Communication

5-Fluorouracil Incorporation into Human Breast Carcinoma RNA Correlates with Cytotoxicity*

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Donald W. Kufe† and Pierre P. Major‡

From the Divisions of Pharmacology and Medicine, Sidney Farber Cancer Institute, Harvard Medical School, Boston, Massachusetts 02115.

We demonstrate a highly significant relationship (p < 0.0001) between the incorporation of 5-fluorouracil in total cellular RNA and loss of clonogenic survival of the human MCF-7 breast carcinoma cell line. The extent of 5-fluorouracil incorporation in RNA is concentration- and time-dependent. Identical results are obtained in experiments employing thymidine to bypass the block of thymidylate synthetase and reverse inhibition of DNA synthesis. These studies suggest that the incorporation of 5-fluorouracil in RNA is the major mechanism of cytotoxicity in this human cell line.

5-Fluorouracil is a pyrimidine analog effective in the treatment of several human epithelial tumors (1-3). Several mechanisms of action may be responsible for the cytotoxic effects of 5-FUra: 1) the conversion of 5-FUra to FdUMP which binds irreversibly to thymidylate synthetase and thereby inhibits DNA synthesis (4) and 2) the conversion of 5-FUra to FUTP, which is incorporated into RNA and thereby disrupts RNA function (5-12). The relative importance of each cytotoxic mechanism may be dependent upon varying patterns of intracellular 5-FUra metabolism (13).

5-FUra incorporates to a significant extent in all species of RNA and interferes with their function (5-12). The formation of (5-FUra)RNA is enhanced by the simultaneous administration of 10^{-3} M dThd and certain antimetabolites (14, 15). This effect is associated with increased antitumor activity (14, 15). These findings suggest that the amount of 5-FUra incorporated into RNA under varying conditions of drug concentration and exposure time should be related to cytotoxicity as measured by colony formation. This direct correlation, however, has not been previously established. Moreover, previous attempts to correlate 5-FUra cytotoxicity of human tumor cells with inhibition of DNA (16, 17) or of ribosomal RNA (18) synthesis have been unsuccessful.

The experiments described here demonstrate a highly significant relationship between incorporation of 5-FUra into total cellular RNA and loss of clonogenic survival of the human MCF-7 breast carcinoma cell line as determined by colony formation. This correlation is maintained in similar experiments employing thymidine to bypass the block of thymidylate synthetase and completely reverse inhibition of DNA synthesis. The results suggest that the incorporation of 5-FUra in RNA is the major mechanism of cytotoxic action in this human cell line.

MATERIALS AND METHODS

Cell Culture—The human breast carcinoma cell line, MCF-7, was obtained from the Michigan Cancer Foundation, Detroit, MI. Cells were grown as a monolayer in α-minimal Eagle's medium with 10% heat-inactivated, dialyzed fetal calf serum, 1% l-glutamine, 100 μg of penicillin/ml, and 100 units of streptomycin/ml at 37 °C in a 5% CO₂ atmosphere.

Incorporation of 5-FUra into Nucleic Acids—MCF-7 cells in logarithmic phase at a concentration of 1 × 10⁶ cells/tissue culture dish (100 × 30 cm) (Palloncica, Oxnard, CA) were washed twice with phosphate-buffered saline and then incubated in 4 ml of complete medium with 50 μCi of [³H]5-FUra (2.8 Ci/mmol; Amersham Corporation, Arlington Heights, IL) and 5 μCi/ml of H₂¹⁷O (carrier-free; New England Nuclear) for varying time periods (3.0 to 24.0 h). Following drug exposure, the cells were harvested with a rubber policeman and washed three times with 5 ml of phosphate-buffered saline. Cells were then counted, resuspended in phosphate-buffered saline and digested with the addition of 2.5 mg of pronase B (Calbiochem-Behring Corp., La Jolla, CA) in 2 ml of 0.01 M Tris (pH 7.4), 0.01 M EDTA, and 0.05% sodium dodecyl sulfate. Subsequent purification was accomplished by phenol extraction. The nucleic acids were then precipitated by the addition of a 1:10 volume of 4 M NaCl and two volumes of absolute ethanol. The nucleic acids were then analyzed by cesium sulfate gradient centrifugation (19). The [³H]5-FUra and ³P counts banding in the RNA region (between density 1.62 and 1.68 gm/ml) and DNA region (between density 1.42 and 1.48 gm/ml) of a gradient were determined and used as a measure of the incorporation of 5-FUra into RNA as well as the relative synthetic rates of RNA and DNA. Similar incorporation studies were done with 10⁻⁵ M dThd present in the incubation media.

