Quiescent human diploid cells can inhibit entry into S phase in replicative nuclei in heterodikaryons

(cell cycle/cell fusion/cellular senescence/simian virus 40/chemical transformation)

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ABSTRACT Serum-deprived quiescent human diploid cells (HDC) were fused to replicative HDC, and DNA synthesis was monitored in the resulting heterodikaryons. Quiescent HDC had an inhibitory effect on DNA synthesis in replicative HDC nuclei in heterodikaryons. The timing of the inhibitory effect suggests that entry into S phase was inhibited but ongoing DNA synthesis was not inhibited in the replicative HDC nuclei. When quiescent HDC were fused to SV40-immortalized human fibroblasts, it was similar to that observed when quiescent HDC were fused to simian virus 40-transformed human cells, adenosivirus 5-transformed human cells, or HeLa cells, DNA synthesis was induced in the quiescent HDC nuclei. A simple hypothesis to explain these results is that quiescent HDC contain an inhibitor of entry into S phase. Transformed cells with a dominant replicative phenotype may have gained a factor that overrides the putative inhibitor, perhaps through viral transformation, whereas recessive transformed cells may have lost the normal inhibitory mechanism, perhaps through mutation. Senescent HDC behave like quiescent HDC in heterodikaryons formed with the same types of replicative cells, which suggest that senescent HDC and quiescent HDC share elements of a common mechanism for cessation of proliferation.

Previous studies with HeLa cells suggest that entry into S phase is controlled by a positive effector in cycling cells. HeLa cells in S phase induced DNA synthesis in HeLa cells in G1 phase after cell fusion (1). In contrast, DNA synthesis was not induced in HeLa cells in G2 phase fused to cells in S phase. These data suggest that S-phase cells contain an inducer of DNA synthesis that stimulates entry into S phase in G1-phase cells. This possibility is supported by other experiments showing that when HeLa cells in early, middle, and late G1 phase were fused together in various combinations, both nuclei in the resulting binucleates entered S phase prematurely, except in early G1 × early G1 fusions (2). Cells in late G1 phase contributed the greatest acceleration of entry into S phase. Therefore, these data suggest that cells in G1 phase accumulate some factor that is necessary for entry into S phase, and this could be the putative inducer found in S-phase cells themselves.

In contrast to these observations on cycling cells, it appears that in noncycling senescent human diploid cells (HDC), regulation of entry into S phase may be negatively controlled. When senescent HDC were fused to replicative young HDC, DNA synthesis was inhibited in the young HDC nuclei in heterodikaryons (3). Further analysis indicated that ongoing DNA synthesis in young HDC in S phase at the time of fusion was not inhibited but that entry into S phase was inhibited (4). Similar results were obtained when senescent HDC were fused to SUSM-1 chemically transformed human cells, CT-1 radiation-transformed WI-38 cells, and several human tumor cell lines (5, 6). In each case, ongoing DNA synthesis was not inhibited in the replicative cell nuclei, but entry into S phase was blocked. A simple explanation for these results is that nonreplicative senescent HDC contain an inhibitor of entry into S phase. This hypothesis is consistent with the observation that senescent HDC themselves are blocked from entering S phase (7, 8).

Normal human cells, such as WI-38 fetal lung fibroblasts, become quiescent when they are deprived of serum; the cells almost completely cease to proliferate and yet they maintain high viability (9). These quiescent HDC have G1-phase DNA contents, indicating that they cannot enter S phase (10). This paper analyzes heterodikaryons formed between quiescent HDC and replicative cells to determine whether entry into S phase is positively or negatively controlled in quiescent HDC.

MATERIALS AND METHODS

Our procedures for cell culture, cell fusion, autoradiography, and identification of heterodikaryons have been described in detail (5, 11). Only an outline of these procedures is given here.

