medium/10% fetal bovine serum in 24-well plates with and without coverslips. After 24 hr of incubation, 0.25 ml of EF medium/10% fetal bovine serum containing various concentrations of membranes in ~25 μl of Tris-buffered saline was added to the medium as described (17). Control cultures received 25 μl of Tris-buffered saline in 0.25 ml of EF medium/10% fetal bovine serum.

For analysis by autoradiography, triplicate samples on coverslip cultures were labeled with [3H]thymidine (2.5 μCi/ml) for 2 hr, beginning 48 hr after membrane addition, and then fixed and processed for autoradiography. Percentage inhibition was determined by comparing the percentage labeled nuclei in membrane-treated versus untreated control cultures. At least 1000–1500 cells were counted per coverslip.

For analysis by scintillation counting, six replicate samples were labeled with [3H]thymidine (2.5 μCi/ml) for 24 hr, beginning 24 hr after membrane addition. The relative amounts of trichloroacetic acid insoluble [3H]thymidine were determined by scintillation counting and were used to calculate percentage inhibition of thymidine incorporation.

Tryptsin, Heat, and Periodate Treatments. Aliquots of membranes were diluted to 4–8 μg per ml of protein in EF medium with no serum. For trypsin treatment, the membranes were incubated with crystalline trypsin (Sigma) at 0.01 mg of trypsin per mg of membrane protein for 10 min at 37°C and then treated with an equal amount of soybean trypsin inhibitor (Sigma) as described (17). For heat inactivation, the membranes were heated to 80°C for 10 min and then cooled to 4°C as described (17). For periodate treatment, the membranes were incubated with 50 mM sodium periodate for 24 hr at 4°C as described (21). Control samples received untreated membranes. In addition, it was shown that cells given no membranes but exposed to either trypsin plus trypsin inhibitor or sodium periodate plus glycercol (to stop the reaction) were not inhibited.

RESULTS

DNA Synthesis Inhibitory Activity in a Subcellular Fraction of Senescent HDF. A plasma membrane-enriched fraction was prepared from senescent HDF and tested for its ability to inhibit DNA synthesis in young HDF. When senescent HDF membranes were added to the medium of replicating young HDF for 48 hr, they caused a reduction in the percentage of cells synthesizing DNA. A dose–response curve (Fig. 1) shows that the inhibitory activity of the senescent HDF membranes reached a plateau of ~35% inhibition at a dose of 4–8 μg/ml. The efficacy of six different preparations of senescent HDF membranes varied from 31% to 42% inhibition. The dose–response curves for the inhibitory activity present in both serum-deprived quiescent HDF membranes and density-inhibited quiescent HDF membranes (21) are very similar to that of senescent HDF membranes (Fig. 1). In contrast, membranes from replicating young HDF have a very low level of inhibitory activity. These results indicate that (i) senescent HDF possess a membrane-associated inhibitor of entry into S phase and (ii) the senescent HDF inhibitor could be the same as the quiescent HDF inhibitor.

Effect of Membranes from Senescent HDF and Quiescent HDF on Rate of Incorporation of [3H]Thymidine. When the inhibitory activity of senescent HDF or quiescent HDF membranes was measured as the decrease in total incorporation of [3H]thymidine in membrane-treated versus untreated replicating young HDF (Fig. 2), the percentage inhibition

![Fig. 1. Inhibition of entry into S phase by membrane fractions prepared from senescent HDF (○), serum-deprived quiescent young HDF (●), density-inhibited quiescent young HDF (●), and replicating young HDF (○). Percentage inhibition was determined by comparing the percentage [3H]thymidine-labeled nuclei in membrane-treated cultures of replicating young HDF vs. untreated cultures of replicating young HDF. The percentage labeled nuclei in the untreated cultures of replicating young HDF varied from 38% to 46% in these experiments.](image-url)
was greater than that based on the fraction of cells in S phase (55–60% vs. 35%). This difference may occur because membrane treatment can reduce both the percentage of cells in S phase at any given time and the total number of cells in the population after 48 hr of exposure to membranes. The total amount of \(^{3}H\)thymidine incorporated in membrane-treated versus untreated cultures would be reduced by both of these effects, whereas the percentage of \(^{3}H\)thymidine-labeled nuclei is not affected by a decrease in cell number.

**Inhibitory Activity in Membranes from Replicating Old HDF.** Membranes were prepared from a population of old HDF that had a \(^{3}H\)thymidine labeling index (24) of 30% at 1 day after subcultivation and 20% at the time of harvest. Sister cultures to the ones used for membrane isolation underwent one or more PD. These replicating old HDF membranes had \(\approx\)5 times as much inhibitory activity as replicating young HDF and one-half as much inhibitory activity as fully senescent HDF (Fig. 2). The high level of inhibitory activity in these replicating old HDF probably comes from the large fraction of nonreplicating cells in the population. However, these data do not rule out the possibility that the level of inhibitor is also increased in the replicating cells in the population.

