Evidence for Endogenous Polypeptide-mediated Inhibition of Cell-cycle Transit in Human Diploid Cells

GLENNA C. BURMER, CAROL J. ZEIGLER, and THOMAS H. NORWOOD
Department of Pathology, University of Washington, Seattle, Washington 98195

ABSTRACT Previous studies have shown that the senescent phenotype is dominant with respect to DNA synthesis in fusions between late passage and actively replicating human diploid fibroblasts. Brief postfusion treatments with the protein synthesis inhibitor cycloheximide (CHX) or puromycin have been found to significantly delay (by 24–48 h) the inhibition of entry into DNA synthesis of young nuclei in heterokaryons after fusion with senescent cells. A significant fraction of the senescent nuclei incorporated tritiated thymidine in CHX-treated heterokaryons. The optimal duration of exposure to CHX was 1–3 h immediately after fusion, although treatments beginning as late as 9 h after fusion elevated the heterokaryon labeling index. Prefusion treatments with CHX were without a significant effect. These results are consistent with the interpretation that regulatory cell cycle inhibitor(s) which are dependent upon protein synthesis may be present in heterokaryons between senescent and actively replicating cells.

The finite in vitro replicative lifespan of human diploid fibroblastlike (HDFL) cells has been well documented (1, 2). Studies have demonstrated that the proportion of nondividing or slowly cycling cells increases with serial passage until the entire population becomes arrested predominantly in the G1 phase of the cell cycle (3, 4). The mechanism(s) responsible for limiting the growth potential remains unclear. Rabinovitch and Norwood (5) and Stein and Yanishevsky (6) have proposed that cessation of proliferation or "senescence" results from the constitutive synthesis of an inhibitory factor(s). This hypothesis is based primarily upon observations derived from cell fusion studies carried out in these laboratories. These investigators observed that initiation of DNA synthesis in the young, actively proliferating cell is inhibited in heterokaryons after fusion with senescent HDFL cells, but ongoing DNA synthesis was not inhibited. In addition, Rabinovitch and Norwood (5) demonstrated that inhibition of entry into DNA synthesis occurs if fusion with a senescent cell is complete while the young nucleus is in the G1 phase of the cell cycle at a point earlier than 3 h before the onset of the S phase. Similar results were observed in parallel studies where actively proliferating HDFL cells were fused with cells rendered quiescent by serum deprivation (5). While the proposal that senescent and quiescent cells elaborate a cell cycle inhibitory factor(s) is a reasonable interpretation of these observations, alternative hypotheses cannot be excluded. For example, it is possible that fusion between actively proliferating low passage and late passage senescent cells results in dilution of factors necessary for cell cycle progression and initiation of DNA synthesis, resulting in passive inhibition of DNA synthesis.

We further investigated these alternative interpretations in studies in which the effect of transient inhibition of protein synthesis on DNA synthetic activity was examined in heterokaryons between old and young HDFL cells. We observed inhibition of entry into DNA synthesis of young nuclei upon fusion with senescent cells to be significantly delayed by brief treatments with protein synthesis inhibitors. These results are interpreted to be consistent with the presence of a regulatory inhibitor of cell cycle function in the senescent cell.

MATERIALS AND METHODS

Cells and Culture Conditions

HDFL cells described in these studies were initiated in this laboratory from primary explants. The sources, population doubling levels, and labeling indices of these strains are described in Table I.

Cells were maintained in Eagle's minimum essential medium (EMEM) with Earle's Salts (Gibco Laboratories, Grand Island Biological Co., Santa Clara, CA), buffered with 26 mM sodium bicarbonate, supplemented with 10% heat-inactivated fetal bovine serum (Gibco Laboratories; and Reheis Chemical Co., Div. Armour Pharmaceutical Co., Kankakee, IL), penicillin (100 U/ml), streptomycin (100 μg/ml), and incubated at 37°C in an atmosphere of 5% CO2 in air. Early passage cells were maintained in an actively cycling state by passaging twice a week. Tests for mycoplasma by aerobic and anaerobic cultures were negative (7).

Cell Fusion Procedure

To facilitate identification of heterokaryons, the early passage, actively proliferating HDFL cells were prelabeled for 24 h with 2.5 μCi/ml [3H]methionine.
TABLE I
Origin and In Vitro Age of Cell Strains Used in These Studies

| Strain          | Source                      | Population doubling index | [%] |
|-----------------|-----------------------------|---------------------------|-----|
| Early passage   |                             |                           |     |
| 76-109          | Newborn foreskin            | 14 (70)                   | 94  |
| 75-69           | Newborn foreskin            | 7 (58)                    | 87  |
| 75-73           | Fetal upper arm skin        | 18 (59)                   | 92  |
| 72-166          | Adult upper arm skin        | 8 (58)                    | 90  |
| Late passage    |                             |                           |     |
| 72-117          | Adult upper arm skin        | 60 (60)                   | <5  |
| 75-69           | Newborn foreskin            | 56 (58)                   | <5  |
| 72-166          | Adult upper arm skin        | 58 (58)                   | <5  |

* These strains were used at the indicated population doublings from the time of the initial passage after explanation. The maximum lifespan is shown in parentheses.