In these experiments, the HDC were IMR-90 human fetal lung fibroblasts at 16–30 population doublings (12). Transforming virus 5-transformed human fibroblasts (13), SUSM-1 chemically transformed human liver cells (M. Namba and L. Hayflick, personal communication), 90VE-VI (simian virus 40 [SV40]-transformed IMR-90 cells; AC2904 from the Institute for Medical Research, Camden, NJ), HeLa human cervical carcinoma cells (14), and 293 adenovirus 5-transformed human kidney cells (15). Replicating cultures of these cells were subcultivated 2 days before fusion. Latex beads (1-μm diameter; Polysciences, Warrington, PA) were added to the cells to label their cytoplasm. HeLa and T98G cells did not take up beads well unless they were stimulated by the addition of DEAE-dextran (10 μg/ml) to the medium. The DEAE-dextran was removed at least 15 hr before fusion.

Quiescent HDC were prepared in the following manner. IMR-90 cells from a replicating culture were seeded at approximately 1.5–2 × 10⁴ cells per cm² in Eagle’s basal medium containing 10% (vol/vol) fetal calf serum. To label the cytoplasm of the cells, 2-μm diameter latex beads (Dow) were added. After 1.5 days, the medium was changed to Eagle’s basal medium containing 0.5% fetal calf serum and additional 2-μm diameter beads. The cells were incubated in Eagle’s basal medium/0.5% fetal calf serum for 5 or 8 days before fusion.

Quiescent HDC were fused to cycling cells of various types by using either polyethylene glycol (16) or UV-inactivated Sen-

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Abbreviations: HDC, human diploid cells; SV40, simian virus 40.* Present address: Cancer Biology Program, National Cancer Institute, Frederick Cancer Research Center, P. O. Box B, Frederick, MD 21701.

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dai virus (17). No differences were noted in the results obtained with the two fusion agents. After fusion, the cells were incubated in medium containing 10% (vol/vol) fetal calf serum. At different time intervals after fusion, the fraction of cells synthesizing DNA was measured by labeling the cells with 0.01 μCi (1 Ci = 3.7 × 10^10 Becquerels) of [3H]thymidine (50 Ci/mmol) per ml. At the end of the labeling period, the cells were fixed and processed for autoradiography (11). The fraction of the cycling cells in S phase at the time of fusion was measured in a separate aliquot of the fusion mixture by a 10-min pulse of [3H]thymidine (2 μCi/ml). This 10-min pulse was followed by a 10-hr chase in medium containing 0.1 mM thymidine. Each experiment was repeated at least once with similar results.

Analysis of the autoradiograms has been described (5). Briefly, the monokaryons and homodikaryons of each parental cell type were identified by the presence of at least 10 beads of the correct size. Heterodikaryons were identified by the presence of at least 10 beads of both sizes. The percentage of labeled nuclei of each parental type were scored in monokaryons, homodikaryons, and heterodikaryons. In all heterodikaryons except quiescent HDC-replicative HDC, it was possible to distinguish the quiescent HDC nuclei from the replicative cell nuclei based on morphological differences. When a quiescent HDC-replicative HDC heterodikaryon contained only one labeled nucleus, it was assumed to be the replicative HDC nucleus.

RESULTS

Quiescent HDC were fused to six types of replicative cells to determine whether quiescent HDC have an inhibitory effect on entry into S phase in replicative cell nuclei. The replicative cell types used were normal HDC, chemically transformed HDC, SV40-transformed HDC, adenovirus 5-transformed HDC, and two human tumor cell lines of unknown etiology. Fusion of Quiescent HDC with Replicative HDC. Quiescent HDC that had been deprived of serum for 8 days were fused to replicative HDC (IMR-90). [3H]Thymidine was added to the fusion mixture at 0–10, 10–24, and 24–48 hr after fusion, and the fraction of quiescent HDC nuclei or replicative HDC nuclei that synthesized DNA in monokaryons, homodikaryons, and heterodikaryons was determined by autoradiography. After fusion, the cells were incubated in medium containing 10% fetal calf serum; consequently, the quiescent HDC were stimulated to reenter S phase, albeit after a lag longer than 24 hr (Fig. IA, monokaryons). By 72 hr after fusion, half of the quiescent HDC monokaryons had synthesized DNA (data not shown).

