Combined Actions of 5-Fluorouracil and 1-(2-Chloroethyl)-3-(4-methylcyclohexyl)-1-nitrosourea on Human Colonic Carcinoma Cells in Vitro

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ABSTRACT

Two lines of human colonic carcinoma cells have different sensitivities to 5-fluorouracil and 1-(2-chloroethyl)-3-(4-methylcyclohexyl)-1-nitrosourea (methyl-CCNU). The growth of the BE line is 50% inhibited by 330 μM 5-fluorouracil and 15 μM methyl-CCNU, and the HT-29 line is 50% inhibited by 85 and 140 μM, respectively. On cloning, 50% of BE cells are killed by 500 μM 5-fluorouracil and 6 μM methyl-CCNU, and the HT-29 cells are killed by 250 and 120 μM, respectively. When the drugs were combined, there were additive effects which occurred on cell growth of both lines and killing of BE cells. Synergism occurred in killing HT-29 cells when a low concentration of 5-fluorouracil was combined with methyl-CCNU. The synergism did not increase with increasing concentrations of 5-fluorouracil.

Both drugs caused growth delay of spheroids (HT-29 cells), an effect that was additive when the drugs were combined.

Growth inhibition of both lines in monolayer culture by 5-fluorouracil was more sensitive than was cell killing or inhibition of spheroid growth but, with methyl-CCNU, killing of BE cells was more sensitive than was growth inhibition. HT-29 cells showed similar sensitivity to methyl-CCNU in all three systems.

The type and sensitivity of drug effect seen in vitro depends on the particular drug used, the cell line tested, and the parameter measured.

INTRODUCTION

Patients with advanced colon carcinoma have a higher response rate to a combination of 5-fluorouracil and methyl-CCNU than to 5-fluorouracil alone in some (2, 13) but not all studies. Corbett et al. (6, 7) have found the combination active in vivo against some mouse colonic carcinomas, although others are not responsive (7). Two cell culture lines of human colonic carcinomas show differing sensitivities to methyl-CCNU (10). We report here the effects of combining methyl-CCNU and 5-fluorouracil on the growth and viability of monolayer cultures and on the growth of spheroids from one of the 2 lines.

MATERIALS AND METHODS

Cell Culture. Human colonic tumor lines BE and HT-29 were obtained from Dr. K. W. Kohn of the National Cancer Institute, the latter with Dr. Fogh’s permission. Dr. Giovanella had established the BE line from an omental metastasis of a male patient, and Dr. Fogh had established the HT-29 line from a primary adenocarcinoma of a female patient (11). Both lines were passaged once a week in Eagle’s α-minimal essential medium with 15% fetal calf serum, bicarbonate, L-glutamine, and phenol red at 37°C in an atmosphere of 10% CO2 and 90% air. We added gentamicin for all experiments but not to stock cultures. Cells were detached with 0.01% Pronase after rinsing with phosphate-buffered saline (containing, per liter: NaCl, 8 g; KCl, 0.2 g; Na2HPO4 (anhydrous), 1.15 g; KH2PO4, 0.2 g; d-glucose, 0.2 g; disodium EDTA, 0.2 g), washed with balanced salt solution plus 2% fetal calf serum, and resuspended in fresh medium. Under these conditions, the HT-29 line doubled in 19 hr and the BE line doubled in 21 hr. Both lines showed no evidence of Mycoplasma when tested regularly with a modification (17) of the method of Schneider et al. (14), which measured the ratio of the specific activities of [3H]uridine-labeled RNA to [3H]uracil-labeled RNA. Cells originally frozen in liquid nitrogen were regularly thawed to start new stock cultures.

Drug Treatment. Stock solutions of 5-fluorouracil (Sigma Chemical Co.) were prepared in distilled water 0.5 hr before use. After appropriate dilution, 25 μl were added to 1 ml of culture or 100 μl to 5 ml of culture. Methyl-CCNU (Drug Development Branch, National Cancer Institute) was stored at −15°C, and a stock solution was prepared in 95% ethanol 0.5 hr before use. After appropriate dilution in 95% ethanol, 2 μl were added to 1 ml of culture, or 10 μl were added to 5 ml of culture. All drug treatments were for 1 hr. This time was chosen to approximate to the concentration × time curve after rapid i.v. injections of drugs. Control cultures treated with similar volumes of diluents alone showed no change in cell growth.

