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Selective Killing of Transformed Fibroblasts by Combined Treatment with Cycloheximide and Aphidicolin

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The possibility of selective killing of transformed cells in a mixed population of untransformed and transformed cells was examined using a cell culture system of rat 3Y1 fibroblasts (parental 3Y1 cells, 3Y1 cells transformed with either SV40, polyoma virus, Rous avian sarcoma virus, E1A gene of adenovirus type 12, or H-v-*ras* oncogene). The principle of the selective killing is as follows. Under suboptimal culture conditions, untransformed cells are inhibited from progressing through G1 phase and retain viability, while transformed cells are not arrested. When DNA synthesis is inhibited for a long period, both types of cells in S phase die. Therefore, if we administer inhibitors of G1 progression and of DNA synthesis simultaneously to a cell population consisting of untransformed and transformed cells, most untransformed cells are arrested in G1 phase, retaining viability, while transformed cells leak from the G1 phase, cease DNA synthesis, and gradually die. The present study shows that all types of transformants in stationary-phase cultures (consisting of cells mainly with a G1 DNA content) were killed to higher extents compared with untransformed cells, during incubation at lower cell densities with a combination of cycloheximide (G1 inhibitor) and aphidicolin (DNA-synthesis inhibitor). However, cycloheximide reduced the killing effect of aphidicolin by changing the irreversible DNA-synthesis inhibition to a reversible inhibition. The availability of G1 inhibitors that do not interfere with the irreversibility of inhibition of DNA synthesis is required for the treatment of cancer based on this idea.

Key words: Transformed cells — Cell killing — Cell cycle — Cycloheximide — Aphidicolin

In cancer chemotherapy it is desirable to kill selectively cancer cells, without cytotoxic effect on normal cells. Cells in most tissues stop proliferating in response to inhibitory signals from the extracellular environment, and reside in the resting state. When proliferation-stimulating signals are conveyed to the resting cells, they initiate the processes required for the initiation of DNA synthesis and resume proliferation.[1] On the other hand, cancer cells are relatively insensitive to the inhibitory signals, and a large fraction of them remain in the proliferating state.[1] If we can hold normal cells in the resting state while chasing cancer cells into the proliferating state, we can selectively kill the cancer cells by using a drug that has a lethal effect specifically on proliferating cells.

It has been reported that transformed cells in culture are less sensitive to cycloheximide, an inhibitor of protein synthesis, in terms of inhibition of the progression of the G1 phase of the cell cycle, than the untransformed counterparts.[2,3] Prolonged inhibition of S-phase progression by inhibitors of DNA synthesis results in cell killing.[4,5] In this study, the possibility of selective killing of transformed cells by a combination of cycloheximide and aphidicolin, an inhibitor of DNA polymerase α,[6] was tested using a cell culture system of rat fibroblasts, untransformed 3Y1 and their transformed derivatives induced by various viral agents.

MATERIALS AND METHODS

Cell lines A clonal isolate (clone 1-6) of rat 3Y1-B diploid fibroblasts[7] (referred to as 3Y1) and its transformed derivatives induced with SV40 (SV-3Y1-C66,[8] SV-3Y1-C60[8]), polyoma virus (Py-3Y1-S2[8]), Rous avian sarcoma virus (SR-3Y1-1[8]), E1A gene of adenovirus type12 (E1A-3Y1-1[9]), and H-v-*ras* oncogene (HR-3Y1-2[8]) were used.

Chemicals Aphidicolin was purchased from Wako Pure Chemical Industries, Osaka.

Cell culture The regular culture medium was Dulbecco's modified Eagle's medium containing 10% fetal calf serum. All cultures were incubated at 37°C in a humidified atmosphere of 10% CO_2/90% air. For stationary-phase cultures, cells were seeded in plastic dishes at approximately 1/20 of the respective saturation density, and incubated for 5–7 days (until 1 day after reaching the saturation density). For proliferating exponential-phase cultures, cells seeded as above were incubated for 2–3 days (until 2–3 days before reaching the saturation density).