The results show that quiescent HDC had an inhibitory effect on DNA synthesis in replicative IMR-90 nuclei in heterodikaryons (Fig. IB) and that quiescent HDC nuclei were not induced to synthesize DNA in these heterodikaryons (Fig. IA). At 0–10 hr after fusion, there was a small reduction in the fraction of labeled replicative IMR-90 nuclei in heterodikaryons compared to monokaryons (27% versus 33%). At 10–24 and 24–48 hr after fusion, the difference between heterodikaryons and monokaryons was large (12% versus 83% at 10–24 hr and 15% versus 89% at 24–48 hr). This relative inhibition of DNA synthesis in replicative nuclei in heterodikaryons is conveniently expressed as a “reduction index.” We define the reduction index as the percentage of labeled replicative cell nuclei in heterodikaryons minus the percentage of labeled replicative cell nuclei in monokaryons, normalized to the percentage of labeled replicative cell nuclei in monokaryons. For the experiment illustrated in Fig. 1, the reduction index is −18% at 0–10 hr, −85% at 10–24 hr, and −83% at 24–48 hr after fusion. The reduction index is useful for comparing the results of different experiments (see Fig. 3).

The inhibition of DNA synthesis in replicative IMR-90 nuclei in heterodikaryons was not an effect of fusion per se because there was no comparable reduction in the fraction of labeled IMR-90 nuclei in homodikaryons at 0–10 and 10–24 hr after fusion. Between 24 and 48 hr after fusion, the fraction of labeled IMR-90 nuclei in homodikaryons was significantly decreased in comparison to the monokaryons. We suggest that this reduction may have occurred because most of the nuclei in homodikaryons had already completed one S phase by 24 hr after fusion and either were not able to reenter S phase in the absence of mitosis or went through mitosis and consequently were no longer binucleate cells when the cells were fixed at 48 hr after fusion.

The timing and amount of DNA synthesis that took place in replicative IMR-90 nuclei in heterodikaryons suggests that ongoing DNA synthesis was not inhibited, but entry into S phase was inhibited after fusion to quiescent HDC. Replicative IMR-90 nuclei in heterodikaryons synthesized DNA immediately after fusion but were inhibited at later times, as expected if only entry into S phase was inhibited. The fraction of replicative IMR-90 nuclei that synthesized DNA in heterodikaryons labeled immediately after fusion approximately equaled the fraction of replicative IMR-90 cells that were in S phase at the time of fusion (27% versus 32%). This result suggests that IMR-90 cells that were in S phase at the time of fusion were able to continue to synthesize DNA in heterodikaryons for a limited period of time. The amounts of DNA synthesized by replicative IMR-90 nuclei in heterodikaryons and monokaryons were compared by counting the number of silver grains over these nuclei in the 0- to 10-hr sample. Of the labeled nuclei in heterodikaryons, 43% had >20 grains/nucleus, whereas 57% of the labeled monokaryons had >20 grains/nucleus. These data indicate that the labeled IMR-90 nuclei in heterodikaryons synthesized almost as much DNA as did the labeled nuclei in monokaryons. This observation is consistent with the hypothesis that replicative IMR-90 in S phase were able to complete the ongoing round of replication as opposed to the hypothesis that ongoing DNA synthesis continued for only a short time after fusion. The extensive inhibition of DNA synthesis in the replicative nuclei at later times indicates that new rounds of replication were not initiated.

