Efficient Deletion of Normal Brca2-Deficient Intestinal Epithelium by Poly(ADP-Ribose) Polymerase Inhibition Models Potential Prophylactic Therapy

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Abstract

The genes encoding the BRCA1 and BRCA2 tumor suppressors are the most commonly mutated in human familial breast cancers. Both have separate roles in the maintenance of genomic stability through involvement in homologous recombination, an error-free process enabling cells to repair DNA double-strand breaks. We have previously shown that cre-mediated conditional deletion of Brca2 within the mouse small intestine sensitizes the tissue to DNA damage. Eventually, the tissue repopulates via stem cells in which recombination at the floxed Brca2 allele has not taken place. In this study, we have treated Brca2-deficient small intestine with a potent small-molecule inhibitor of poly(ADP-ribose) polymerase 1 (PARP1), an enzyme predominantly involved in the recognition of DNA single-strand breaks. Brca2 deficiency rendered otherwise normal cells exquisitely sensitive to PARP inhibition, resulting in very high levels of apoptosis as early as 6 hours after treatment, with evidence for repopulation of the tissue at 12 hours. Furthermore, the intestines of animals treated with serial injections of the inhibitor repopulated very rapidly in comparison with those from untreated mice. Our results represent the first in vivo demonstration that inhibition of PARP1 activity confers exquisite sensitivity to death in physiologically normal Brca2-deficient cells, suggesting that such a regimen may be extremely potent prophylactically in women heterozygous for the BRCA2 gene, as well as against established tumors lacking functional BRCA2.

Introduction

It is currently estimated that familial cases account for ~10% of human breast cancers (1). Of these, up to half are believed to be due to deficiency in either of the tumor suppressors BRCA1 or BRCA2 (1). The identification of new drug regimens against such tumors is therefore of paramount importance in the current fight against the disease. BRCA1 and BRCA2 have separate roles in the homologous recombination pathway, a process by which DNA double-strand breaks are conservatively repaired (2). Cells lacking either protein are prone to genomic instability due to the alternative employment of the error-prone nonhomologous end-joining pathway of DNA repair (2). Cells lacking Brca2 have been shown to be sensitive to a variety of DNA damaging agents, either in vitro or in vivo, particularly following treatment with DNA cross-linking agents such as mitomycin C (3–11).

The enzyme poly(ADP-ribose) polymerase 1 (PARP1) is activated by binding to DNA strand breaks (12) and is known to have a variety of roles, including participation in base excision repair (13). Through its ability to modify proteins by the synthesis and elongation of ADP-ribose polymers, PARP1 reduces cellular pools of ATP and NAD+, thereby affecting many energy-dependent processes (13). As PARP1 is known to actively participate in DNA repair, inhibition of its activity can lead to enhanced cell death either alone or in combination with DNA damaging agents (12). As such, PARP1 inhibitors have been discussed as potential chemotherapeutic agents, either alone or by potentiating other cytotoxic treatments (12–15). Indeed, studies have already shown the effectiveness of PARP inhibition in combination with either radiotherapy or chemotherapy in a range of human tumor mouse xenograft models (16, 17). We have previously shown that conditional deletion of the Brca2 gene, using a well-characterized CYP1A1-driven cre-loxP approach (18, 19), sensitizes the mouse small intestine to DNA damage and eventually leads to repopulation by stem cells in which recombination of the floxed Brca2 allele has not taken place (11). In this study, we have used our model Brca2-deficient system to analyze whether PARP inhibition further sensitizes the intestine to DNA damage in vivo. We show that apoptosis is dramatically increased and that stem cell repopulation proceeds very rapidly compared with untreated tissue, showing that cells lacking Brca2 are indeed highly sensitive to PARP inhibition. As such, we concur with previous studies (12–17) which suggest that treatment with PARP inhibitors may be extremely potent against human tumors in which BRCA2 is mutated. Based on our current data, we now propose that PARP inhibition may also be considered as a potential prophylactic treatment of nonneoplastic BRCA2-deficient cells in women heterozygous for BRCA2, as well as a primary follow-up treatment following surgery in patients in whom BRCA2 deficiency has led to tumorigenesis.

Materials and Methods

Experimental mice. Mice carrying the floxed Brca2 allele (Brca2f) were provided by Tak Mak at the University of Toronto (Toronto, Ontario, Canada) and details of these mice can be found in ref 11. Mice bearing the intestinal inducible Cre vector, Ah-cre, were provided by Douglas Winton at Cambridge University (Cambridge, United Kingdom; ref. 18). Mice carrying the Rosa26R transgene were already in house and details can be found in ref 20. Mice were fed standard diet and water ad libitum and all animal experiments were carried out in accordance with current UK Home Office regulations.