Digestion of [³H]5-FUra-labeled RNA—MCF-7 cells were labeled with [³H]5-FUra for 6 h in a manner similar to that described above. The total cellular nucleic acids were purified by pronase digestion, phenol extraction, and ethanol precipitation. The RNA fraction was degraded to nucleosides by previously described techniques (20, 21) using RNAse A, snake venom phosphodiesterase, and alkaline phosphatase. The nucleosides were analyzed by high pressure liquid chromatography on a Micropak AX-10 ion exchange column (Varian Associates, Palo Alto, CA) using 80% acetonitrile, 20% 0.01 M K₂HPO₄, pH 2.85, as eluent. Eluents were performed after the addition of appropriate markers. Fractions (0.4 ml) were collected and assayed for tritium counts.

Clonogenic Survival of MCF-7 Cells—MCF-7 cells in logarithmic growth phase were washed twice in phosphate-buffered saline and incubated for various time periods in medium containing 5-FU at final concentrations ranging from 10⁻⁷ to 10⁻⁴ M. Cells were exposed to these drug concentrations for 3, 6, 12, and 24 h. Following drug exposure, the cells were harvested by trypsinization, washed, and resuspended in culture dishes (60 × 15 mm) (Palloncica) at a concentration of 1 × 10⁴ cells/plate. After 3 weeks, the cells were fixed with ethanol and air dried. Colonies greater than 50 cells were counted after staining with 10% Giemsa (Harleco, Gibbstown, N.J.). Viability was determined by the ratio of colonies formed by 5-FUra-treated cells as compared to untreated cells × 100. Similar clonogenic survival studies were done with 10⁻³ M dThd present in the incubation media. Thymidine suicide experiments were performed as previously described (22).

RESULTS

Cesium sulfate gradient analysis was employed to monitor the incorporation of 5-FUra into cellular nucleic acids. This
methodology permits a distinct separation of RNA (banding between density 1.62 and 1.68 gm/ml) and DNA (banding between density 1.42 and 1.48 gm/ml). Fig. 1 shows the incorporation of [3H]5-FUra and 32P into RNA and DNA during incubation periods of 3, 6, and 12 h. Significant amounts of [3H]5-FUra are detectable within the RNA regions of these gradients, while less than 10% of the tritium radioactivity incorporated into total nucleic acids is associated with the DNA region. The labeling with 32P measures newly synthesized RNA and DNA, and the ratio [3H]5-FUra/32P in the RNA region of the gradients serves as a measure of the relative incorporation of 5-FUra into RNA and corrects for variable RNA synthesis rates when results are compared under varying experimental conditions or with different cells.

It is important to demonstrate that all the tritium label detectable in RNA represents incorporation of [3H]5-FUra as opposed to possible metabolites. MCF-7 cells were labeled with [3H]5-FUra and the RNA fraction resulting from Cs2SO4 centrifugation was purified and digested to nucleosides for analysis by high pressure liquid chromatography. The nucleoside profile is illustrated in Fig. 2 with the addition of appropriate markers. The tritium counts clearly co-migrate with 5-FUrd, and there is no significant incorporation of label in other nucleosides such as uridine. Thus, the tritium-labeled radioactivity detectable is specific for the incorporation of [3H]5-FUra. The incorporation of tritium label in the DNA region of the gradient will be considered elsewhere.

The relevance of 5-FUra incorporation into cellular RNA to the biologic effects of the drug were studied by comparing the amount of 5-FUra incorporation with the clonogenic survival of MCF-7 cells. Cells were exposed to 5-FUra. The use of this concentration of dThd does not affect incorporation of 5-FUra in RNA and permits the specific determination of the effect of (5-FUra)RNA formation on cytotoxicity.

Fig. 3 illustrates the effects of 5-FUra exposure on the clonogenic survival of MCF-7 cells. Cells were exposed to concentrations of 5-FUra (10-7 to 10-4 M) alone and in combination with 10-5 M dThd for periods of 3, 6, and 12 h. Simultaneous exposure of MCF-7 cells to 10-5 M dThd had no effect on the cytotoxicity of 5-FUra. Loss of clonogenic survival was a function of both drug concentration (C) and time (T) of exposure. The drug concentrations resulting in 50% clonogenic survival (ID50) were proportionately less as a function of increasing duration of drug exposure. The product (C × T) of the ID50 concentration and the duration of exposure for each of the three different exposure periods was approximately constant as stated in the legend to Fig. 3. The dotted lines represent the percentage of control survival for cells treated with high levels of [3H]dThd in "thymidine-suicide" experiments (22). Thus, approximately 30% of MCF-7 cells were in S-phase during the 3-h exposure, while greater than 80% were in S-phase during the 12-h exposure.