Fusion of Quiescent HDC with SV40-Transformed HDC. Quiescent HDC that had been deprived of serum for 8 days were fused to SV40-transformed HDC (90VA-VI) in the same
manner as described above. The purpose of this experiment was to determine whether quiescent HDC would inhibit entry into S phase in a transformed cell that does not enter quiescence itself. The results showed that DNA synthesis was not inhibited in 90VA-VI nuclei in heterodikaryons (Fig. 2B). Rather, DNA synthesis was induced in quiescent HDC nuclei in heterodikaryons. There was a small amount of induction at 0–10 hr after fusion, and a large amount of induction at 10–20 and 20–44 hr after fusion (Fig. 2A). Only a small fraction of the quiescent HDC nuclei in monokaryons and homodikaryons had reentered S phase by 20–44 hr after fusion, indicating that the induction of DNA synthesis in heterodikaryons was due to the presence of 90VA-VI. There was no significant reduction in the fraction of 90VA-VI nuclei that synthesized DNA in homodikaryons or heterodikaryons. These results indicate that fusion with 90VA-VI cells can overcome the inhibitory mechanism that prevents quiescent HDC from entering S phase.

Previous experiments have shown that SV40-transformed HDC can induce DNA synthesis in senescent HDC and that normal HDC are inhibited by senescent HDC (3, 4, 18). The present experiments show that quiescent HDC behave like senescent HDC in heterodikaryons formed with both SV40-transformed HDC and normal HDC. This parallel between the behavior of quiescent HDC and senescent HDC suggests that they may share a common mechanism for cessation of proliferation.

Fusion of Quiescent HDC with Other Replicative Transformed Cells. Quiescent HDC were fused to a series of replicative transformed cells in order to answer two questions. The first question was whether all transformed cells can induce DNA synthesis in quiescent HDC. The second question was whether quiescent HDC always behave like senescent HDC in heterodikaryons. Four transformed cell types with various properties were chosen for this analysis: T98G glioblastoma cells, SUSM-1 chemically transformed HDC, HeLa cervical carcinoma cells, and 293 adenovirus 5-transformed HDC. In previous experiments T98G (5) and SUSM-1 cells (6) were inhibited by senescent HDC, in contrast to HeLa cells, which were able to induce DNA synthesis in senescent HDC (18).

The fusion procedures and analysis of heterodikaryons were carried out as before. The data from this series of experiments and from experiments done with replicative HDC and SV40-transformed HDC are summarized in Fig. 3, which shows an induction index for the quiescent HDC nuclei and a reduction index for the replicative nuclei in each type of heterodikaryon at 0–10, 10–20 (or 10–24), and 20–44 (or 24–48) hr after fusion. A reduction index was defined earlier to describe the behavior of the replicative nuclei in heterodikaryons formed with quiescent HDC. Similarly, an induction index can be defined to describe the behavior of the quiescent HDC nuclei in heterodikaryons formed with various types of replicative cells. The induction index is percentage of labeled quiescent HDC nuclei in heterodikaryons minus the percentage of labeled quiescent HDC nuclei in monokaryons, normalized to the percentage of unlabeled quiescent HDC monokaryons. This summary of the data indicates that HeLa and 293 cells behaved like 90VA-VI cells in heterodikaryons with quiescent HDC. They were not inhibited from entering S phase and they induced DNA synthesis in quiescent HDC nuclei. In contrast, T98G and SUSM-1 cells behaved like replicative HDC in heterodikaryons with quiescent HDC: they did not induce DNA synthesis in quiescent HDC, and their own ability to enter S phase was inhibited. These data indicate that not all transformed cells can induce DNA synthesis in quiescent HDC. They also indicate that quiescent HDC behave like senescent HDC in heterodikaryons formed with five types of cells.

In the previous series of experiments, the quiescent HDC monokaryons did not begin to reenter S phase for more than 20–24 hr after they were replated in medium containing 10% serum (e.g., Figs. 1A and 2A). Maizel et al. (19) found that confluent quiescent HDC that were merely trypsinized and replated in medium containing 10% serum had a lag period approximately 18 hr long. The longer lag period that we observed may be due to the trauma of treatment with a fusion agent or to a difference between confluent quiescent cells and serum-deprived quiescent cells. Because the lag period in our fusion experiments was relatively long, we investigated whether quiescent HDC serum starved for only 5 days and having a shorter lag period for recovery would also have an inhibitory effect on the replication of IMR-90, T98G, and SUSM-1 cells.