SV40 infection Resting 3Y1 cells were infected with SV40 (strain SV68C) at 1300 plaque-forming units per cell for 90 min as has been described.[10]

Kinetics of entry into S phase Stationary cells were reseeded with the regular medium containing or not

containing 0.75 μM cycloheximide at 1/5 of the saturation density and incubated in the continuous presence of [^{3}H]thymidine (37 kBq/ml; 37 kBq=1 μCi). At intervals, the cells were fixed for autoradiography.

Colony-forming ability Single cells dispersed with trypsin-EDTA were plated with the regular medium at an input of 10^2–2×10^4/5.3-cm dish. After a 10-day incubation, cultures were fixed and stained with Giemsa, and visible colonies were counted. For each data point, 3 dishes were prepared.

Progression of S phase Stationary-phase SV-3Y1-C60 cells were reseeded with medium containing 15 μM aphidicolin at 1/3 of the saturation density, and incubated for 9 h. The cells were further incubated in the presence of aphidicolin (15 μM) plus cycloheximide (0.75 μM) or aphidicolin alone for 12 h. The media of these cultures were changed to the inhibitor-free regular medium. At intervals, cells were stained with propidium iodide and the DNA content distribution was analyzed by flow cytometry as has been described.[3)]

RESULTS

Determination of the cycloheximide dose which selectively inhibits entry into S phase in 3Y1 cells without effect on those abortively transformed by infection with SV40 In order to determine the dose of cycloheximide that selectively prevents 3Y1 cells from entering the S phase, we compared the dose-response relationship between 3Y1 cells and those infected with SV40. Because the cells compared are derived from the same cloned line, the difference in the dose-response relationship reflects only the transforming effect of SV40. When stationary-phase 3Y1 cells were reseeded sparsely with fresh medium, almost all cells entered the S phase within 20 h.[11)] As shown in Fig. 1, in the presence of cycloheximide entry into the S phase was inhibited in a dose-dependent fashion. This inhibition was surmounted when the stationary-phase cells had been infected with SV40. Similar results have been reported previously.[3)] From

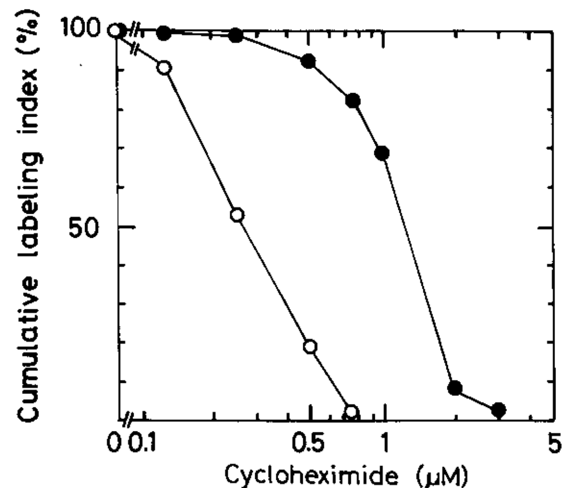

Fig. 1. Inhibition of entry into the S phase by cycloheximide after sparse reseeding of stationary-phase 3Y1 cells, and the inhibition-overcoming effect of SV40 infection. Stationary-phase 3Y1 cells were uninfected (○) or infected with SV40 (●) and reseeded at 1/5 of the saturation density in the presence of the indicated concentrations of cycloheximide. The fractions of cells that had entered the S phase by 40 h after reseeding were determined by autoradiography.

