p53 Is Essential for Chemotherapy-induced Hair Loss

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Abstract

Anticancer drugs stimulate apoptosis in the hair follicles (HF) and cause hair loss, the most common side effect of chemotherapy. In a mouse model for chemotherapy-induced hair loss, we demonstrate that p53 is essential for this process: in contrast to wild-type mice, p53-deficient mice show neither hair loss nor apoptosis in the HF keratinocytes that maintained active proliferation after cyclophosphamide treatment. HF in p53 mutants are characterized by down-regulation of Fas and insulin-like growth factor-binding protein 3 and by increased expression of Bcl-2. These observations indicate that local pharmacological inhibition of p53 may be useful to prevent chemotherapy-associated hair loss.

Introduction

Cancer treatment with chemotherapeutic agents is associated with severe side effects due to the occurrence of apoptosis in several sensitive tissues (such as the hematopoietic system or epithelia of digestive tract) as a result of drug cytotoxicity (1). This apoptosis largely depends on p53, a key mediator of cellular mechanism of stress response (2). p53 accumulation in sensitive cells after a variety of stresses results in growth arrest at one of the cellular checkpoints or induction of programmed cell death (3). p53 acts as a nuclear transcription factor altering expression of multiple p53-responsive genes, the activity of which is, at least in part, responsible for cell reaction to stress (2, 3). The major role of p53 in mediating the side effects of cancer treatment was confirmed by the isolation and application of a chemical inhibitor of p53 that reduced the toxicity of cancer treatment in experimental animals by temporary reversible suppression of p53 (4).

Temporary hair loss (alopecia) is a common side effect of chemotherapy. HF are “hair shaft-producing miniorgans” showing during postnatal life a unique pattern of cyclic activity with periods of relative resting (telogen), active growth (anagen), and apoptosis-driven involution (catagen) (5). HF are strongly affected by many chemotherapeutic agents because of the rapid proliferative rate of hair matrix keratinocytes during anagen. In the mouse model of chemotherapy-induced hair loss, the active hair growth phase was first induced by depilation, and cyclophosphamide administration during new anagen phase causes complete alopecia imitating changes seen in human chemotherapy-induced hair loss (6, 7)...

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3 The abbreviations used are: HF, hair follicle(s); IGF-BP3, insulin-like growth factor-binding protein 3; TUNEL, terminal deoxynucleotidyl transferase-mediated nick end labeling.

4 Anticancer drugs stimulate apoptosis in the hair follicles (HF) and cause hair loss, the most common side effect of chemotherapy. In a mouse model for chemotherapy-induced hair loss, we demonstrate that p53 is essential for this process: in contrast to wild-type mice, p53-deficient mice show neither hair loss nor apoptosis in the HF keratinocytes that maintained active proliferation after cyclophosphamide treatment. HF in p53 mutants are characterized by down-regulation of Fas and insulin-like growth factor-binding protein 3 and by increased expression of Bcl-2. These observations indicate that local pharmacological inhibition of p53 may be useful to prevent chemotherapy-associated hair loss.

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6 P53 induces dystrophic changes in growing HF and, in more severely damaged follicles, premature regression as a result of massive apoptosis in the entire proximal hair bulb, with subsequent hair shedding (6, 7).

7 Apoptosis of hematopoietic cells and cells of the digestive tract associated with cancer treatment is known to be p53 dependent (2–4, 8). Radiation- or chemotherapy-induced DNA damage leads to the rapid accumulation of p53 protein in the susceptible cells (3, 9), followed by up-regulation of Fas, IGF-BP3, and Bax, encoded by the corresponding p53-responsive genes (10–12). Moreover, it was demonstrated that Fas and Bax are up-regulated in the HF during cyclophosphamide treatment (13) and that p53 is involved in the ionizing radiation-induced apoptosis in the HF (14).

8 To explore the role of p53 in the hair loss induced by chemotherapeutic agents, we used a mouse model for chemotherapy-induced hair loss: cyclophosphamide treatment of C57BL/6 mice after hair cycle synchronization in anagen by depilation (6, 7, 13). We analyzed the expression of p53 in the HF of drug-treated wild-type mice and then compared the dynamics of HF of wild-type versus p53-deficient mice after cyclophosphamide treatment. We demonstrate here that p53 is indeed essential for the development of chemotherapy-induced hair loss in mice.

Materials and Methods

Animal Models and Tissue Collection. Female 8-week-old C57BL/6 mice (n = 20), 8–to-10-week-old p53 knockout (n = 25), and wild-type mice (n = 25) were purchased from Charles River (Boston, MA) and The Jackson Laboratory (Bar Harbor, ME). p53 knockout mice generated on C57BL/6 background were viable, showed apparently normal fur, and developed spontaneous tumors not earlier than 12 weeks after birth (15), i.e., after the end of experiment. Mice were housed in community cages at the animal facilities of the Boston University School of Medicine and University of Illinois at Chicago. All mice were fed water and murine chow ad libitum and were kept under 12-h light/dark cycles.