† The percentage of labeled cells in subconfluent cultures after a 24-h pulse with [3H]thymidine (0.5 μCi/ml).

The kinetics of recovery of protein and DNA synthesis were determined after treatment with CHX and puromycin. In these studies, ~1 x 10⁶ cells were plated into 60-mm tissue culture dishes (Corning Glass Works), and, after overnight attachment, some dishes were treated with medium containing CHX or puromycin for 1 h. The medium was then aspirated, the dishes were rinsed three times with HBSS, and medium lacking inhibitors was added. At various times after release, replicate cultures were pulsed for 1 h with [3H]thymidine (4 μCi/ml, S.A. = 200 mCi/mM, New England Nuclear) for determination of protein synthesis or with [3H]thymidine (10 μCi/ml, S.A. = 6.7 Ci/mM, New England Nuclear) for determination of DNA synthesis. Immediately after the pulse, the cells were rinsed three times in ice-cold phosphate-buffered saline, dissolved in 1 ml of 1% (wt/vol) SDS (Bio-Rad Laboratories, Richmond, CA) containing 100 μg/ml carrier DNA (Type I: calf thymus DNA; Sigma Chemical Co.), and precipitated with 0.5 ml of ice-cold 50% TCA. The precipitate was pelleted, resuspended in 1 ml of 0.3 N NaOH, and re-precipitated with 0.5 ml of 50% TCA two more times before adding 1 ml of 0.1 N NaOH and incubating at 60°C for 10 min. The solution was placed in a scintillation vial in 10 ml of Aquasol (New England Nuclear), with 75 μl of acetic acid, and counted on a Packard TriCarbon liquid scintillation counter (Packard Instrument Co., Inc., Downers Grove, IL). To obtain baseline values of amino acid incorporation in the presence of inhibitor, cultures were exposed to CHX or puromycin simultaneously with [3H]thymidine for 1 h, and immediately rinsed and dissolved in SDS.

Cell counts were obtained from duplicate dishes using a Coulter Counter (Coulter Electronics Inc., Hialeah, FL).

RESULTS

Initial studies were performed to determine the effect of a short postfusion treatment with CHX on the nuclear [3H]thymidine labeling index in heterokaryons at various times after release from the protein synthesis inhibitor. A concentration was used which inhibits protein synthesis by >98% (10 μg/ml). A representative result from multiple experiments is shown in Fig. 1. As we have observed in previous studies (5), the fraction of [3H]thymidine-labeled nuclei in heterokaryons in the untreated cultures began to decline after a 3-h delay after fusion, and by 9 h was similar to that enumerated in old homokaryons (Fig. 1a). In contrast, the heterokaryon labeling index in the CHX-treated cultures was elevated throughout the labeling period, while the young and old homokaryon labeling analysis. To minimize the possibility of observer bias, randomly selected preparations were analyzed by another individual familiar with the prelabeling system. The percentage of labeled nuclei in the homokaryons and heterokaryons were calculated by dividing the number of [3H]thymidine-labeled nuclei by the total number of nuclei multiplied by 100.

Measurements of Amino Acid and Thymidine Incorporation

The percentage of labeled nuclei in the homokaryons and heterokaryons were calculated by dividing the number of [3H]thymidine-labeled nuclei by the total number of nuclei multiplied by 100.

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indices were unaffected by the treatment (Fig. 1b). In the absence of markers the parental origin of the nuclei in the heterokaryons cannot be identified. However, the fraction of heterokaryons with both nuclei labeled provides an estimate of the extent to which thymidine incorporation occurs in the senescent nuclei. In all cell strains examined in this study the proportion of double-labeled heterodikaryons was significantly increased in the CHX-treated cultures (Table II).

To determine whether the elevation in the heterokaryon labeling index observed following postfusion CHX treatment was specifically the result of inhibition of protein synthesis or an action unique to CHX, studies were conducted using puromycin, which has a different mode of action than CHX (10). Dose-response studies carried out with both inhibitors demonstrated that similar results were obtained when the cultures were treated with puromycin after fusion (Fig. 2). An approximate linear increase in the percentage of labeled heterokaryons was observed with a 1-h postfusion treatment, correlating with exponential increases in the concentration of CHX in the range of 0.05 to 2 μg/ml and with puromycin in the range of 1 to 50 μg/ml.