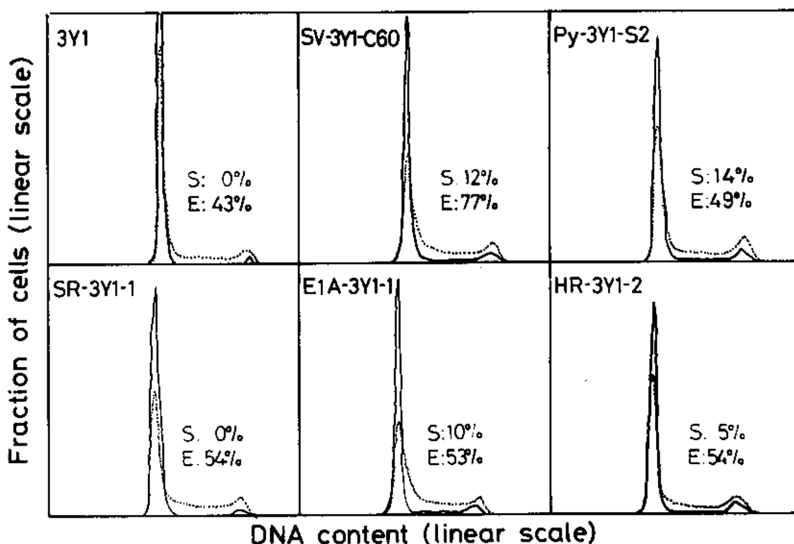

Fig. 2. DNA content distribution of stationary-phase (——) and exponential-phase (······) cultures of 3Y1 cells and transformed derivatives. All the cytograms are normalized so that the total cell number is equal. The labeling indices with [^{3}H]thymidine for 4 h are presented for stationary-phase (S) and exponential-phase (E) cultures.

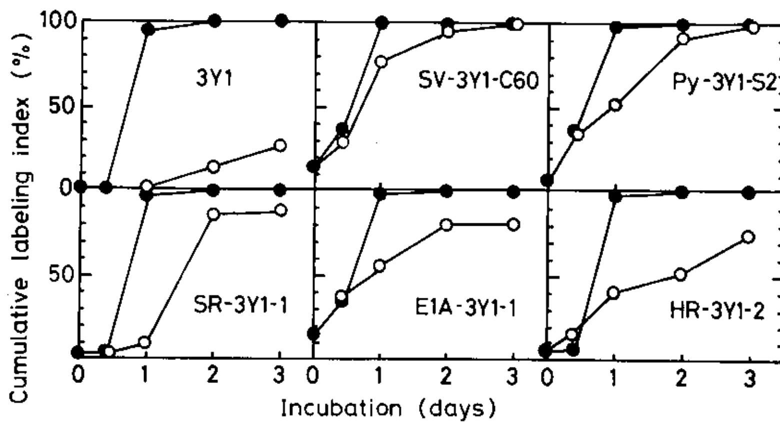

Fig. 3. Entry into S phase in the presence of cycloheximide in 3Y1 and its transformed derivatives. Stationary-phase cells were reseeded at 1/5 of the saturation densities in the presence (○) or absence (●) of cycloheximide (0.75 μM). The fractions of cells that had entered the S phase by the indicated times after reseeding were determined by autoradiography.

