Combination Therapy with 5-Fluorouracil and IFN-α2a Induces a Nonrandom Increase in DNA Fragments of Less Than 3 Megabases in HT29 Colon Carcinoma Cells

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ABSTRACT

We have used pulsed-field gel electrophoresis to examine 5-fluorouracil (5FU)-induced DNA double-strand breaks (DSBs), both with and without modulation by IFN-α2a (IFNα), in HT29 human colon adenocarcinoma cells. Although 24-h treatment with either 10 μM 5FU or 500 units/ml IFNα did not result in significant DNA fragmentation, the combination of 5FU + IFNα resulted in a significant increase in DNA DSBs versus either drug alone (P < 0.05). The pattern of fragmentation induced by treatment with 5FU + IFNα was compared to that induced by γ-irradiation, which generates lesions at random sites, digestion with NotI restriction endonuclease, which cleaves at the specific sequence 5'...GCGGCCGC...3', and HhaI restriction endonuclease, which cleaves at the specific sequence 5'...GCGC...3'. 5FU + IFNα resulted in a specific pattern characterized by the accumulation of fragments of <3 Mb in the absence of fragments of >3 Mb, which differed from that of γ-irradiation and restriction endonuclease digestion. Because neither morphological nor DNA fragmentation characteristic of apoptosis was observed after 5FU + IFNα treatment, the nonrandom pattern of DSBs that was observed did not appear to be the result of the initiation of programmed cell death within these cells.

INTRODUCTION

The primary biochemical target of 5FU is TS, which is inhibited by the 5FU anabolite,FdUMP (1). TS inhibition results in thymidine depletion and deoxyuridine accumulation, which can interfere with DNA synthesis and repair (2, 3). DNA single-strand breaks (4, 5) and DSBs (5, 6) ultimately result.

IFNα augments the activity of 5FU both in vitro (7) and in vivo (8) by inducing thymidine phosphorylase and increasing 5FU activation (9). We chose to examine the effects of IFNα modulation on the induction of DSBs using PFGE because of its ability to separate fragments clearly on the basis of size and its improved sensitivity compared to the older techniques of sucrose sedimentation and neutral elution (10). Our previous study of the effects of 5FU + IFNα on DNA fragmentation in the 3–5-Mb range demonstrated that there was no increase in DSBs with 5FU alone, but with the combination of 5FU and IFNα, a 3-fold increase in DSBs was observed (11). Interestingly, the greatest relative increase was observed in the fragments <3 Mb in size, as opposed to RT, which produced predominantly larger DNA fragments (>5 Mb), suggesting a difference in fragmentation pattern. We now report the results of further studies of 5FU-induced DNA fragmentation in the 1–3 Mb-range, both with and without IFNα modulation. In addition, we compared the fragmentation pattern induced by 5FU + IFNα to that induced by RT, which results in lesions at random sites (12–14), and digestion with either NotI restriction endonuclease, which recognizes the infrequently occurring sequence 5'...GCGGCCGC...3', or HhaI restriction endonuclease, which recognizes the sequence 5'...GCGC...3', to better characterize the distribution of DNA DSBs induced by 5FU + IFNα.

MATERIALS AND METHODS

Cell Culture. HT29 cells were maintained in standard medium (RPMI 1640; Life Technologies, Inc., Grand Island, NY) with 10% FBS (Life Technologies) and 1% penicillin-streptomycin at 37°C in 5% CO₂. Exponentially growing cells were used for all experiments. Twenty-four h prior to drug exposure, cells were cultured in labeling medium (RPMI 1640 with 10% dialyzed FBS, 1% penicillin-streptomycin, and 1 μCi/ml [methyl-3H]thymidine). For drug exposures, cells were incubated in treatment medium [folate-free RPMI 1640 (Life Technologies) with 10% dialyzed FBS, 1% penicillin-streptomycin, and 80 nm 5-methyl-tetrahydrofolate; Ref. 15].

Drugs and Reagents. Recombinant IFNα was a gift of Hoffman-LaRoche (Nutley, NJ). 5FU was from Lyphomed (Rosemont, IL). The NotI and HhaI restriction endonucleases were from New England Biolabs (Beverly, MA). Schizosaccharomyces pombe and Hansenula wingei DNA size markers as well as chromosomal grade agarose were from Bio-Rad (Her...
cules, CA). All other reagents were from Sigma Chemical Co. (St. Louis, MO).