The degree of inhibition of protein synthesis corresponding to these concentrations of inhibitors was determined by liquid scintillation counting of [3H]methionine incorporation into acid-precipitable material (Fig. 3). Increased concentrations of inhibitors resulted in an increased inhibition of amino acid uptake in both young and senescent cultures. A 1-h exposure to 10 μg/ml puromycin or 2 μg/ml CHX was sufficient to reduce [3H]methionine incorporation by ~90%. Recovery of synthetic activity was observed to be 90–100% of controls within 2 h in both early and late passage cultures. No consistent significant differences were observed in the kinetics or extent of recovery after treatment with CHX at the various concentrations (Fig. 3a and b). In contrast, treatment of young cultures with concentrations of puromycin necessary to inhibit [3H]methionine uptake by ~90% (50–100 μg/ml) and maximally stimulate DNA synthesis in heterokaryons resulted in a significantly decreased recovery in the incorporation of the labeled precursor, which is in agreement with a previous report (11) (Fig. 3c). A large proportion of cells, exposed to the highest concentration, were judged nonviable by their inability to exclude trypan blue dye. Similar studies carried out with senescent cells at concentrations of puromycin of <10 μg/ml displayed complete recovery of incorporation of [3H]methionine (Fig. 3d).

The kinetics of recovery of protein synthesis as a function of duration of exposure to CHX (2 or 10 μg/ml) in young and senescent cultures was also examined. No differences were observed in cultures exposed from 1 to 24 h (data not shown).

Recovery of DNA synthesis in young cultures as assessed by [3H]thymidine incorporation into acid-precipitable material was also observed to be independent of the concentration of CHX used in these studies (Fig. 4). Within 4 h after release from CHX, the cultures had recovered to within 80% of the values for untreated controls.
To further characterize the effect of inhibition of protein synthesis on the DNA synthetic activity in these heterokaryons, the effect of duration of exposure to CHX immediately after fusion was examined (Fig. 5). Exposure periods of 1 to 3 h resulted in the greatest elevation of the nuclear thymidine in the heterokaryons. Increasingly lowered values were observed with longer exposure periods. Treatment periods of 15 or 30 min produced no elevation in the fraction of labeled nuclei in these cells (data not shown).

The time of initiation of the CHX treatment in relation to cell fusion proved to be a significant variable. Exposure for 3-h intervals within the first 24 h after fusion resulted in elevations in the [3H]thymidine labeling index in the heterokaryons (Fig. 6b, c, and d). Stimulation of thymidine incorporation was observed when the treatment was initiated as late as 9 h after fusion. When the protein synthesis inhibitor was introduced at 24 h after fusion for either 3- (Fig. 6e) or 12-h (Fig. 6f) intervals, no increase in the fraction of labeled nuclei in the heterokaryons was observed. Note also that the labeling index remains elevated for at least 24 h after termination of the treatment with the inhibitor.
In contrast to the dramatic increase in the heterokaryon labeling indices observed with postfusion CHX treatments, exposures of both young and senescent cells to the inhibitor for various durations before fusion did not result in significant increases in the percentage of labeled heterokaryons (Fig. 7). Similarly, prefusion treatment of senescent cells alone with CHX for various durations of exposure had no effect on the subsequent heterokaryon labeling index (Fig. 7, open circles).

**DISCUSSION**

The experiments described here have demonstrated that brief treatments with protein synthesis inhibitors after cell fusion results in a significant but temporary elevation of the nuclear thymidine-labeling index in heterokaryons derived from the fusion of young, actively proliferating, and senescent human diploid cells. The transient nature of this effect (24–48 h) and the observation that introduction of the inhibitors 24 h after fusion fails to stimulate nuclear thymidine incorporation in the heterokaryons suggest that inhibition of protein synthesis permits the young nuclei to progress into the S phase in the hybrid environment but does not stimulate sustained sequential cell cycle activity. In addition, the increased frequency of heterokaryons with both nuclei labeled after treatments with these inhibitors indicates a modest stimulation of initiation of DNA synthesis in the senescent nuclei. Identical results have also been observed in parallel experiments using Sendai virus as a fusogen (results not shown), demonstrating that the stimulatory effects of postfusion treatments with protein synthesis inhibitors were not specific to the polyethylene glycol (PEG)-mediated fusion protocol.