When quiescent HDC were deprived of serum for only 5 days, they began to reenter S phase much sooner after the fusion procedure (8–20% labeled nuclei at 10–20 or 10–24 hr after fusion, 35–80% labeled nuclei at 20–44 or 24–45 hr after fusion). As before, the quiescent HDC had an inhibitory effect on entry into S phase in IMR-90, T98G, and SUSM-1 nuclei in heterodikaryons (Fig. 4B). The inhibitory effect was less dramatic in these experiments than in the experiments shown in Fig. 3 because the quiescent HDC cells were beginning to reenter S phase just as the replicative cells were finishing their ongoing S phase. Consequently, there was no interval when the full inhibitory effect could be observed.

In the experiments with more rapidly recovering quiescent HDC, there was a small amount of induction of DNA synthesis in quiescent HDC nuclei in heterodikaryons formed with T98G and SUSM-1 (compare Figs. 3A and 4A). These data suggest that the inhibitory effect may be almost gone in quiescent HDC nuclei that are about to enter S phase on their own, thereby allowing some premature induction of DNA synthesis in these nuclei by cells that are sensitive to the inhibitory effect. The induction of DNA synthesis in quiescent HDC nuclei by 90VA-VI and HeLa cells was also greater and occurred more rapidly when the quiescent HDC were less ‘‘deep’’ in quiescence.

In summary, the results of these experiments with more rapidly reversible quiescent HDC confirm the conclusion that quiescent HDC have an inhibitory effect on entry into S phase in normal HDC and certain types of transformed cells. They also suggest that the inhibitory effect may increase when cells are held quiescent for longer times.

![Fig. 2. Fusion of quiescent HDC to 90VA-VI cells. The percentage of labeled nuclei of each type were scored in monokaryons (□), homodikaryons (■), and heterodikaryons (▲) that were labeled with [3H]thymidine at 0–10, 10–20, and 20–44 hr after fusion. (A) Percentage of labeled quiescent HDC nuclei. (B) Percentage of labeled 90VA-VI nuclei.](image)
DISCUSSION

Quiescent serum-deprived HDC had an inhibitory effect on DNA synthesis in replicative IMR-90, T98G, and SUSM-1 nuclei in heterodikaryons. The timing and the magnitude of the inhibitory effect suggest that ongoing DNA synthesis in the replicative cell nuclei was not inhibited, but entry into S phase was inhibited. When the cells were labeled with [3H]thymidine immediately after fusion, 10–50% of the replicative cell nuclei in heterodikaryons synthesized DNA in the first 10 hr after fusion. In each experiment, the fraction of replicative cell nuclei that synthesized DNA in the first 10 hr after fusion approximately equaled the fraction of replicative cell nuclei that were in S phase at the time of fusion. By 10 hr after fusion, when most replicative cells would have finished the ongoing S phase, few replicative cell nuclei synthesized DNA in heterodikaryons containing nonreplicative quiescent HDC. The hypothesis that quiescent HDC inhibit entry into S phase but do not inhibit ongoing DNA synthesis is also consistent with the observation that quiescent HDC themselves are blocked from entering S phase—i.e., they have G1 phase DNA contents (10).

Quiescent HDC did not inhibit DNA synthesis in 90VA-VI, HeLa, and 293 cell nuclei in heterodikaryons; rather, DNA synthesis was induced in the quiescent HDC nuclei in these heterodikaryons. These data indicate that in some cell types their replicative phenotype is dominant over that of quiescent HDC, whereas in others, such as replicative HDC, the replicative phenotype behaves in a recessive fashion. A simple hypothesis to explain these two sets of results is that quiescent HDC contain a diffusible inhibitor of entry into S phase. We propose that in the recessive case, entry into S phase is blocked by the putative inhibitor in the same way that quiescent HDC themselves are blocked from entering S phase. We propose further that, in the dominant case these cells contain a factor that inactivates or overrides the putative inhibitor allowing both nuclei in heterodikaryons to enter S phase.