**Characterization of the Senescent HDF Inhibitory Activity.**

The inhibitory activity in senescent HDF membranes was inactivated by incubation at 80°C for 10 min (Fig. 3). This result distinguishes the senescent HDF inhibitor from the type \(\beta\) transforming growth factor/growth inhibitor, which is heat stable (25). The senescent HDF inhibitor was also sensitive to inactivation by trypsin and periodate, which cleave proteins and carbohydrates, respectively (Fig. 3). These data suggest that the inhibitory activity depends on a glycoprotein. However, some residual inhibitory activity remained after each of these treatments, especially trypsinization. Increasing the concentration of trypsin 10-fold halved the remaining inhibitory activity but did not completely eliminate it. This result may mean that some of the inhibitory activity is inaccessible to the trypsin (e.g., in clumps of membrane), or it may mean that there is a second inhibitory element in the membrane preparations. The membrane-associated inhibitory activity of quiescent HDF is also heat, trypsin (11, 17, 19), and periodate sensitive (Fig. 3), in keeping with the possibility that senescent HDF and quiescent HDF have the same inhibitor.

**Effect of Senescent HDF Membranes on SV40-Transformed HDF.** Senescent HDF membranes were added to cultures of post-crisis ("immortalized") SV40-transformed HDF (90VA-VI), and inhibition of DNA synthesis was measured as described in Fig. 1. There was no significant decrease (0–3%) in the percentage \(^{3}H\)thymidine-labeled nuclei in SV40-transformed HDF treated with 4, 8, 16, or 32 \(\mu\)g of senescent HDF membrane protein per ml (data not shown). These results indicate that SV40-transformed HDF are refractory to inhibition by senescent HDF membranes, just as they have previously been shown to be refractory to inhibition by senescent HDF in heterokaryons (5, 6). These data also indicate another similarity between the senescent HDF and quiescent HDF membrane-associated inhibitors, because the latter were also unable to inhibit SV40-transformed HDF (17).

**Effect of Serum Stimulation on the Membrane-Associated Inhibitory Activity of Quiescent HDF.** Density-inhibited quiescent HDF cultures were refed with fresh serum-containing medium to determine how this would affect both the level of membrane-associated inhibitory activity and the concomitant level of DNA synthesis in these cells. For ease of discussion, we will call this procedure serum stimulation.

When young density-inhibited quiescent HDF were serum-stimulated, the majority of the membrane-associated inhibitory activity was still present 12 hr later but was completely gone by 20 hr (Fig. 4A). Conversely, there was little induction of DNA synthesis in these cells during the first 12 hr after stimulation, but 37% of the cells entered S phase by 20 hr. Previous studies (ref. 26; unpublished results) have shown that the peak of DNA synthesis in serum-stimulated quiescent young HDF is reached by 20 hr after stimulation and usually involves entry into S phase in 35–40% of the cells. Thus, the stimulation of DNA synthesis obtained in this experiment is quite typical of serum-stimulated quiescent HDF. The fact that all of the membrane-associated inhibitory activity was abolished, while only 37% of the cells entered S phase, indicates that loss of the membrane-associated inhibitor may be a necessary but not sufficient prerequisite for initiation of DNA synthesis in these cells. The timing of the loss of inhibitory activity in these cells suggests that elimination of the inhibitor is a late \(G_1\) event.
because they do not inactivate or eliminate their membrane-associated inhibitor.

Effect of Serum Stimulation on Quiescent Old HDF. The effect of serum stimulation on the level of membrane-associated inhibitory activity in quiescent old HDF was examined to determine whether there was a transition between the all (100% removal) or none (0% removal) responses of quiescent young HDF and senescent HDF, respectively. Density-inhibited quiescent old HDF (2–3 PD remaining) were prepared in exactly the same way as density-inhibited quiescent young HDF. After serum stimulation, the quiescent old HDF lost two-thirds of their membrane-associated inhibitory activity, and 24% of the cells entered S phase by 24 hr after stimulation (Fig. 4B). The time course of this experiment was extended to 24 hr because our previous experiments showed that the peak of DNA synthesis occurs between 21 and 24 hr in serum-stimulated quiescent old HDF (data not shown). These data show that both the extent of loss of inhibitory activity and the extent of increase of cells in S phase were reduced by ~1/3rd in quiescent old HDF compared to quiescent young HDF. Thus, these results support the hypothesis that (i) removal of the membrane-associated inhibitor is necessary but not sufficient for entry into S phase; and (ii) as HDF approach the end of their life-span, they become less and less able to remove the inhibitor in response to serum growth factors. Consequently, old HDF eventually become completely nonreplicative because they are totally unable to remove the membrane-associated inhibitor, regardless of their culture conditions.

As mentioned above, the timing of entry into S phase is slower in quiescent old HDF than in quiescent young HDF. The data in Fig. 4B show that the timing of removal of the membrane-associated inhibitor is likewise slower in quiescent old HDF, such that at 16 hr after stimulation, there was no loss of inhibitory activity and no induction of DNA synthesis. These data further support the idea that elimination of the membrane-associated inhibitor is a late G1 event, whose timing is intimately related to the timing of entry into S phase.