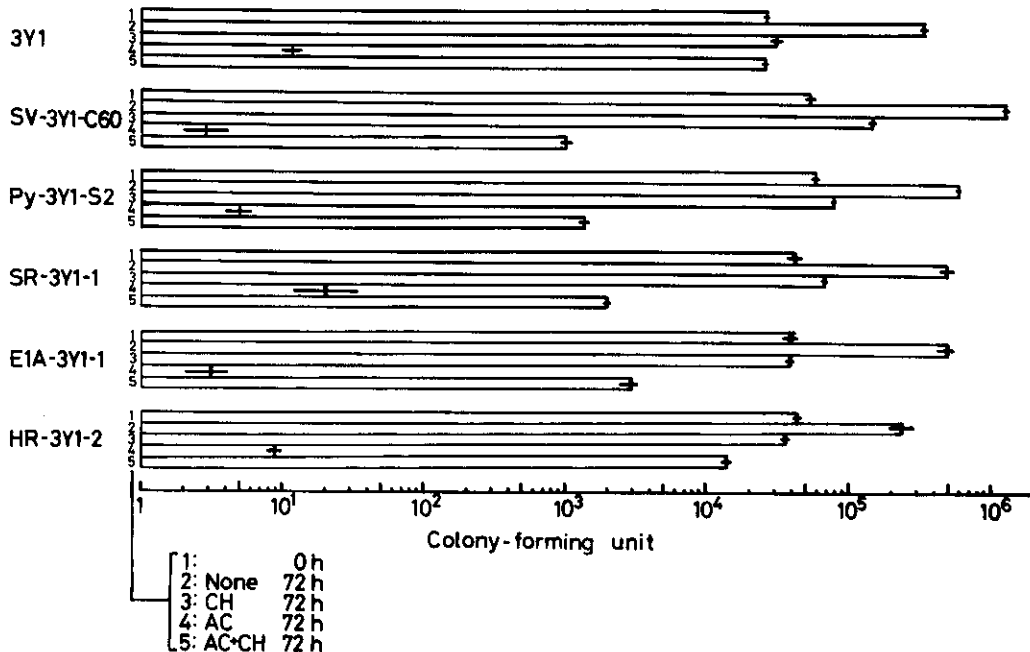

Fig. 4. Selective killing of transformed cells by combined treatment with cycloheximide (0.75 μM) (CH) and aphidicolin (15 μM) (AC). Stationary-phase cells were reseeded at 1/5 of the saturation densities in 3.3-cm dishes, and incubated in the presence of the indicated combinations of drugs for 72 h. The number of surviving cells per culture was determined by colony-formation assay. Bar, standard error for 3 cultures.

a number of experimental results,[3, 10, 12] we have concluded that the process required for entry into the S phase mediated by growth factors is different from that mediated by SV40 large T antigen.[3] At a cycloheximide dose of 0.75 μM, entry into the S phase was almost completely inhibited in the uninfected cells, while as many as 80% of the infected cells entered the S phase during a 40-h incubation. Hereafter, we used cycloheximide at the concentration of 0.75 μM.

Effect of cycloheximide on entry into S phase after mitotic stimulation of stationary-phase transformed lines As can be seen from the cytograms in Fig. 2, the stationary-phase cell population of each transformed line contained fewer cells in the S and G2 phases than the exponential-phase cell population, as in the case of the untransformed counterpart. This was also confirmed by the autoradiography of [^{3}H]thymidine-incorporated cells (labeling indices shown in Fig. 2). As shown in Fig.

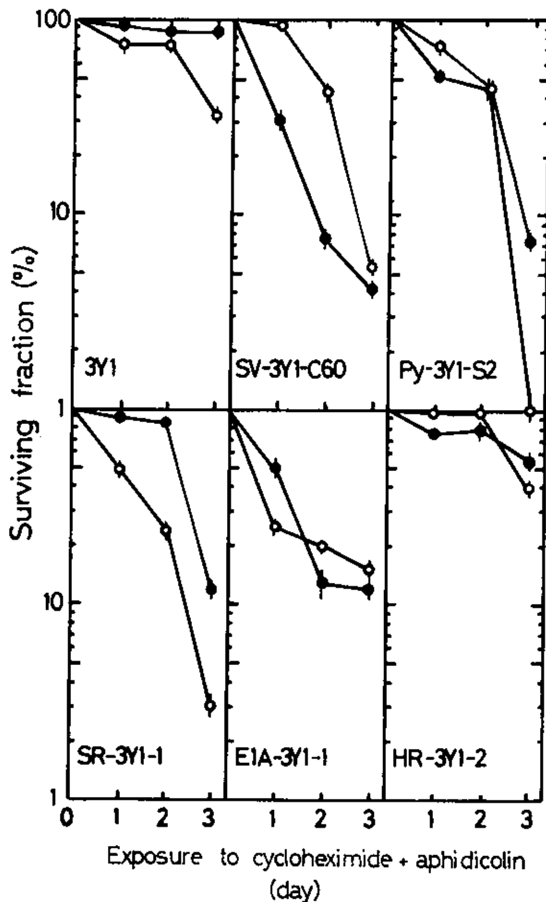

Fig. 5. Comparison of the killing effects of combined treatment with cycloheximide and aphidicolin of 3Y1 and its transformed derivatives between stationary-phase cultures (●) and exponential-phase ones (○). Exponential-phase or stationary-phase cultures were reseeded at an input of 100–1000 cells/5.3-cm dish and incubated in the presence of cycloheximide (0.75 μM) and aphidicolin (15 μM), for the indicated periods. The drugs were removed from these cultures, which were then incubated for another 10 days for determination of the efficiency colony formation. Bar, standard error for 3 cultures.

