The Importance of p53-Independent Apoptosis in the Intestinal Toxicity Induced by Raltitrexed (ZD1694, Tomudex): Genetic Differences between BALB/c and DBA/2 Mice

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ABSTRACT

The thymidylate synthase inhibitor raltitrexed (ZD1694, Tomudex) induces greater intestinal toxicity, manifested as diarrhea and weight loss, in BALB/c than in DBA/2 mice. No convincing pharmacokinetic or pharmacodynamic reason for this strain difference has been established. We have investigated whether this strain difference in response to raltitrexed is related to differential susceptibilities of intestinal mucosae to undergo apoptosis and also whether p53 expression, a critical factor in 5-fluorouracil-induced intestinal apoptosis and toxicity, modulates this response. Ten mg/kg or 100 mg/kg raltitrexed were administered as single or double i.p. injections 24 h apart to BALB/c, DBA/2, and p53−/− mice. Apoptosis, mitosis, and tissue damage were assessed in intestinal epithelium, and animal weight was recorded. BALB/c mice developed diarrhea and weight loss following 100 mg/kg × 2 raltitrexed, whereas DBA/2 mice did not. BALB/c mice were more sensitive than DBA/2 to induction of small-intestinal and colonic apoptosis 24 h following 100 mg/kg raltitrexed. Inhibition of mitosis was equivalent in both strains. Both strains showed histopathological damage to the small intestine after 100 mg/kg × 2 raltitrexed, but only BALB/c mice demonstrated colonic damage. p53-null mice showed the same level of small intestinal apoptosis as their wild-type counterparts 24 h after 100 mg/kg × 1 raltitrexed and also the same levels of intestinal toxicity 3, 5, and 7 days after 100 mg/kg × 2 raltitrexed. Thus, BALB/c mice were more susceptible to induction of intestinal apoptosis by raltitrexed than DBA/2 mice and also demonstrated more histopathological damage in the colon correlating with the induction of diarrhea and weight loss. In contrast to 5-fluorouracil, the intestinal apoptosis and toxicity induced by raltitrexed were p53-independent.

INTRODUCTION

Raltitrexed (ZD1694, Tomudex) is a novel quinazoline pure TS1-inhibitor that has been assessed in Phase III trials in advanced colorectal cancer (1, 2). Raltitrexed has similar efficacy in this disease to 5-FU modulated with leucovorin but demonstrates a different profile of side effects. One particularly troublesome side effect is diarrhea. Grade III/IV diarrhea occurs in ~14% of patients given this drug and if they have coexisting neutropenia, it may be life-threatening (2). A murine model has been established previously to identify determinants of the gastrointestinal toxicity of raltitrexed (3, 4). One inbred mouse strain (BALB/c) is much more sensitive to sequential administration of raltitrexed than another (DBA/2), with a maximum tolerated dose in BALB/c mice of 5–10 mg/kg/d × 5, compared with >500 mg/kg/d × 5 in DBA/2. This difference between the strains is primarily attributable to different susceptibilities to gastrointestinal toxicity. We have demonstrated differences between the two strains in histopathological gut damage and in induction of diarrhea and weight loss following raltitrexed treatment. However, to date, no convincing pharmacodynamic or pharmacokinetic explanation for this difference between the two strains has been established (3). The intestinal toxicity caused by raltitrexed administration can be reversed by leucovorin (4).

In addition to so-called “upstream” factors, which are directly related to the mechanism of action of a chemotherapeutic drug, the toxic effects of a cytotoxic agent are also influenced by “downstream” factors. These include the genes that modulate the thresholds at which particular cells undergo cell death (5, 6). Previous experiments have not demonstrated any differences in the baseline levels of thymidine, deoxyuridine, folate, and TS between the two strains (3). Also, the concentrations of raltitrexed in gut scrapes of intestinal mucosa were the same in BALB/c and DBA/2 mice at early time points after drug administration (3). We have therefore investigated whether the different susceptibilities of BALB/c and DBA/2 mice to intestinal toxicity following raltitrexed treatment are related to differential
Strain Differences in Raltitrexed-induced Gut Toxicity

We have therefore investigated whether intestinal epithelia from BALB/c and DBA/2 mice show different susceptibilities to the induction of apoptosis after raltitrexed administration congruent with the different levels of gastrointestinal toxicity demonstrated in the two strains. We have also used p53-null mice to examine whether, as with 5-FU, the apoptosis and intestinal toxicity induced by raltitrexed are influenced by p53 expression.

MATERIALS AND METHODS

Materials. Raltitrexed was a gift from Zeneca Pharmaceuticals (Macclesfield, United Kingdom) and was dissolved in 0.05 M sodium bicarbonate and the pH altered to 8.5–9.0.