The relationship between the incorporation of 5-FUra into

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\text{Fraction Number} \quad \text{RCM} \times 10^3
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\text{Fraction Number} \quad \text{cpm} \times 10^3
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\text{Fraction Number} \quad \text{cpm} \times 10^3
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Cytotoxicity and Formation of (5-Fluorouracil)RNA

Fig. 3. Clonogenic survival of MCF-7 cells following exposure to 5-FU. MCF-7 cells were exposed to 5-FU alone (©) or in combination with $10^{-5}$ M dThd (©) for 3, 6, and 12 h at concentrations ranging from $10^{-7}$ M to $10^{-4}$ M. After the appropriate drug exposure, the cells were washed, trypsinized, and reseeded in drug-free medium. Viability as determined after 21 days by scoring colonies greater than 50 cells. Per cent viability was determined by the ratio of colonies formed by 5-FU treated cells compared to untreated cells x 100. The C x T values for the drug concentration resulting in 50% survival are: A, $2.7 \times 10^{-4}$ M x h; B, $1.8 \times 10^{-4}$ M x h; and C, $1.7 \times 10^{-4}$ M x h.

Fig. 4. Relationship between MCF-7 clonogenic survival and incorporation of 5-FU into cellular RNA. Relationship of MCF-7 clonogenic survival and $[^3H]$5-FU incorporation into RNA (picomoles/4 x 10^6 cells) at 5-FU concentrations of 1 x $10^{-7}$ M (©), 5 x $10^{-7}$ M (©), 1 x $10^{-6}$ M (©), 5 x $10^{-6}$ M (©), 1 x $10^{-5}$ M (©), 5 x $10^{-5}$ M (©), 1 x $10^{-4}$ M (©), and time periods ranging from 3 to 24 h. A, no thymidine present during incubation with 5-FU; B, $10^{-4}$ M dThd present during 6-FU incubation.

MC7 cellular RNA with the effect of the drug on clonogenic survival was determined by measuring the amount of $[^3H]$5-FUra incorporation into RNA over a wide range of C x T values. Incorporation studies were performed with $[^3H]$5-FUra alone at concentrations ranging from 1 x $10^{-7}$ to 1 x $10^{-2}$ M and in combination with $10^{-5}$ M dThd, and the amounts incorporated into RNA during the various incubation periods (3.0 to 24.0 h) was determined by cesium sulfate gradient analysis as shown in Fig. 1. Clonogenic survival for each 5-FUra concentration was derived from the cloning data shown in Fig. 3, as well as other data derived from experiments performed during a 24-h incubation. Using a probit model (23) the log (C x T) correlated significantly with picomoles of 5-FUra incorporated into RNA (5-FUra alone: coefficient $[R] = 0.982, p < 0.0001$; 5-FUra and $10^{-3}$ M dThd: coefficient $[R] = 0.983, p < 0.0001$) and with log per cent survival (5-FUra alone: coefficient $[R] = -0.797, p < 0.0001$; 5-FUra and $10^{-3}$ M dThd: coefficient $[R] = -0.794, p < 0.0001$). There was also a highly significant relationship between incorporation of 5-FUra in RNA and survival; the probit analysis of this relationship is illustrated in Fig. 4 (5-FUra alone: coefficient $[R] = -0.896, p < 0.0001$; 5-FUra with $10^{-5}$ M dThd: coefficient $[R] = -0.892, p < 0.0001$). This relationship is best illustrated over a 5-FUra concentration range of $10^{-4}$ to $10^{-3}$ M, while the remaining points cluster at the ends of the curves.

DISCUSSION

The results of this study demonstrate a highly significant relationship between the incorporation of 5-FUra in RNA and loss of clonogenic survival. This relationship had not been previously established, although several studies have suggested that the incorporation of 5-FUra into RNA is one mechanism responsible for producing lethal cellular events (8-10). Our studies confirm these observations and demonstrate that there is a concentration- and time-dependent incorporation of 5-FUra in RNA, and that the loss of clonogenic survival also follows a C x T relationship.

The loss of MCF-7 clonogenic survival was not a function of cells in S-phase as determined by the thymidine-suicide experiments. This analysis suggests that cells in all phases of the cell cycle were susceptible to the effects of 5-FUra, which is consistent with disruption of RNA function, rather than inhibition of DNA synthesis. Furthermore, 5-FUra incubations were performed with simultaneous administration of $10^{-5}$ M thymidine. This concentration of thymidine reverses the inhibitory effect of 5-FU on DNA synthesis as determined by $^{32}$P incorporation into DNA. Under these conditions, identical results were obtained for both clonogenic survival of the MCF-7 cells and the extent of 5-FU incorporation into RNA, giving further support to incorporation of 5-FU in RNA as the primary mechanism responsible for cytotoxicity in the MCF-7 cell line.

Our studies correlating the extent of 5-FU incorporation
Cytotoxicity and Formation of (5-Fluorouracil)RNA into total cellular RNA with cytotoxicity suggests that this is one process responsible for cell death which represents an end point in drug activation and obviates the need to monitor the various enzyme activities involved in drug metabolism (21). This approach might be employed as a useful clinical marker to monitor lethal events induced by 5-FUra and to study approaches with modulating agents designed to enhance the effectiveness of 5-FUra by increasing the formation of (5-FUra)RNA.

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