**Radioisotopes.** [methyl-3H]Thymidine (20 Ci/mmol) was obtained from New England Nuclear (Boston, MA).

**Preparation of Agarose Plugs for PFGE.** HT29 cells were seeded such that, at the time of harvest, each 25-cm² flask contained approximately 4 × 10⁶ cells in logarithmic growth phase. The cells were incubated with 2 ml of labeling medium for 24 h. After labeling, cells were exposed to 10 μM 5FU, 500 units/ml IFNa, or the combination of 10 μM 5FU and 500 units/ml IFNα, or to no drug treatment for 24 h in 4 ml of treatment medium. Cells were washed and placed into agarose plugs, and DNA was isolated as described previously (11). Following DNA isolation, the plugs were washed four times without agitation for 30 min with 50 ml of wash buffer (20 mM Tris, pH 8, and 50 mM EDTA) with 1 mM phenylmethylsulfonyl fluoride added to the second wash to neutralize any residual proteinase K. The plugs were protected from direct light throughout preparation.

**Irradiation of Plugs.** To determine the incidence and pattern of DSBs with RT, plugs of non-drug-treated cells were irradiated at room temperature in wash buffer using 0.66-MeV photons from a 37 Cs source (Picker International, Cleveland, OH) at the Radiation Biology department of the Albert Einstein College of Medicine.

**Restriction Endonuclease Digestion.** To determine the incidence and pattern of DSBs with restriction endonuclease digestion, plugs of non-drug-treated cells were digested with 0.5–10 units of Nol or 0.1–1.0 unit of HhaI for 1 h at 37°C.

**PFGE.** The plugs were sealed in 0.6% chromosomal grade agarose and electrophoretically size-fractionated in a CHEF-DR II (Bio-Rad) for 50 h at 3 V/cm, with a linearly ramped switch time from 250 to 900 s in 40 s intervals. The resulting agarose cubes were melted and counted by liquid scintigraphy (11). This PFGE protocol resulted in adequate separation of H. wingei marker chromosomes (1–3 Mb) and >99% recovery of tritiated DNA from the plugs.

**PFGE Data Analysis.** The amount of DNA in each slice was expressed as the cpm%slice (100 × cpm_slice/cpm_lane). The fraction of activity released from the well into the gel matrix has been used as a measure of DSBs (16–18). For this study, the FAR% for a treatment is defined as the mean value for the cpm%slice minus the cpm%well. The slope is defined as the change in cpm%slice with increasing electrophoretic mobility (decreasing fragment size). Multiple regression analysis was used to compare treatments in terms of their FAR% or slope. Two-tailed Student’s t tests were used to compare cpm% between individual slices and size ranges. Bonferroni criteria were used when multiple comparisons were made.

**Measurement of Apoptosis.** Death by apoptosis was assessed using two methods. HT29 cells were treated with 10 μM 5FU + 500 units/ml IFNα for 24 h or with 10 μM 5FU + 500 units/ml IFNα for 24 h, followed by washing twice with medium and then incubating for an additional 24 h in drug-free medium. Results from the drug-treated samples were compared to matched controls. First, the generation of oligosome-sized DNA fragments (19) was analyzed as described (20, 21).

Briefly, low molecular weight DNA was isolated from adherent cells and electrophoretically size-fractionated using 1.7% agarose gels containing ethidium bromide. Second, the percentage of cells exhibiting morphological alterations characteristic of apoptotic death was quantified using uptake of acridine orange/ethidium bromide as an index (22). In brief, cell monolayers grown on chamber slides were stained in situ by overlaying with 25 μl of 1× PBS, containing 100 ng of acridine orange and 100 ng of ethidium bromide. Eight individual chambers for each of three experimental conditions were examined using fluorescence microscopy, and the percentage of cells in the final stages of apoptosis, which stain bright orange and exhibit highly condensed or fragmented chromatin, was determined.