Animals. Male BALB/c and DBA/2 mice and p53 wild-type (+/+ ) and null (−/−) mice on a mixed genetic background (11) of either sex were used. Mice were aged 10–12 weeks, and there were four mice in each experimental group. They were housed under conventional animal house conditions with food and water ad libitum and a 12-h dark/light cycle (lights on at 6 a.m. and off at 6 p.m.).

Treatment and Sampling. Raltitrexed was administered by bolus i.p. injection to 38 BALB/c and 38 DBA/2 mice on four schedules: (a) 10 mg/kg at 9 a.m. on day 0; (b) 100 mg/kg at 9 a.m. on day 0; (c) 10 mg/kg at 9 a.m. on day 0 and again at 9 a.m. on day 1; and (d) 100 mg/kg at 9 a.m. on day 0 and again at 9 a.m. on day 1. One group of 10 mice for each treatment schedule was weighed daily at 9 a.m., and records of state of health, viability, and nature of feces were kept. Four mice in each group were sacrificed by cervical dislocation at times ranging from 12 h to 10 days after administration of the last dose of raltitrexed, and their intestines were dissected and fixed in Carnoy’s fixative. Three-μm transverse sections of paraffin-embedded mid-small intestine and middle third of colon were stained with H&E.

Groups of four p53 wild-type and null mice received either a single injection of 100 mg/kg raltitrexed at 9 a.m. or two injections of 100 mg/kg raltitrexed at 9 a.m. on consecutive days and were sacrificed at time intervals ranging from 24 h to 7 days. Tissues were processed as for BALB/c and DBA/2 mice.

Scoring. Apoptosis was scored by a single observer (L. B.) who was blinded to the treatment administered. A proportion of the slides (~5%) were also scored in a blinded manner by a second observer (D. M. P.) to ensure reproducibility of the scoring procedure. Apoptosis and mitosis were scored on a cell-positional basis, analyzing 50 half-crypts/mouse using a light microscope as described in detail previously (12). Each cell position was scored as showing either a normal cell, apoptotic event, or mitotic event, with cell position 1 being at the crypt base (12). An apoptotic event was defined as the presence of one or more apoptotic bodies at a particular cell position. Apoptotic bodies are characterized by condensed chromatin and are easy to distinguish in sections of intestinal epithelium fixed in Carnoy’s fixative (12). Data are presented as mean and SD of the apoptotic/mitotic index (calculated as the percentage of counted cells that were apoptotic/mitotic) for a group of four mice and as plots smoothed over three cell positions (12) of apoptotic/mitotic cell index percentage against cell position along the crypt.

The number of cells per hemicypt and per hemivillus were used as indicators of tissue damage induced by the drug. These parameters were measured in 10 small intestinal crypts, 10 small intestinal villi (where possible, crypts and villi in continuity were used because these were most likely to represent a true longitudinal section), and 10 midcolonic crypts per mouse as described previously (10). Data are presented as means and standard errors in a group of four mice.

Clonogenic crypt survival was assessed 96 h following 2 doses of 100 mg/kg raltitrexed as described previously (13). Crypt width correction was applied (13).

Statistical analysis was by Student’s t test assuming unequal variance of the groups and by the modified median test described in detail previously (14, 15). A significance level of P < 0.05 was used for both tests.

RESULTS

Whole Animal Toxicity of Raltitrexed. None of the doses of raltitrexed used in current experiments were lethal to BALB/c or DBA/2 mice. Single or double doses of 10 mg/kg raltitrexed and a single dose of 100 mg/kg raltitrexed did not cause significant weight loss in either strain (Fig. 1). However, two doses of 100 mg/kg raltitrexed caused significant weight loss, maximal at 4 days in BALB/c mice (Fig. 1a), but no significant weight loss in DBA/2 mice (Fig. 1b), in a similar fashion to the experiments described in Ref. 3. The difference between the two strains at the time point of maximal weight loss (4 days) was highly statistically significant following two doses of 100 mg/kg raltitrexed (P < 0.0001). Additionally, BALB/c mice that received two doses of 100 mg/kg raltitrexed developed diarrhea between days 3 and 7–8, manifested as brown fecal staining around the anal margin, whereas DBA/2 mice produced formed fecal pellets throughout the experiment.