The elevation in the heterokaryon labeling index after treatment with CHX or puromycin correlated with the degree of inhibition of protein synthesis (as measured by the incorporation of [3H]methionine) produced by different concentrations of the inhibitors. Also, treatments of <1 h (15 and 30 min) produced no effect on the [3H]thymidine labeling indices. These results are consistent with the interpretation that a labile factor(s) dependent upon protein synthesis may be involved in the inhibition of DNA synthesis in heterokaryons derived from fusions between senescent and actively replicating human diploid cells. The DNA synthesis-inhibiting activity becomes insensitive to interruption of protein synthesis if the introduction of CHX is delayed by at least 24 h after fusion. This suggests this product(s) of protein synthesis is not required once inhibition of DNA synthesis is completed.

CHX has been shown to produce a reduction in the rate of DNA replication in cultured mammalian cells (12–14). Thus, it is possible that these results could reflect a prolonged residual recovery from the action of the inhibitor on the young nucleus and not a real increase in the fraction of labeled heterokaryons. However, this interpretation appears to be unlikely in view of our observation that recovery of [3H]thymidine incorporation was complete within 3–4 h after release from treatment in actively cycling early passage cells (Fig. 4). This is consistent with the rapid recovery of DNA synthesis reported by other investigators after withdrawal of this inhibitor from cells in early S phase (15–18).

Several investigators have hypothesized the presence of an inhibitor(s) of DNA synthesis which acts to block entry into S phase in senescent cells (5, 6, 19). This idea is further supported by reconstruction experiments between cytoplasts derived from senescent cells and karyoplasts (nuclei) from early passage cells. The reconstructed cells formed from these fusions are incapable of extended replicative activity, which is consistent with the idea that a diffusible cytoplasmic regulatory inhibitor may be present in late passage cytoplasts (20). However, it is unlikely that protein regulatory inhibitors are solely responsible for preventing senescent cells from initiating DNA synthesis.

Stimulation of entry into S phase was never observed in senescent monokaryons or homokaryons after release from prolonged treatments with CHX, although recovery of [3H]methionine uptake was rapid under these conditions. The presence of a positive regulatory factor(s) from the actively replicating parental cells is apparently necessary for the induced stimulation of DNA synthesis in senescent nuclei observed in a small fraction of double-labeled heterokaryons after inhibition of protein synthesis (Table II).

A somewhat surprising observation was that treatment with CHX immediately before fusion had minimal, if any, effect on the post-treatment [3H]thymidine-labeling indices in the heterokaryons. The interpretation of this result is not clear at the present time. However, as indicated in Results, the putative inhibitor(s) must be depressed for at least 1 h. If the half-life of the effector molecule(s) is significantly shorter than 1 h, then functional levels may be restored rapidly enough to achieve inhibition of DNA synthesis in the heterokaryons. Alternatively, a unique fusion specific product which is dependent upon polypeptide synthesis may be produced by the heterokaryons immediately after fusion.

While a number of investigators have postulated the presence of an inhibitor(s) of DNA synthesis to explain the mechanism of inhibition of DNA synthesis in heterokaryons between senescent and low passage, actively dividing HDFL cells, there are other interpretations of this observation. It is possible that initiation factors present in the young cell are diluted below a minimum-required threshold after fusion. The results presented here are inconsistent with the latter hypothesis, which would not predict that inhibition of protein synthesis would elevate the [3H]thymidine-labeling indices in the heterokaryons. However, transient elevations of protein synthesis above pretreatment values following recovery from exposure to CHX have been reported in cultured primary chick cells and in vivo (21, 22). It is possible that activators of DNA synthesis are synthesized in excess immediately after release from inhibition of protein synthesis, resulting in the initiation of DNA synthesis in a larger fraction of heterokaryons. Although we were unable to consistently observe this overshoot of protein synthesis in our monokaryon cultures after CHX treatment, this interpretation cannot be ruled out on the basis of our studies.

In addition to its dramatic inhibitory effect on protein synthesis, CHX has also been shown to inhibit both basal and induced autophagic proteolysis in cultured cells (23–25). Another possible explanation for our results is that CHX treatment produces an increase in the turnover rate of the polypeptide initiation factors in the young cell. This might stimulate the heterokaryons to progress through one round of DNA synthesis.

While studies presented in this paper are consistent with the interpretation that an inhibitor(s) is involved in the regulation of cell cycle activity in human diploid cells derived from the skin, clearly this hypothesis can be confirmed only by the isolation and biochemical characterization of this factor(s). This will require the development of suitable bioassays for this factor(s). We are currently attempting to develop an assay system which will detect physiologic inhibition of cell cycle activity by polypeptides. Manual microinjection may provide...
a potential technical approach to the development of such a bioassay system for these putative regulatory peptides.

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