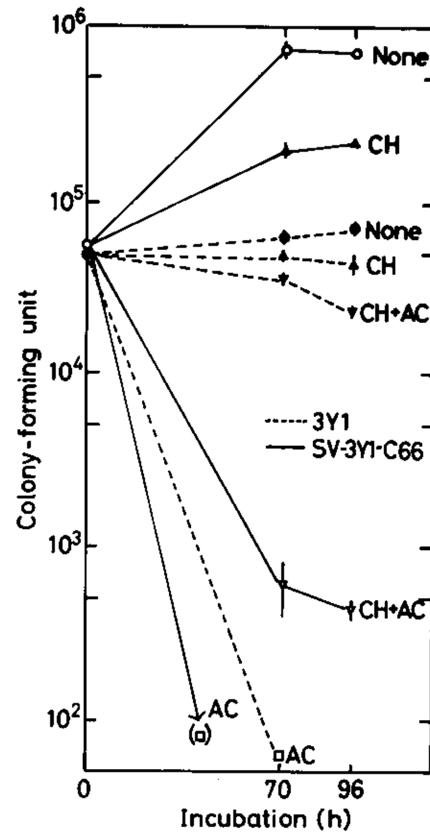

Fig. 6. Selective killing of transformed cells by combined treatment with cycloheximide (0.75 μM) (CH) and aphidicolin (15 μM) (AC) in mixed cultures of 3Y1 and SV-3Y1-C66. Both types of cells in the stationary phase (8×10^4 each) were mixed, reseeded in 3.3-cm dishes, and incubated in the presence of the indicated combinations of drugs. At the indicated times the number of surviving cells was determined by colony-formation assay. The colonies of 3Y1 and those of SV-3Y1-C66 were easily distinguished by the cell density of each colony. Bar, standard error for 3 cultures.

3, when stationary-phase cells of each transformed cell line were reseeded sparsely with fresh medium, entry into the S phase was less inhibited in the presence of 0.75 μM cycloheximide than in the case of parental 3Y1 cells. Stationary-phase cells of SR-3Y1-1 and HR-3Y1-2 seem to be in a resting state similar to that of the stationary-phase 3Y1 cells, because when they were stimulated to proliferate in the absence of cycloheximide, there were lag times (approximately 0.5 day for the 3 cell lines) before appearance of S-phase cells.

Selective killing of transformed cells by combined treatment with cycloheximide and aphidicolin Stationary-phase cells of each cell line were reseeded sparsely with fresh medium, and either cycloheximide or aphidicolin or both were administered to the culture. After a 72-h incubation, viable cells were counted by colony-formation assay. As shown in Fig. 4, the number of viable cells did not markedly change during the 72-h incubation in the presence of cycloheximide for any of the cell lines. The efficiencies of colony formation did not change when the assay cultures were exposed to cycloheximide for 80 h (data not shown), indicating that cycloheximide had no cell killing effect on any of the cell lines. In the case of aphidicolin exposure, cells of all the lines were killed drastically. When cycloheximide and aphidicolin were administered simultaneously, 3Y1 cells were not killed