Effects of Raltitrexed upon Apoptosis and Mitosis. Fig. 2a and b demonstrate that for 72 h following a single injection of 100 mg/kg raltitrexed, BALB/c mice developed significantly more apoptosis in both small intestinal and midcolonic epithelium than DBA/2 mice. This difference was most marked at 24 h, the time point of peak apoptosis (P = 0.001 in
The zero time point represents the level of baseline apoptosis in untreated control mice. There were no statistically significant differences between the two strains in the levels of baseline small intestinal or midcolonic apoptosis. Previous experiments that analyzed small intestinal apoptosis at 3-hour intervals from 3–48 h after administration of raltitrexed in BDF1 mice indicated that 24 h was the time point of peak apoptosis.
apoptosis (data not shown). Analysis of the cell positional
distribution of apoptosis in small intestinal epithelium 24 h
following 100 mg/kg raltitrexed (Fig. 3a) demonstrated that cell
positions 8–12 showed peak levels of apoptosis in the BALB/c
strain, whereas cells higher up the crypt hierarchy (cell positions
14–16) developed peak levels of apoptosis in DBA/2 mice.
Modified median test analysis confirmed significant differences
between the two strains at cell positions 4–12. In midcolonic
epithelium, apoptosis was induced at cell positions near the
crypt base (cell positions 1–8) in BALB/c mice, whereas no
apoptosis above control levels was induced by 100 mg/kg ralti-
trexed in DBA/2 mice (Fig. 3b). The modified median test
confirmed statistically significant differences between the mid-
colons of the two strains between cell positions 1 and 9.

There were no significant differences in small intestinal ap-
optosis between BALB/c and DBA/2 mice after a single injection
of 10 mg/kg raltitrexed, and this dose caused no apoptosis above
baseline levels in the midcolon of either strain (data not shown).
Analysis of the apoptotic index percentage after two injections 24 h
apart of 10 mg/kg or 100 mg/kg raltitrexed revealed only minor
differences between BALB/c and DBA/2 mice (data not shown).
The second injection of raltitrexed was administered at 24 h when
crypt cells had stopped proliferating as a result of the first injection
(Fig. 2). As the cytotoxic effect of raltitrexed depends upon cells
undergoing active cell division, the effects of the second injection
are thus difficult to interpret.

One hundred mg/kg raltitrexed caused a similar suppression
of proliferation (mitosis) in small intestinal (Fig. 2c) and
midcolonic (Fig. 2d) epithelia of BALB/c and DBA/2 mice. The
elevated mitotic index in BALB/c midcolon at 72 h suggests that
the epithelium is attempting to repair the apoptotic damage
manifested at 24 h; this increase in proliferation was absent in
DBA/2 mice.

Changes in Crypt and Villus Cellularity after Ralti-
trexed. Two doses of 100 mg/kg raltitrexed caused changes in
the cellularity of small intestinal and midcolonic crypts and villi
(Fig. 4). There were minor differences in hemicrypt and hemiv-
illus cell numbers in control animals [small intestinal crypt 20.0
(BALB/c) versus 18.4 (DBA/2), P = 0.09; small intestinal villus
38.5 (BALB/c) versus 45.0 (DBA/2), P = 0.08; and midcolonic
crypt 30.8 (BALB/c) versus 24.5 (DBA/2), P = 0.0004], hence
data are presented as the percentage of change from control.
BALB/c mice demonstrated a greater loss of cellularity of small
intestinal (Fig. 4a; P = 0.012 at 72 h) and midcolonic crypts
(Fig. 4c; P = 0.007 at 72 h) than DBA/2 mice, but no differ-
ences were seen in the cellularity of small intestinal villi (Fig. 4b; P = 0.059 at 72 h). Examination of crypt histology (Fig. 5) confirmed this statistical analysis. Fig. 5g demonstrates particularly that changes in colonic crypt architecture were seen 72 h after two doses of 100 mg/kg raltitrexed in BALB/c mice, whereas the histology of DBA/2 colonic crypts 72 h after the same treatment was normal (Fig. 5h).

**Effects of Raltitrexed on Clonogenic Crypt Survival.** Clonogenic crypt survival was assessed in small intestinal epithelium as described previously (13), 96 h after two doses of 100 mg/kg raltitrexed. The surviving fraction was significantly (P = 0.004) less in BALB/c mice [45.62% (n = 4)] than DBA/2 mice [70.78% (n = 4)]. This suggests either that there is an inherent difference in small intestinal clonogenic and stem cell properties between the two strains, or that more clonogenic cells are sterilized by the same dose of raltitrexed in BALB/c compared with DBA/2 mice.