Spheroids. To grow spheroids from HT-29 cells, we modified the method of Yuhas et al. (19) by suspending exponentially growing cells in 100-mm untreated plastic Petri dishes at a concentration of 10⁶ cells/15 ml in Eagle’s α-minimal essential medium. These cells formed spheroids without the agar base required in the experiments of Yuhas et al. (19). Independently, Barone et al. (4) have prepared spheroids of HT-29 cells in spinner flasks pretreated with silicone antifoam spray. After 8 to 10 days of incubation, groups of 20 spheroids were isolated and incubated a further 24 hr in 5 ml of fresh medium in 50-mm untreated plastic Petri dishes. We then added drugs for 1 hr, washed the spheroids twice, and transferred them one to a well in a cluster dish coated with agar. Each 16-mm well contained 1 ml of medium, which was changed twice a week. Spheroids were measured with the calibrated eyepiece of an inverted microscope. Spheroids with an approximate initial diameter of 350 ± 25 (S.E.) μm and which remained spherical throughout the experiment were included in the study. Their average diameter (2 values measured at right angles) was plotted against time. The BE tumor line did not form spheroids of a uniform shape and could not be measured, even when agar-coated dishes were used. Spheroids of HT-29 cells of 350 μm in diameter were labeled with tritiated thymidine for 24 hr, fixed, and sectioned, and autoradiographs were prepared (19). These showed active DNA incorporation in the nuclei of outer cells and reduced incorporation in central cells, with no necrotic cells present. These develop in spheroids of 450 to 500 μm diameter.

Growth Inhibition Studies. Cells from exponential cultures were harvested, and 2.5 × 10⁶ HT-29 cells and 1 × 10⁶ BE cells were distributed in 1 ml of medium in the 16-mm wells of cluster dishes. After 3 days to allow exponential growth, we refed the cells with 1 ml...
of fresh medium 3 hr prior to addition of drug. Drugs were added for 1 hr, and a sample was counted to determine cell number. After 1 hr, the cells were washed twice to remove the drug, refed, and incubated for 4 more days, when they were counted on a Coulter Counter. We did not observe any detachment of cells due to the drug treatment.

For each experiment, duplicate wells were set up with increasing concentrations of one drug in the presence of various levels of the second drug. These data were plotted as cell number against dose of the one varying drug, with a separate curve for each concentration of the second drug. Each curve gave a concentration of the first drug which produced 50% growth inhibition in the presence of a fixed concentration of the second drug. A plot of this inhibitory concentration plotted against the corresponding concentration of the second drug gave an isobologram (9) to show the type of drug interaction.

Colony Formation. Cells, harvested as before, were plated in 5 ml of medium in 25-sq cm flasks at a concentration of 2 x 10^4 cells/ml, incubated for 3 days, and refed with 5 ml of fresh medium 3 hr prior to drug addition. Drugs were added as above, and after 1 hr the cells were washed, and a single-cell suspension prepared by Pronase treatment. We counted the cells in a hemocytometer and made appropriate dilutions so that 50 to 100 colonies were obtained in 50-mm Petri dishes treated for tissue culture after incubation for 8 days (BE cells) or 11 days (HT-29 cells). Five dishes were prepared for each sample. After this time, we washed and fixed the colonies and stained them for 5 min with aqueous 0.01% crystal violet (BE cells) or 0.002% aqueous methylene blue (HT-29 cells). Colonies containing 50 or more cells were counted using a dissecting microscope. Plating efficiency was 52 ± 4% for HT-29 cells and 52 ± 3% for BE cells. These were taken as 100% survival for comparison with the drug-treated cultures. Survival curves were plotted for each drug alone and in combination. Each point represents the mean of at least 3 experiments. The predicted additive effect was calculated as the product of the fractional survivals obtained with each drug alone, expressed as a percentage.

To assess the effect of cell number on plating efficiency, untreated cells were plated with viable cells or with lethally irradiated cells in the same dish. Plating efficiency did not change in either case over a wide range of cell concentrations. We tested whether drug would carry over into dishes when large numbers of cells were added. Control cells plated in the presence of large numbers of cells exposed to lethal doses of drugs did not show a change in plating efficiency compared with control cells plated alone.

RESULTS

Growth Inhibition. The results of a typical growth inhibition experiment using monolayer cultures of HT-29 cells are shown in Chart 1. From data shown in Chart 1, isobolograms (Chart 2) were prepared for each cell line as described in "Materials and Methods." The straight line between 1.0 on each axis of the isobologram shows the drug interaction to be additive for both cell lines. Synergism would have produced a curve to the left, and antagonism would have given a curve to the right. Fifty % growth inhibition of the HT-29 and BE lines with 5-fluorouracil occurred at 85 and 330 μM, respectively and with methyl-CCNU at 140 and 15 μM, respectively.