Preparation and injection of DNA damaging drugs. β-Naphtoflavone was prepared as previously described (18). Mice were given an ip. injection of 80 mg/kg four times over 4 successive days. KU0058948 was provided by KuDOS Pharmaceuticals (Cambridge, United Kingdom; see Fig. 1A for
After a single i.p. injection of 15 mg/kg KU0058948, mice were killed and sections of small intestine were removed. Samples were homogenized for 30 seconds in 3 volumes of ice-cold extraction buffer [1 tablet of Complete EDTA-free protease inhibitor cocktail (Roche) in 20 mL 1% NP40/1× PBS], incubated on ice for 5 minutes, and snap frozen on dry ice. Following thawing, the drug was extracted from the homogenate by protein precipitation with acetonitrile and supernatant was isolated by centrifugation at 13,000 rpm for 10 minutes. Samples were analyzed by liquid chromatography-tandem mass spectrometry (LC-MS/MS) with the chromatography done using a Phenomenex Synergi MaxRP (50 × 2 mm) column with a gradient elution from 5% acetonitrile:95% formic acid (0.01%) to 95% acetonitrile:5% formic acid (0.01%) over a 6.5-minute period. The mass spectrometer used was a Sciex 2000 with TurboIonSpray ionization, operating in positive ion mode and using multiple reaction monitoring of ions 381.1→281.3. Calibration standards (20 ng/mL-20 μg/mL) were prepared in mouse plasma as a proxy matrix.

Removal and preparation of small intestines. All small intestinal preparations, including whole mounts, were carried out as previously described (18).

PCR for the recombined Brca2 allele. Recombined Brca2 PCR was done on DNA from small intestinal tissue as previously described (11).

Statistical analysis. All statistical analyses were done using nonparametric Mann-Whitney test with a confidence level of 95%.

Results and Discussion

Bioavailability of poly(ADP-ribose) polymerase inhibitor in small intestine after a single i.p. injection of 15 mg/kg. To ascertain the levels of KU0058948 in the small intestine after a single i.p. dose of 15 mg/kg, LC-MS/MS analysis for the presence of the PARP inhibitor was done on tissue extracts from duplicate mice killed over a 24-hour period following treatment. As shown in Fig. 1B, high levels of the molecule were detected 1 hour after treatment, after which the inhibitor was cleared relatively quickly from the tissue. It should be noted that a concentration of 100 ng/mL equates to ~250 nmol/L, which has been shown to be extremely effective at killing Brca2-/−/ mouse embryonic stem cells in vitro (14). It was therefore clear that the PARP inhibitor was present in the small intestine at high levels almost immediately following dosing and was retained at an effective level for at least 12 hours. On the basis of this data, we assessed the apoptotic response at both 6 and 12 hours after treatment.

Increased apoptosis following treatment with 15 mg/kg poly(ADP-ribose) polymerase inhibitor. Mice were killed 6 or 12 hours after a single i.p. dose of 15 mg/kg KU0058948 and their intestines scored for apoptotic bodies and mitotic figures. At 6 hours, the levels of apoptosis had increased significantly in mice treated with the PARP inhibitor compared with control mice that had been injected with saline or were untreated (Fig. 2A; P = 0.04). In addition, the levels of mitosis in the experimental mice were significantly reduced (Fig. 2A; P = 0.04), presumably as a reflection of the reduced numbers of viable cells available to drive proliferation in the affected crypts. At 12 hours, the level of apoptosis had increased to around 35% in Brca2-deficient mice, which is highly significant when compared with controls (Fig. 2B; P = 0.0004). This is a high level of apoptosis, and the representative image in Fig. 2C shows that the majority of apoptotic bodies were confined to the stem cell compartment and the proliferative zone in the crypts of these mice. This level of apoptosis seems to reflect the peak response as levels were significantly reduced to ~13% by 24 hours (P = 0.014; data not shown). At 12 hours, we also tested the levels of apoptosis in mice heterozygous for Brca2 and found that although there was a significant increase in apoptotic levels at this time point compared with untreated controls (Fig. 2B; P = 0.015), the level of increase was very small compared with the induction of death in the homozygotes. These data show that the PARP inhibitor–induced death was highly specific to the Brca2-−/− cells.

Close inspection of intestinal sections from Brca2-deficient mice revealed subtle evidence that the tissue was starting to repopulate with unrecombined cells at 12 hours after inhibitor treatment. Figure 2D shows a representative image from a Brca2-deficient intestine treated with the inhibitor and shows a crypt which contains very few apoptotic bodies but many mitotic figures (arrow). These apparently healthy crypts contrasted the many unhealthy or “dead” crypts, which contained many apoptotic bodies and virtually no numbers of viable cells available to drive proliferation in the affected crypts. At 12 hours, the level of apoptosis had increased to around 35% in Brca2-deficient mice, which is highly significant when compared with controls (Fig. 2B; P = 0.0004). This is a high level of apoptosis, and the representative image in Fig. 2C shows that the majority of apoptotic bodies were confined to the stem cell compartment and the proliferative zone in the crypts of these mice. This level of apoptosis seems to reflect the peak response as levels were significantly reduced to ~13% by 24 hours (P = 0.014; data not shown). At 12 hours, we also tested the levels of apoptosis in mice heterozygous for Brca2 and found that although there was a significant increase in apoptotic levels at this time point compared with untreated controls (Fig. 2B; P = 0.015), the level of increase was very small compared with the induction of death in the homozygotes. These data show that the PARP inhibitor–induced death was highly specific to the Brca2-−/− cells.