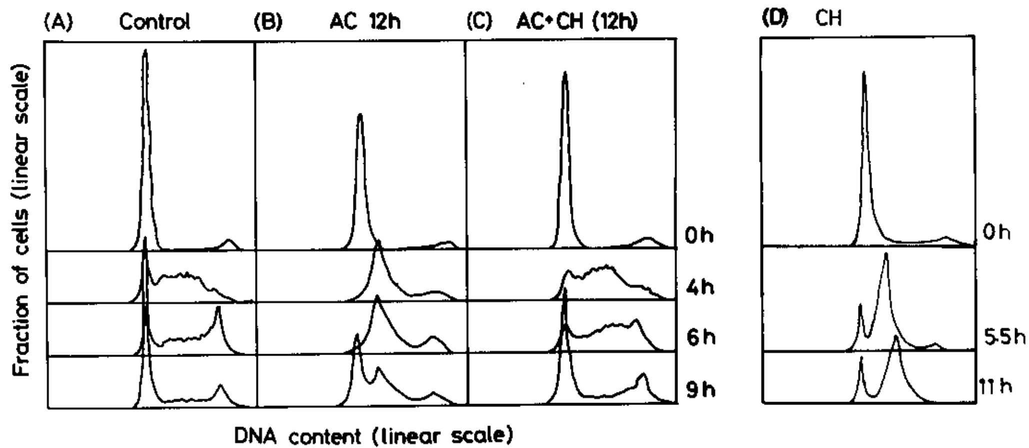

Fig. 7. Inhibition of S-phase progression after release from prolonged arrest at early S phase by aphidicolin (AC), and the inhibition-overcoming effect of simultaneous exposure to cycloheximide (CH) during the prolonged arrest in SV-3Y1-C60 cells. Stationary-phase cells were reseeded sparsely and incubated in the presence of aphidicolin (15 μM) for 9 h. Then the cultures were divided into 4 groups as follows. After removal of aphidicolin, cells were incubated for the indicated periods (A). The incubation was continued in the presence of aphidicolin for another 12 h, and then after removal of aphidicolin, cells were incubated for the indicated periods (B). The incubation was continued in the presence of aphidicolin and cycloheximide (0.75 μM) for another 12 h, and then after removal of both drugs, cells were incubated for the indicated periods (C). After removal of aphidicolin, cells were incubated in the presence of cycloheximide (0.75 μM) for the indicated periods (D). At the end of the last incubation, the DNA-content distribution was determined by flow cytometry. The results of (A–C) are from a set of experiments performed in parallel. All the cytograms are normalized so that the total cell number is equal.

significantly, while cells of all the transformed lines were killed markedly. In this case, however, the killing effects were decreased compared with the case of administration of aphidicolin alone.

The killing effects of combined treatment with cycloheximide and aphidicolin on exponential-phase (proliferating) cultures were examined. As shown in Fig. 5, exponential-phase 3Y1 cells were more sensitive to the combined treatment with aphidicolin and cycloheximide than stationary-phase ones. Except for HR-3Y1-2, exponential-phase transformed cells were more sensitive than exponential-phase parental 3Y1 cells. It should be noted that in contrast to 3Y1 and SR-3Y1-1, in the case of SV-3Y1-C60 stationary-phase cells were more sensitive than exponential-phase cells.

Selective killing of transformed cells in a mixed culture of 3Y1 and SV-3Y1-66 by cycloheximide and aphidicolin Since the interactions mediated by cell-cell contact and medium conditioning between untransformed cells and transformed cells may protect transformed cells from the combined treatment, the killing effect was examined in a mixed culture. Equal numbers of stationary-phase 3Y1 cells and SV-3Y1-C66 cells were mixed and reseeded sparsely into medium containing aphidicolin and cycloheximide. As shown in Fig. 6, after a 70-h incubation the number of viable transformed cells decreased to 1/100, whereas that of viable 3Y1 cells decreased marginally. However, in both cases, the decrease in the viable cell number was small compared with the case of administration of aphidicolin alone. All these results show that the transformed cells are not protected through cell-cell interactions by the untransformed cells.

Irreversible arrest in early S phase by prolonged exposure to aphidicolin and its reduction in the simultaneous presence of cycloheximide As was shown in Fig. 4, the killing effect of aphidicolin on the transformed cultures was reduced when cycloheximide was added simultaneously. We examined the possibility that prolonged aphidicolin exposure causes cells to be irreversibly arrested in the S phase and that simultaneous addition of cycloheximide diminishes the irreversible effect. Stationary-phase SV-3Y1-C60 cells were reseeded sparsely with fresh medium containing aphidicolin. After a 9-h incubation in the presence of aphidicolin, cells retained the reversibility, because subsequent withdrawal of aphidicolin resulted in the normal progression of S, G2 and M phases (Fig. 7A). When the aphidicolin exposure was prolonged for another 12 h, the progression through S phase was partially inhibited after removal of aphidicolin (Fig. 7B). This inhibition was overcome when cycloheximide had been added during the prolonged period (12 h) of aphidicolin exposure (Fig. 7C).

The normal progression of the S phase was inhibited in the presence of cycloheximide (Fig. 7D). These results suggest that inhibition of the progression of the S phase in an unbalanced condition — although DNA-chain elongation is inhibited, other cellular processes occur normally — will cause cell damage.