**RESULTS**

**Effect of Chemotherapy on the Induction of DSBs.** Previously, it was demonstrated that treatment of HT29 cells with 5FU + IFNα resulted in a significant increase in DNA fragmentation, whereas fragmentation following treatment with 5FU alone was indistinguishable from that of untreated cells (11). Therefore, the effect of the combination of 5FU + IFNα was studied to confirm the results of the previous study and to rule out IFNα as the sole cause of this increase. In the current study, 24-h exposure of HT29 cells to either 10 μM 5FU or 500 units/ml IFNα did not result in an increase in DNA fragmentation over control, as measured by their FAR%. While either drug alone produced no increase in fragmentation, the combination 5FU + IFNα resulted in a significant increase in FAR% over either drug alone (P < 0.05) and control (P < 0.01; Figs. 1 and 2, inset). Furthermore, the increase in FAR% with 5FU + IFNα was entirely due to an increase in fragments of <3 Mb (Fig. 2).

**Effect of Duration of Exposure on 5FU + IFNα-induced DSBs.** DNA fragmentation was measured at sequential time points following exposure to 5FU + IFNα. A significant
increase in FAR% was not observed until 16 h after exposure ($P < 0.05$). At 16 h, the greatest relative increase in DNA fragmentation was observed in the 1–3-Mb (Fig. 3B) and <1-Mb fragments (Fig. 3C), as compared to the >3-Mb fragments (Fig. 3A). Further DNA fragmentation was observed for the 1–3-Mb and >1-Mb fragments between 16 and 24 h, but not for the larger fragments.

**Effect of RT on DSB Induction.** To determine whether the distribution of fragments induced by 5FU + IFNα differed from the random pattern induced by RT, direct comparisons were made. As shown in Fig. 4, RT resulted in a dose-dependent increase in the >3-Mb and 1–3-Mb fragments, without an increase in the <1-Mb fragments, whereas 5FU + IFNα resulted in an increase in the 1–3-Mb and <1-Mb fragments, without an increase in the >3-Mb fragments. For example, cells exposed to 10 Gy of RT exhibited an approximately 3-fold increase in the percentage of DNA fragments in the >3 Mb and 1–3-Mb size range, without an increase in fragments <1 Mb. In contrast, treatment with 5FU + IFNα resulted in the percentage of DNA fragments in the 1–3-Mb and <1-Mb range increasing by approximately 3-fold, without an increase in the >3-Mb fragments.

**Effect of Restriction Endonuclease Digestion on DSB Induction.** Nonrandom, site-specific DSBs were induced by digestion with either the NotI restriction endonuclease, which has an 8-nucleotide recognition sequence, or the HhaI restriction endonuclease, which has a 4-nucleotide recognition sequence. The patterns induced by these nonrandom damaging treatments differed from that induced by RT, as expected (Fig. 4), with increases in all fragment sizes, but more profound increases (2–11-fold) in the smaller DNA fragments. NotI induced a 1.5–6.1-fold change in 1–3-Mb fragments and a 2.0–5.7-fold change in <1-Mb fragments in a dose-dependent manner. HhaI induced a 1.7–4.3-fold change in 1–3-Mb fragments and a 2.5–11-fold change in the <1 Mb fragments in a dose-dependent manner. The percentage of DNA fragments in the >3 Mb fragments were also increased, but to a lesser degree (1.8–2.9-fold). Thus, both treatment with 5FU + IFNα and the restriction endonucleases differed from RT in that the greatest relative increase was in the fragments <3 Mb in size. However, in contrast to 5FU + IFNα, treatment with the restriction endonucleases also resulted in an increase in the >3-Mb fragments.

**Effect of Pattern on Determination of DNA Damage.** To control for the effect treatment intensity with different modalities on the pattern of DNA damage, treatment with 5FU + IFNα was also compared with doses of RT, NotI, or HhaI, which resulted in an equivalent amount of total DNA fragmentation (Fig. 5). 5FU + IFNα resulted in a FAR% ± SE equal to 16.7 ± 1.5%. In terms of FAR%, 1 Gy of RT (19.8 ± 2.2%), 0.5 unit of NotI (17.7 ± 4.3%), and 0.1 unit of HhaI (18.7 ± 1.3%) generated comparable quantities of DNA fragmentation (Fig. 5, inset). Although equivalent quantities of DNA fragments were generated by these treatments, the slope of the 5FU + IFNα-induced fragmentation curve was significantly larger than that induced by RT, NotI, or HhaI ($P < 0.01$) due to the increased
ratio of intermediate (1–3 Mb) and small (<1 Mb) fragments to larger (>3 Mb) fragments, as compared to the other treatments, confirming that the pattern induced by SFU + IFNα differed from that induced by the other treatments at equivalent treatment intensities.