**Apoptosis and Crypt/Villus Cellularity in p53-Null Mice.** We have demonstrated previously that p53 expression played a major role in controlling the amount of apoptosis and intestinal toxicity induced in murine intestinal epithelium by another cytotoxic drug that is an inhibitor of TS, 5-FU, although this drug was acting via an inhibition of RNA metabolism (9, 10). We therefore investigated whether p53 expression played any role in the differences observed in apoptosis and intestinal toxicity between BALB/c and DBA/2 mice following administration of raltitrexed. There was no statistically significant difference in small intestinal apoptotic yield by Student’s t test or by modified median test analysis between p53 wild-type and null mice 24 h after 10 mg/kg or 100 mg/kg raltitrexed (data not shown). One hundred mg/kg of raltitrexed caused no apoptosis above control levels in the midcolonic crypts of p53 wild-type and null mice (data not shown). There was no significant difference in small intestinal crypt cell number between p53 wild-type and null mice at various time points after two injections 24 h apart of 100 mg/kg raltitrexed (Fig. 6). No changes in colonic crypt cell number were observed in either p53 wild-type or null mice following this dose regimen of raltitrexed (data not shown). The apoptosis and changes in crypt and villus cellularity induced in intestinal epithelia by raltitrexed are therefore independent of p53 expression.

**DISCUSSION**

BALB/c mice developed more diarrhea and lost more weight than DBA/2 mice after sequential administration of the
TS-inhibitor, raltitrexed (Fig. 1), as we described previously (3, 4). This difference in overall toxicity between two mouse strains has been shown to result primarily from the effects of the drug in the gastrointestinal tract rather than in other organs such as the bone marrow (3, 4). However, previous experiments have not identified any upstream factors (directly related to the mode of drug action) which were responsible for this strain difference in toxicity (3). In particular, there were no differences between the strains in the pretreatment levels of thymidine, deoxyuridine, folate or TS and, at early times after drug administration (up to day 3), there were no significant differences in the tissue levels of raltitrexed and its polyglutamates in intestinal mucosa. We have therefore investigated whether any downstream factors (not directly related to the mechanism of drug action), particularly the engagement of apoptosis, were important determinants of this differential response. Indeed, BALB/c mice developed significantly more small-intestinal and colonic apoptosis than DBA/2 mice 24 h after a single injection of 100 mg/kg raltitrexed (Fig. 2). Interestingly, raltitrexed induced apoptosis preferentially in cells at different positions in the small intestinal crypts of the two mouse strains (Fig. 3). Cells nearer the small-intestinal crypt base underwent more apoptosis in BALB/c than in DBA/2 mice. The cells located nearer the crypt base are more rapidly dividing and represent cells that are earlier in the cell lineage than those more differentiated cells near the crypt-villus junction. It is possible that the higher levels of apoptosis in the proliferative compartment of BALB/c small intestine compared with DBA/2 might result in more disruption of crypt function, and one would predict that this might have more profound consequences upon the overall architecture of the epithelium, especially as proliferation is suppressed by this drug. This is supported by the clonogenic crypt survival assay, which showed significantly less crypt survival in BALB/c compared with DBA/2 mice 96 h following two doses of 100 mg/kg raltitrexed (see “Results”). Why, on a cell-by-cell basis, there is a difference in positional apoptosis in the two strains is difficult to answer. This may have a pharmacodynamic explanation, but is difficult to test on a cell-by-cell basis.

In our study of the p53-dependent pathology induced by 5-FU in the murine intestine in vivo (10), we concluded that toxicity was dependent upon apoptosis occurring against a background of inhibition of proliferation. Scoring apoptosis alone showed that the amount of apoptosis was not dependent on the dose of 5-FU past a certain threshold, with toxicity emerging above that threshold (10). Instead, inhibition of proliferation was 5-FU dose-dependent so that cell loss, by apoptosis, could not be replenished once proliferation was also inhibited. Raltitrexed also had profound effects upon cellular proliferation within the intestinal crypt compartment (Fig. 2), and so the increased apoptosis observed in the BALB/c strain occurred against a background of inhibited proliferation, increasing overall toxicity.