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apoptosis after deletion of Brca2, the small intestine repopulates very slowly, with ~50% of recombined stem cells replaced by those which had failed to recombine 6 months after Brca2 deletion (11). To test whether treatment with the PARP inhibitor increased the rate of repopulation in Brca2-deficient tissue, we used mice which carried the Rosa26R reporter allele (20) as well as the Ah-Cre and floxed Brca2 genes. Mice were subjected to twice-weekly injections of 15 mg/kg KU0058948 for 3 weeks and then scored for recombined crypts using a simple whole-mount stain for the reporter allele (11). Figure 3A shows representative areas of small intestinal crypts from Ah-cre+ Brca2fl/fl mice 12 hours after treatment with either saline or 15 mg/kg PARP inhibitor. Bar, 50 μm. D, representative image from Ah-cre+ Brca2fl/fl small intestine 12 hours after treatment with 15 mg/kg PARP inhibitor. Arrow, a healthy, highly proliferating crypt. Bar, 50 μm.

Overall, the presented data report two phenomena: First, they confirm our previous data indicating that Brca2-deficient cells, including stem cells, are removed from the small intestine following DNA damage, and that this effectively prevents the accumulation of cells with the potential to acquire further cancer-causing mutations (11). In this study, where the detection and subsequent repair of DNA damage is reduced through inhibition of PARP, fewer recombined crypts remain in Brca2-deficient mice treated with the PARP inhibitor compared with untreated controls (P = 0.04). As we have previously shown (11), a simple PCR strategy confirmed that the recombined (“white”) areas of intestine contained no recombined floxed Brca2 allele (data not shown).

**Figure 2.** Apoptotic response of Brca2-deficient small intestine following a single i.p. injection of 15 mg/kg KU0058948. A and B, levels of apoptosis and mitosis 6 (A) or 12 (B) hours after treatment. Open columns, apoptosis; closed columns, mitosis; y axis, percentage of apoptotic bodies/mitotic figures per crypt. C, representative images of small intestinal crypts from Ah-cre+ Brca2fl/fl mice 12 hours after treatment with either saline or 15 mg/kg PARP inhibitor. Bar, 50 μm. D, representative image from Ah-cre+ Brca2fl/fl small intestine 12 hours after treatment with 15 mg/kg PARP inhibitor. Arrow, a healthy, highly proliferating crypt. Bar, 50 μm.
one of the key proteins involved in damage recognition (12), both the apoptotic response and the rate of repopulation are increased, presumably due to the increased number of cells in which the defective homologous recombination pathway is required but unavailable. Second, we have modeled for the first time the in vivo consequences of drug challenge on otherwise physiologically normal Brca2-deficient cells. We observe highly efficient and selective deletion of Brca2<sup>−/−</sup> cells with no apparent deleterious effect on surrounding Brca2-functional cells or whole animal physiology. It is interesting to note that the effects of the PARP inhibitor seem to be more highly specific to cells lacking Brca2 compared with other DNA-damaging agents we have tested in our model system. Thus, we have previously shown that a low concentration of mitomycin C (0.1 mg/kg) specifically kills Brca2-deficient cells in the small intestine, but at higher concentrations this specificity was reduced, with levels of cell death also increased in cells with functional Brca2 (11). In contrast, our current data show that PARP inhibition delivers even higher levels of Brca2<sup>−/−</sup> cell deletion and yet has no deleterious effect on Brca2<sup>+/−</sup> (wild-type) cells.

Taken together, our results go beyond previous studies (12–17) to show that PARP inhibition is highly effective at specifically deleting nonneoplastic Brca2-deficient cells, and thus may be effective for prophylactic therapy or therapy subsequent to surgery in Brca2 mutation carriers, as well as against Brca2-deficient tumors as previously suggested. Deficiency of Brca2 in our model intestinal system does not predispose to tumorigenesis (11) and thus we cannot address whether Brca2-deficient tumors regress, or can be prevented, in this system. We are now pursuing studies within the mouse mammary epithelium which will allow us to directly test the efficacy of PARP inhibitors for both tumor and prophylactic therapy.

**Acknowledgments**

Received 4/6/2005; revised 9/1/2005; accepted 9/21/2005.

**Grant support:** American Institute for Cancer Research grant no. 03-336, KuDOS Pharmaceuticals Ltd., and Wales Gene Park.

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**Brca2 Deficiency Sensitizes Cells to PARP Inhibition**

In the article on how *Brca2* deficiency sensitizes cells to PARP inhibition in the November 15, 2005 issue of *Cancer Research* (1), the grant support in the Acknowledgment should have read as follows: Association for International Cancer Research grant no. 03–336, KuDOS Pharmaceuticals Ltd., and Wales Gene Park.

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