DISCUSSION

Untransformed 3Y1 cells were inhibited from entering the S phase in the presence of cycloheximide, while cells of transformed 3Y1 cell lines were less inhibited. It is reasonable to suppose that such a differential effect of cycloheximide on the cell cycle progression between 3Y1 and its transformants causes the observed selective killing of the transformants after combined treatment with cycloheximide and aphidicolin. The doubling times of the cells used are 16.3 h (3Y1), 12.4 h (SV-3Y1-C60), 13.4 h (Py-3Y1-S2), 25.4 h (SR-3Y1-1), 17.9 h (E1A-3Y1-1) and 19.2 h (HR-3Y1-2).[8] Therefore, the alternative explanation that the differential effect of cycloheximide on aphidicolin-treated transformed cells and 3Y1 cells simply reflects the difference in growth rates is not adequate.

The suppression of the aphidicolin-induced killing of 3Y1 cells in combination with cycloheximide was less in exponential-phase cultures than in stationary-phase cultures. This is because the proliferating cells contained appoximately 40% of S-phase cells, most of which would not share the rescue benefit. On the other hand, in SV-3Y1-C60, stationary-phase cells were more sensitive to the combined treatment than exponential-phase cells. Stationary-phase SV-3Y1-C60 cells do not stably stop proliferation as stationary-phase 3Y1 cells do (see below), and probably accumulate damage during the inhibition of proliferation. The injured transformed cells will show increased susceptibility to the treatment. HR-3Y1-2 cells exhibited low sensitivity to the combined treatment compared with other transformed cells. This may be due to the marked inhibition of entry into the S phase by cycloheximide in HR-3Y1-2 cells (see Fig. 3).

The killing effects of aphidicolin on transformed cultures were reduced by cycloheximide but to lesser extents compared with that observed in the untransformed counterpart. The arrest in the early S phase became irreversible after prolonged (over 9 h) exposure to aphidicolin, but this irreversibility was diminished by simultaneous exposure to cycloheximide. Exposure of DNA synthesis inhibitor for a long period has been supposed to cause cell killing by inducing unbalanced growth (increase in cell size without increase in DNA content).[13-15] Cycloheximide inhibits cell size increase by inhibiting protein synthesis, and therefore will diminish the imbalance that is brought about by aphidicolin.

It is generally assumed that transformed cells do not cease proliferation and do not enter the resting state under the conditions under which normal cells do so. In tumor tissues, a substantial fraction of cells is in the non-proliferating (resting) state, and the resting cells are refractory to chemotherapeutic drugs that act by killing cycling cells.[16] In the present study, stationary-phase cells of SR-3Y1-1 and HR-3Y1-2 were arrested in the resting state, because there were lag phases before entry into the S phase when these cells were reseeded sparsely with fresh medium, as observed in stationary-phase 3Y1 cells. On the other hand, in the stationary phase cells of SV-3Y1-1, Py-3Y1-S2 and E1A-3Y1-1, the G1 period was merely increased (as opposed to G1-arrest) resulting in a decrease in the fraction of cells with S-phase and G2-phase DNA contents, because the fractions of cells which had entered the S phase increased continuously with incubation time after reseeding. When resting transformed cells (SR-3Y1-1 and HR-3Y1-2) entered the S phase after being reseeded sparsely with fresh medium, cycloheximide inhibited entry into the S phase to a greater extent than in the case of resting 3Y1 cells. Probably, the pathway to the S phase mediated by growth factors is bypassed in cells expressing the transforming genes. A similar situation has been described for the G1-phase cells expressing SV40 large T antigen.[3] Therefore, there is a possibility that some drugs can be developed that selectively drive resting cancer cells to enter the cell-cycle phase. The combination of these drugs and cells cycle-dependent anticancer drugs may offer good results in cancer treatment.

Combinations of caffeine and hydroxyurea,[17] and of dibutyryl cyclic AMP and hydroxyurea[18] have been proposed for cancer therapy based on a theory similar to that presented here. However, the effective concentrations of these drugs for protecting untransformed cells were relatively high (m*M* order), compared to that of cycloheximide (μM order). Cycloheximide inhibited the progression through the S phase and changed the irreversible arrest in the S phase induced by aphidicolin to reversible arrest, resulting in protection from aphidicolin-induced killing of transformed cells. If one can find or develop a G1-specific inhibitor that is effective at low doses, it should be useful to protect normal cells when used in combination with cell-cycle-dependent anticancer drugs.

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