Cell-Killing Effects. Fifty % of HT-29 cells were killed by 250 μM 5-fluorouracil or 120 μM methyl-CCNU, and 50% of the BE cells were killed with 500 μM 5-fluorouracil or 6 μM methyl-CCNU. The killing effects on HT-29 cells of the 2 drugs combined are shown in Chart 3. The concentration of methyl-CCNU was constant at 185 μM, while that of 5-fluorouracil increased. This concentration of methyl-CCNU for 1 hr killed 75 ± 3% of HT-29 cells, a point on the linear portion of the killing curve for increasing concentrations of this drug alone (data not shown). The observed killing by both drugs (lowest curve) shows marked synergism occurring at a very low concentration of 5-fluorouracil, less than the lowest concentration tested (2.5 x 10^-4 M). Increasing concentrations of 5-fluorouracil did not increase the cell killing in more than an additive way. The effect observed, therefore, is not true synergism but rather a single synergistic effect of a low concentration of 5-fluorouracil. The combination of a constant concentration (750 μM 5-fluorouracil) with increasing concentrations of methyl-CCNU was also synergistic (data not shown).

A concentration of 1 mm 5-fluorouracil reduced survival of BE cells by 77 ± 3% and was on the linear portion of the killing curve for increasing concentrations of this drug alone (data not shown). The observed and predicted survival curves of BE cells
treated with this concentration of 5-fluorouracil together with increasing concentrations of methyl-CCNU were similar, indicating an additive effect of the 2 drugs on this cell line (data not shown).

**Spheroids.** Exposure of HT-29 spheroids of 350 ± 25 µm in diameter to 5-fluorouracil and methyl-CCNU affected their growth (Chart 4B). After an initial delay, spheroids grew at the same rate as untreated controls. This delay increased with increasing concentrations of drug. An increase in the size of drug-treated spheroids occurred during the first 2 days, probably due to drug-induced increase in cell volume observed microscopically at this time. Exposure to both drugs produced a lag period equal to the sum of the lag periods for each drug alone (6 + 4 = 10 days; Chart 4B). The lag period was the growth delay shown by the time required for drug-treated spheroids to reach the same size as the control, in this case 800 µm, since growth was linear with all treatments at this size of spheroid. Also, the distance of the combined drug line parallel to control was the sum of the distances of each single drug line from the control (Chart 4B). A lower concentration of methyl-CCNU gave a similar additive effect (Chart 4A).

**Comparison of Different Systems.** The data for growth inhibition of monolayer cultures, cell killing in monolayers as shown by colony formation, and inhibition of spheroid growth were normalized as percentages and plotted against increasing drug concentrations for each cell line (Chart 5). For inhibition of monolayer cultures, the exponential growth portion of the inhibition curve from Day 3 to Day 7 was used, and the difference in cell numbers on these days was expressed as a percentage of the cell number on Day 3. For spheroids, the starting volume was subtracted from the volume on Day 14 and expressed as a percentage of the starting volume, calculated from the mean diameter.

The graphs for 5-fluorouracil show that all 3 systems of the HT-29 cell line are more sensitive than the 2 systems of the BE line (Chart 5A). Growth inhibition of each monolayer culture was more sensitive than cell killing, which was as sensitive as inhibition of spheroid growth for the HT-29 cells. The most sensitive curve, that of growth inhibition of monolayers of HT-29 cells, differed also in its sigmoid shape.

With methyl-CCNU, the 2 systems of the BE cell line were more sensitive than the 3 systems of the HT-29 line (Chart 5B). Unlike 5-fluorouracil, however, methyl-CCNU affected colony formation more than growth with the BE line. This was not so with the HT-29 line. Spheroid sensitivity, as in the case of 5-fluorouracil, was similar to that of colony formation for HT-29 cells. The killing curve for the BE cells was linear, unlike the other curves, probably because the methyl-CCNU concentrations used were not low enough to detect a shoulder.

**DISCUSSION**

Our results show that HT-29 cells are 4 times as sensitive to growth inhibition and twice as sensitive to cell killing by 5-fluorouracil as are BE cells; however, HT-29 cells are only one-ninth as sensitive to growth inhibition and one-twentieth as sensitive to cell killing by methyl-CCNU. These results are similar to those of Erickson et al. (10) who proposed that DNA repair in HT-29 cells may account for the differing sensitivities of these cell lines to methyl-CCNU. No reason is known for the difference with 5-fluorouracil.