Effect of SFU + IFNα as an Inducer of Apoptosis. To determine whether the fragments of <3 Mb induced by SFU + IFNα represented a precursor to the nonrandom pattern of DNA fragmentation observed with apoptosis, samples exposed to SFU + IFNα for 24 h and kept in drug-free medium for 24 h after drug washout (see “Materials and Methods”) were analyzed by DNA fluorescent staining. As shown in Table 1, the extent of apoptosis following these treatments was indistinguishable from that of untreated cells. Electrophoretic size fractionation of low molecular weight DNA preparations from these samples also failed to demonstrate DNA laddering on ethidium bromide staining (data not shown).

DISCUSSION

IFNα augments the effects of SFU by increasing the expression of thymidine phosphorylase (9), resulting in higher

**Table 1.** DNA fluorescent staining for apoptotic cells

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**Fig. 4.** 5FU (10 μM) + 500 units/ml IFNα (Δ, n = 16) produced a pattern with a greater slope (quantity/ decrease in fragment size) than treatments with an equivalent FAR% (P < 0.01): 1 Gy of RT (■, n = 6); 0.5 unit of NotI for 1 h at 37°C (▲, n = 11); and 0.1 unit of HhaI for 1 h at 37°C (□, n = 4). Inset, equivalence (P is nonsignificant) of FAR% for SFU + IFNα (FI; ◊), RT (□), NotI digestion (N1; ■), and HhaI digestion (HI; □). Points, means of n determinations; bars, SE.

**Fig. 5.** DNA fragment distribution by size. Cells were either untreated (□) or treated with radiation therapy (■: Column 2, 1 Gy; Column 3, 10 Gy), NotI digestion (□: Column 4, 0.5 unit; Column 5, 1.0 unit; Column 6, 10 units), HhaI digestion (□: Column 7, 0.1 unit; Column 8, 0.5 unit; Column 9, 1.0 unit) or 10 μM SFU + 500 units/ml IFNα (□) for 24 h. Percent of DNA in the following size ranges: from the forward edge of the well to the 3-Mb size marker (>3 Mb); the 3-Mb to the 1-Mb size marker (1–3 Mb); and the 1-Mb size marker to the end of the gel (<1 Mb). Columns, means of at least 4 determinations; bars, SE. *, significantly different from control (P < 0.05); **, significantly different from control (P < 0.005).

FDUMP levels and greater TS inhibition. This mechanism is consistent with the observations that IFNα could not potentiate direct TS inhibition with CB3717 or thymidine deprivation (5). The increased TS inhibition with IFNα modulation of SFU ultimately results in an increase in DNA DSBs, as compared to SFU alone, resulting in a pattern that differs from that induced.
by RT (11). With RT, the greatest relative increase is in the largest DNA fragments (>5 Mb), whereas with 5FU + IFNα, it occurred in the <3-Mb fragments. A similar pattern was reported previously in the HT29 cell line, following treatment with 5-fluorodeoxyuridine (6) and CB3717, a folate analogue inhibitor of TS (23).

In the current study, although neither 5FU alone nor IFNα alone induced DSBs in HT29 cells, the combination of these two drugs did generate DSBs. An exposure longer than 24 h may have been required to demonstrate DNA DSBs with 5FU alone. For example, in GC/ccl cells, it was shown that modulation of 5FU with IFNα, in addition to increasing single-strand breaks and DSBs, resulted in a more rapid induction of DNA damage (5). Alternatively, IFNα, by increasing levels of thymidine phosphorylase, leading to increased conversion of 5FU to FdUMP (9), may promote cytotoxicity via a DNA-mediated mechanism, whereas with unmodulated 5FU, other mechanisms may predominate.

The time course of 5FU + IFNα-induced DNA fragmentation suggests that it is a late event (>8 h). We have previously shown that TTP pools are maximally depleted by 6–8 h after treatment with 5FU + IFNα at the doses used in this study (11). This delay between maximal TTP depletion and the development of DSBs may represent a passive process, such as the accumulation of single-strand breaks, leading to functional DSBs, or to an active process requiring protein translation or replication.