**DISCUSSION**

The data presented in this paper indicate that senescent HDF contain an inhibitor of DNA synthesis in a plasma membrane-enriched cell extract. The properties of that inhibitor suggest that (i) it is a glycoprotein, (ii) it blocks entry into S phase, and (iii) it is effective on normal HDF but not on SV40-transformed HDF. Previous studies of the behavior of senescent HDF in cell heterokaryons and cybrids suggested the existence of a senescent HDF inhibitory protein with these characteristics (3–10). The finding of this activity in a subcellular extract of senescent HDF has allowed us to test further our hypothesis concerning the mechanism by which HDF age and become arrested in a senescent state.

We have hypothesized that the aging process in HDF involves a progressive decrease in the ability of the cells to respond mitogenically to serum or other growth factors, such that old HDF reach a point where they are functionally serum-deprived even though they are incubated in high serum-containing medium. We suggest that this functional serum deprivation in old HDF triggers production of the same inhibitor of DNA synthesis as is produced in young HDF made quiescent by crowding or serum deprivation (6, 15). This hypothesis makes two clear predictions: (i) the inhibitor of DNA synthesis present in senescent HDF is the same as the inhibitor of DNA synthesis present in quiescent HDF; and (ii) the regulation of this inhibitor by serum will be different in senescent HDF and quiescent HDF — i.e., the inhibitory activity of senescent HDF will not be reversed by serum stimulation and that of quiescent HDF will be reversed.

The membrane-associated senescent HDF inhibitor characterized in this paper behaves in the same way as does the quiescent HDF membrane-associated inhibitor that we have previously characterized (17). The quiescent HDF membrane-associated inhibitors studied by others (11, 18, 19) have different dose–response curves, perhaps owing to procedural differences. Using a uniform set of procedures, we find that the inhibitors from senescent HDF, density-inhibited quiescent HDF, and serum-deprived quiescent HDF all have (i) the same dose response curves; (ii) the same sensitivity to trypsin, heat, and periodate; and (iii) the same inability to inhibit SV40-transformed HDF. These results support the
hypothesis that senescent HDF contain the same inhibitor of DNA synthesis as do quiescent HDF. Serum stimulation of senescent HDF does not affect their membrane-associated inhibitor of DNA synthesis, whereas serum stimulation of quiescent young HDF abolishes their inhibitor. Furthermore, serum stimulation of quiescent old HDF abolishes only a fraction of their inhibitory activity, indicating a progressive decrease in the response to serum with age of the cells. These results are fully supportive of the hypothesis described above.

Recent reports from several laboratories (27–29) have shown that senescent HDF (defined here as cells with >90% life-span completed) can carry out a number of events in the prereplicative pathway from G₀ to S phase, even though only a small fraction of the cells can initiate DNA synthesis. Ritting et al. (27) studied 11 cell-cycle-dependent genes, including the late G₁/S phase genes thymidine kinase and histone H3, and found that each one was expressed in serum-stimulated senescent HDF at levels comparable to those in serum-stimulated quiescent young HDF. Olashaw et al. (28) showed that after serum stimulation the level of thymidine kinase activity and total TTP synthesis also increased as much in senescent HDF as in quiescent young HDF. Finally, Pendergrass et al. (29) showed that serum-stimulated senescent HDF and serum-stimulated quiescent HDF produced equal amounts of DNA polymerase α, which is another enzyme that is induced immediately prior to the onset of DNA synthesis. These results indicate that senescent HDF are still able to respond to some of the signals in serum. On the surface, these data might seem to contradict the hypothesis that there is a progressive loss of responsiveness to serum with age, but instead they refine it. The data presented in this paper show that abolition of the inhibitor in response to serum stimulation is an event that is increasingly defective in old HDF and absent in fully senescent HDF. Therefore, we suggest that abolition of the inhibitor is on a different pathway of response to serum stimulation than are the prereplicative events studied by Ritting et al. (27), Olashaw et al. (28), and Pendergrass et al. (29). An alternative possibility is that abolition of the inhibitor is on the same pathway as induction of DNA polymerase α, thymidine kinase, and histone mRNA synthesis, but it occurs at a later time, which would have to be just at the G₁/S border. Our data are also consistent with this second possibility because they show that abolition of the inhibitor is a late G₁ event. In either case, failure to abolish the inhibitor appears to be responsible for failure to enter S phase in senescent HDF. Therefore, it will be important to learn more about the regulation of this inhibitor.

Note Added in Proof. Recently, Brooks et al. (30) have found that plasma membranes prepared from senescent HDF and quiescent HDF have a stimulatory effect on [³H]thymidine incorporation into senescent HDF. These results suggest that senescent HDF and quiescent HDF plasma membranes contain both stimulatory and inhibitory factors, which can be revealed by different experimental protocols.

This work was supported by National Institutes of Health Grants AG00947 and AG04811.