Viral transformation is one way that cells with a dominant replicative phenotype may have gained an overriding factor that is not present in normal cells. Two of the three "dominant" cell types were virally transformed and the other, HeLa, was derived from a cervical carcinoma of unknown etiology. Some serological and epidemiological evidence suggests that Herpes simplex virus 2 may play a role in the etiology of cervical carcinoma (20). Thus, it is possible that HeLa may have been virally transformed in vivo. On the other hand, the three "recessive" cell lines were normal HDC, SUSM-1 chemically transformed cells, and T98G cells, which were derived from a glioblastoma of unknown etiology. Although transformed to immortality and anchorage independence, the T98G cells appear to exhibit the normal mechanism for quiescence (13). These data suggest that "recessive" transformed cells have lost all or part of the normal mechanism for control of cellular proliferation, perhaps through mutation. Thus, they are sensitive to the putative inhibitor, when it is supplied by quiescent HDC in heterodikaryons.

Studies with quiescent mouse 3T3 cells also have provided evidence that quiescent cells contain an inhibitor of DNA synthesis. Extracts prepared from the cell surface (21) or from cell-surface membranes (22) of quiescent 3T3 cells inhibited DNA synthesis by approximately 50–70% in replicative 3T3 cells. DNA synthesis in SV40-transformed 3T3 cells was not inhibited by the extracts prepared from quiescent 3T3 cells (22, 23). Analysis of the cell cycle specificity of the inhibitory effect suggests that entry into S phase was inhibited but that ongoing DNA synthesis was not inhibited (23). Thus, the results obtained from analyses of quiescent mouse 3T3 cell extracts and from the analysis of quiescent HDC in heterodikaryons both support the hypothesis that quiescent cells contain an inhibitor of entry into S phase.

The evidence presented here for an inhibitor of entry into S phase in quiescent HDC, combined with the evidence for an inducer of entry into S phase in cycling cells, suggests that normal control of cellular proliferation may be governed by a balance between positive and negative effectors. A shift of the balance to the negative effector could coordinate a series of metabolic changes that are necessary for survival when conditions are not adequate for proliferation. A number of reports indicate that transformed cell lines that have lost the normal mechanism for quiescence do not survive well under stationary phase conditions where normal cells survive quite well (24–26). An irreversible shift of the balance to the negative effector could...
also be the basis for the cessation of proliferation associated with terminal differentiation and cellular senescence.

Previous experiments have shown that senescent HDC inhibit entry into S phase in IMR-90, T98G, and SUSM-1 nuclei in heterodikaryons and that senescent HDC are induced to synthesize DNA by fusion to 90VA-VI and HeLa cells. In this paper, the same five cell types were analyzed in heterodikaryons formed with quiescent HDC. The results show that quiescent HDC and senescent HDC behave in the same way in these heterodikaryons. In addition, Rabinovitch and Norwood (27) have shown that the timing of the induction of DNA synthesis in senescent HDC and quiescent HDC fused to HeLa cells is the same and that the timing of the inhibition of entry into S phase in replicative HDC fused to either senescent HDC or quiescent HDC is also the same. These parallels between the behavior of senescent HDC and quiescent HDC suggest that they share a common mechanism for cessation of proliferation (e.g., they may contain the same inhibitor of entry into S phase). Indeed, senescence may be identical to quiescence due to serum deprivation. This would occur if senescent HDC have a defect or a block in their ability to interact with serum mitogens; consequently, senescent HDC in high serum-containing medium would functionally experience the same deprivation of serum as young HDC in low serum-containing medium. Alternatively, senescent HDC and quiescent HDC may activate the same inhibitory mechanism through different signals.

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