Is the strain difference in susceptibility to induction of intestinal apoptosis described here specific to raltitrexed, or does it also occur with other damaging stimuli? Previous experiments have shown differences between the strains after the alternative stimulus of γ-radiation (8). These differences between the same strains were less marked than with raltitrexed. Small-intestinal apoptotic index was 8.6% in BALB/c compared with 6.7% in DBA/2, and midcolonic apoptosis was 3.3% in BALB/c compared with 4.1% in DBA/2 at 4.5 h after 1 Gy γ-radiation (8). As discussed above, the final outcome, in terms of tissue integrity, depends on apoptosis and the extent of suppression of mitosis. In the DBA/2 and BALB/c mice, the suppression of mitosis was 3.1% and 1.6%, respectively, in small intestine and 1.2% and 0.7%, respectively, in midcolon 4.5 h after 1 Gy γ-radiation (8). This suggests that, whereas there may be an inherent difference between the mouse strains in susceptibility to apoptosis and suppression of proliferation, part of the response described here may also be specific to raltitrexed. Direct comparison of these two stimuli is not completely valid because γ-radiation-induced apoptosis occurs in different cell positions from that induced by raltitrexed. This could influence the rate of cell replenishment after a wave of cell death, for example, if many of the cells in the region necessary for cell number amplification, higher up the crypt from the stem cells, the so-called “transit cells,” were lost. In addition, the effects of γ-radiation are completely p53-dependent (16), whereas raltitrexed-induced apoptosis is p53-independent (Fig. 6).

Damage to the small intestine and colon may not contribute equally to the diarrhea induced by raltitrexed, and again, there are differences in these two sites between the strains studied here. The changes in crypt cell number (Fig. 4) and particularly changes in gross crypt histology (Fig. 5) resulting from two injections of 100 mg/kg raltitrexed were more marked in the colon than in the small intestine. The colon is a vital organ for the reabsorption of water. In humans the capacity for reabsorption can increase from 1.5 liters/day to 4–5 liters/day under stressful conditions (17). We hypothesize that the reduction of damage to the colonic epithelium of DBA/2 mice compensates for the increased water-load resulting from small intestinal damage by reabsorbing water. Hence, no diarrhea or weight loss results in the DBA/2 strain. By contrast, the damaged colonic epithelium of BALB/c mice has reduced functional reserve and is not able to reabsorb water fully. This results in diarrhea and weight loss. Although differences in both small intestinal and colonic apoptosis were observed after 100 mg/kg raltitrexed...
(Fig. 1), we propose that the colonic component may be more important. Importantly, the colonic apoptosis that was observed in BALB/c mice occurred maximally at cell positions near the crypt base where the stem cells are believed to be located (Fig. 3b), whereas no midcolonic apoptosis occurred above baseline levels in DBA/2 mice after raltitrexed administration. Moreover, DBA/2 mice did not display any late (>48 h) increase in mitotic index in the colon, which was indicative of an attempt to repair damaged epithelium after cell loss by apoptosis (Fig. 2).

The p53-independence of raltitrexed-induced apoptosis and intestinal toxicity (Fig. 6) was surprising in view of the drug's mechanism of action as a TS-inhibitor leading to strand breaks in DNA and in view of the p53-dependent apoptosis caused by 5-FU (9, 10). However, 5-FU and raltitrexed appear to have different mechanisms of action in this context. Whereas the apoptosis induced by raltitrexed can be prevented by coadministration of thymidine (9), in keeping with a mechanism of action as a TS-inhibitor, 5-FU-induced apoptosis was not affected by thymidine administration, but was partially rescued by uridine administration, suggesting a cytotoxic action by RNA damage (9). We and others have also confirmed these findings in vitro using p53-wild-type HCT116 human colon carcinoma cells (18). The cytotoxicity of raltitrexed to this cell line was reduced by thymidine administration and was maintained in clones of HCT116 cells with reduced levels of functional p53. By contrast, the cytotoxicity of 5-FU to HCT116 was unaffected by thymidine administration, but was partially rescued by uridine administration, and was significantly reduced in cells with reduced levels of p53 (18). Thus the mechanistic basis of strain-dependent differences in apoptosis remains to be determined and is open to genetic investigations.

In summary, we have demonstrated that BALB/c and DBA/2 mice show different thresholds for the induction of apoptosis after raltitrexed administration. We suggest that the colonic component of this apoptosis, coupled with an inhibition of proliferation, is an important determinant of the intestinal toxicity induced by raltitrexed. These findings may have important implications regarding the severe diarrhea caused by raltitrexed chemotherapy in some human patients. For example, it may be possible to predict which patients are most at risk of developing severe diarrhea by measuring apoptotic and proliferation indices in rectal biopsies after drug administration. However, the methodology associated with such an approach is fraught with problems (19), and a genetic basis for the difference is being sought. Also, we would predict that therapeutic interventions aimed at either limiting the degree of colonic damage caused by raltitrexed or at increasing the colon's capacity to reabsorb water might be useful for reducing the severity of diarrhea. The difference between 5-FU and raltitrexed in terms of the p53-dependence of intestinal apoptosis and toxicity also raises interesting possibilities for targeting chemotherapy based on the p53 status of colonic carcinomas and of considering combination chemotherapy with both agents in certain settings.

REFERENCES

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