The drugs together showed additive effects on cell growth of both lines and on cell killing of the BE line as might be expected from the apparently unrelated sites of biochemical action of each of the drugs (1, 10, 12). Unexpectedly, killing of HT-29 cells was markedly synergistic when the 2 drugs were used. The HT-29 line may become more sensitive to 5-fluorouracil in the presence of methyl-CCNU, but more likely low concentrations of 5-fluorouracil make the line more sensitive to methyl-CCNU, to which it is only one-twentieth as sensitive as the BE
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line. Since repair of DNA damage is a factor in this lack of sensitivity (10), 5-fluorouracil may inhibit this process. The drug has been reported as enhancing the effects of X-radiation (3, 18). Little is known about the effect of 5-fluorouracil on DNA repair. Even with the synergism observed in HT-29 cells, their sensitivity to methyl-CCNU was still much less than that of the BE line. The synergism occurred only at low concentrations of 5-fluorouracil and was not observed with increasing concentrations of 5-fluorouracil as shown by the marked increase over predicted values of the killing of HT-29 cells by methyl-CCNU at low but not high concentrations of 5-fluorouracil. High concentrations of 5-fluorouracil killed in an additive fashion with methyl-CCNU, as shown by the line parallel to that predicted for an additive effect of the 2 drugs (Chart 3).

Anticancer drugs inhibit cell growth by slowing their progress through the cell cycle and by killing cells. We do not know how these 2 processes interrelate although there is evidence that cell death requires progression of the drug-damaged cell through the cell cycle. Our experiments illustrate the sensitivity to 5-fluorouracil and methyl-CCNU of 3 systems. The first, inhibition of cell growth, is a combined effect of cell kill and slowing of cell growth, although the latter effect may be small when cells are treated for only 1 hr with drug. Cell killing is measured by formation of colonies of more than 50 cells, each assumed to arise from a single surviving cell. This is probably true, but microcolonies are ignored in this system and may represent drug-damaged cells which grow more slowly. Spheroids are more complex, with an outer layer of actively dividing cells, an inner layer of more slowly growing hypoxic cells, and, in the larger spheroids, necrotic central cells (8). Cells of a spheroid show varying times of cell cycle. This, with central necrosis, makes the growth pattern complex, especially after drug treatment. However, both methyl-CCNU and 5-fluorouracil affect oxygenated and hypoxic cells equally (16); this simplifies interpretation of the effects of these drugs on spheroid and monolayer growth.

The sensitivity of the various systems depend on the cell line and drug used. As discussed above, the HT-29 line was more sensitive to 5-fluorouracil and less so to methyl-CCNU. With 5-fluorouracil, growth inhibition was most sensitive with both cell lines, consistent with an effect on the cell cycle as well as in killing cells. The first effect would not be reversed in the time of growth inhibition experiments (4 days) but would be reversible in some cells in the longer period of the cloning experiments (8 or 11 days). With methyl-CCNU, killing of BE cells was the more sensitive parameter rather than growth inhibition. This is difficult to explain except by a reversed situation to that above where all the lethally damaged cells may not die during the time of the growth inhibition experiment (4 days) but did during the longer period (8 or 11 days) of the cloning experiment. This would be consistent with decreased activity of a DNA repair mechanism in this cell line (10). Each drug affected the spheroids of HT-29 cells differently. They were affected by 5-fluorouracil in the same way as colony formation, being less sensitive to inhibition than monolayer growth, perhaps because fewer cells in the spheroid were in sensitive stages (S and late G2) of the cell cycle than in monolayer cultures (8). With methyl-CCNU, on the other hand, growth inhibition was similar in both spheroids and monolayers, perhaps due to the relative non-specificity of the effect of this drug in the cell cycle (8). In investigations of single drugs or drug combinations, our data indicate that it is essential to use several test systems and more than one cell line of similar tissue origin. Even then, generalization is difficult.

Opinions differ on whether methyl-CCNU and 5-fluorouracil together offer any advantage over 5-fluorouracil alone in the treatment of colonic carcinoma. In vitro experiments do not give information on host toxicity and so make extrapolation to in vivo chemotherapy difficult. A peak blood level of 500 μM 5-fluorouracil can be achieved after i.v. bolus injection in humans, falling in 1 hr to 20 μM (5). Peak levels for 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea, a close analog of methyl-CCNU, are 18 μM sustained for 4 hr after p.o. administration (15). These concentrations are of the same order as most concentrations used in our experiments when time is taken into account. Our data suggest that certain patients whose tumors resemble the HT-29 cell line may respond best to either 5-fluorouracil alone or to lower doses of 5-fluorouracil with methyl-CCNU. Those with tumors resembling the BE line would do poorly if methyl-CCNU were not included in their treatment.

REFERENCES

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