In characterizing the pattern of fragmentation, we found that treatment with 5FU + IFNα resulted in a nonrandom pattern of DNA DSBs, in contrast to comparable doses of RT, which induces lesions in deproteinized DNA at random sites (12–14). Treatment with RT at doses of 1–10 Gy resulted in a relative increase in the >3-Mb and 1–3-Mb fragments without a significant increase in the <1-Mb fragments. Treatment with 5FU + IFNα resulted in a relative increase in the 1–3-Mb and <1-Mb fragments without a significant increase in the >3-Mb fragments. We conclude that the DSBs induced by 5FU + IFNα are occurring in a nonrandom pattern because the pattern of fragmentation differs from that induced by a dose of RT, resulting in an equivalent amount of total DNA fragmentation.

To better define the effect of site-specific DNA-damaging agents, the patterns induced by treatment with two restriction endonucleases were also studied. These nonrandom patterns differed from that induced by RT, as expected; however, they also differed from that induced by 5FU + IFNα. Although the restriction endonucleases produced a relative increase in the <3-Mb fragments, as did 5FU + IFNα, they also produced a lesser increase in the >3 Mb fragments which was not observed in the 5FU + IFNα-treated samples. Therefore, 5FU + IFNα induced a DNA fragmentation pattern that was distinct from that induced by equivalent doses of RT, which induces DSBs at random sites, and restriction endonuclease digestion with HhaI or NotI, which induces DSBs at a sequence-specific site.

Furthermore, treatments resulting in an equivalent FAR% may not represent equivalent numbers of DSBs when their patterns of fragmentation are significantly different. For example, comparing the DSBs induced by 5FU + IFNα to 1 Gy of RT on the basis of FAR% would drastically underestimate the number of DSBs induced by 5FU + IFNα, because the fragments induced by 5FU + IFNα are smaller and, therefore, would require a greater number of DSBs for their production.

We also attempted to determine whether the observed nonrandom increase in the 1–3-Mb DNA fragments after treatment with 5FU + IFNα represents a precursor lesion to the nonrandom DNA fragmentation characteristic of apoptosis. Apoptosis has been observed in HT29 cells after treatment with short-chain fatty acids and polar solvents (24). However, an increase in the level of apoptosis was not observed immediately following treatment with 5FU + IFNα or at 24 h after drug washout. The HT29 cell line contains a mutant p53 gene (25), and the lack of functional p53 protein may conceivably have been responsible for the absence of apoptosis in response to the DNA DSBs induced by 5FU + IFNα. However, functional p53 protein was demonstrated not to be a prerequisite for DNA damage-induced apoptosis in the PC/JW colorectal carcinoma cell line after treatment with RT (26).

The mechanism by which 5FU + IFNα induces DSBs at specific sites remains open to speculation. Thymineless death requires a functional DNA replication apparatus (2), and the distribution of DNA replication initiation sites may be responsible for the pattern we have observed. Fragmentation in HT29 cells in response to 5-fluorodeoxyuridine could be prevented by expression of the Escherichia coli dUTPase gene (27), but not with aphidicolin, a DNA polymerase inhibitor (23). This suggests that dUTP misincorporation may play a role in fluoropyrimidine toxicity, and this is supported by recent data from our laboratory which shows a significant increase in the dUTP/TTP ratio in cells treated with 5FU + IFNα.4 As uracil-DNA glycosylase excises deoxycytidine and fluorodeoxycytidine residues from DNA (28), intracellular accumulation of dUTP with an associated decrease in TTP pools could result in repetitive misincorporation of dUTP and saturation of uracil-DNA glycosylase activity, resulting in DNA strand breaks. The sequence specificity (29) of this repair enzyme may be responsible for the nonrandom pattern of DNA DSBs observed. Furthermore, although the pattern of DNA DSBs was nonrandom, the absence of an increase in smaller, oligonucleosomal fragments in the 24–48-h period during and following exposure to drug suggest that this process is at least temporally distinct from apoptosis, which is also associated with nonrandom DNA DSBs, although these processes may not be totally unrelated.

Alternatively, fluoropyrimidine treatment could lead to purine/pyrimidine imbalance in HT29 cells (11). Purine/pyrimidine imbalance led to the activation of an endogenous endonuclease in FM3A mouse mammary tumor cells treated with 5-fluorodeoxyuridine (30, 31). An endogenous endonuclease may play a role in the induction of the nonrandom pattern observed in the current study. Identification of the mechanism and specific site of fluoropyrimidine-induced DNA damage is

4 S. Wadler, unpublished data.
important because it may provide insights into the mechanisms of both cytotoxicity and resistance.

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