Active hair growth (anagen) was induced in the back skin by application of a wax-resin mixture with subsequent depilation, as described before (6, 7, 13). On day 9 after hair cycle induction (at anagen VI stage of the hair cycle), a single i.p. injection of 150 mg/kg cyclophosphamide (Endoxan; Bristol Meyers Squibb, Princeton, NJ) or PBS (vehicle control) was given as described previously (6, 7, 13). Skin samples were harvested at days 1, 3, 5, 7, 9, and 11 after cyclophosphamide administration (at days 10, 12, 14, 16, 18, and 20 postdepilation, respectively). Harvesting of skin and cryosectioning were performed by a special technique to obtain longitudinal sections of the HF, as described previously (6, 7, 13).

Immunohistochemistry and TUNEL Technique. Expression of p53 protein, p55 TNF receptor, and IGF-BP3 was assessed with the use of rabbit polyclonal antiserum against murine p53 (Novocastra, Newcastle upon Tyne, United Kingdom); rat monoclonal antibody against murine p55 TNF receptor (PharMingen, San Diego, CA), and rabbit polyclonal antiserum against IGF-BP3 (Santa Cruz Biotechnology, Santa Cruz, CA), respectively. Tyramide amplification was used for the visualization of these antigens, as described previously (16, 17). Double immunovisualization of p53 and TUNEL or proliferative marker Ki-67 and TUNEL was performed, as described previ...
ously (18). For immunovisualization of Ki-67, a rabbit polyclonal antiserum against murine Ki-67 was obtained from Dianova (Hamburg, Germany). Immunodetection of Fas, p75 neurotrophin receptor, Bax, and Bcl-2 was performed with corresponding monoclonal antibodies and the avidin-biotin-alkaline phosphatase method, as described previously (13, 17). Histoenzymatic detection of the endogenous alkaline phosphatase was performed for the precise identification of dermal papilla morphology in HF of the distinct stages of apoptosis-driven involution (catagen) (6, 7, 13, 16, 17).

Results and Discussion

**p53 Is Up-Regulated in Hair Follicles after Cyclophosphamide Treatment.** To determine the p53 involvement in the pathogenesis of chemotherapy-induced hair loss, we first compared the expression of p53 in actively growing HF in the back skin of vehicle-treated and cyclophosphamide-treated C57BL/6 mice. Active hair growth (anagen) was induced by depilation as described before (6, 7, 13), and cyclophosphamide was administered 9 days postdepilation, i.e., at the time point when all HF have reached the stage of active hair shaft production (anagen VI).

Twenty-four hours after cyclophosphamide administration (10 days postdepilation), HF treated by vehicle control showed a relatively weak expression of p53 in the proximal outer root sheath and hair matrix, associated with absence of TUNEL-positive cells (Fig. 1A). In contrast, cyclophosphamide-treated HF displayed a strong up-regulation of p53 in the proximal outer and inner root sheaths and hair matrix (Fig. 1B). In the HF after cyclophosphamide treatment, many TUNEL-positive apoptotic cells located in the HF matrix were also p53 positive (Fig. 1B).

In line with previous reports (6, 7, 13), 3 days after cyclophosphamide administration (12 days post depilation), HF showed a shortening of their length caused by the massive apoptosis in the proximal hair bulb. This was associated with prominent p53 expression and colocalization of p53 and TUNEL in the regressing outer and inner root sheaths (Fig. 1C). At day 7 after cyclophosphamide administration (day 16 postdepilation), all cyclophosphamide-treated animals showed massive hair loss in the entire back (not shown), and 2 days later the process of cyclophosphamide-driven HF regression was...
Fig. 2. p53 knockout mice treated by cyclophosphamide do not display hair loss. Hair cycle was induced in the back skin of 8-week-old wild-type (n = 25) and p53-null mice (n = 25) by depilation, and cyclophosphamide was administered 9 days after. Skin was harvested at day 7 after cyclophosphamide administration (day 16 postdepilation), and cryosections (8 μm thick) were processed for the histoenzymatic detection of alkaline phosphatase (B and C) and double immunovisualization of proliferative marker Ki-67 and TUNEL (D and E). Cell nuclei in D and E were visualized by TO-PRO-3 staining. A, severe hair loss over the entire back of the wild-type mice treated by cyclophosphamide, with no hair loss visible in the p53 knockout mice. B, shortening of the length and reduction in the volume of proximal hair bulb in the wild-type HF (arrows) is associated with dramatic decrease of skin thickness after cyclophosphamide treatment. C, large volume of the proximal hair bulb (arrows) and dermal papilla (red color) in the p53 null HF after cyclophosphamide treatment. D, numerous TUNEL-positive cells (green fluorescence, arrows) and single Ki-67-positive cells (red fluorescence, arrowhead) in the regressing HF compartments of wild-type skin. E, numerous Ki-67-positive cells in the HF matrix (red fluorescence, arrowheads) and single TUNEL-positive granules in the precortical zone (arrow). Some TUNEL-positive cells are also visible in the subcutis around HF (green fluorescence). Bars, 100 μm.
p53 Knockout Mice Do Not Show Cyclophosphamide-induced Hair Loss. To test the role for p53 in the control of cyclophosphamide-induced apoptosis in the HF and hair loss, we compared the effects of cyclophosphamide on actively growing HF of p53 knockout versus wild-type mice. Animals were treated by cyclophosphamide 9 days postdepilation as described previously (6, 7, 13). Seven days after cyclophosphamide treatment (16 days postdepilation), wild-type mice showed extensive hair loss over the entire back associated with dramatic shortening of HF length and massive apoptosis in the regressing HF compartments (Fig. 2, A, B, and D).

However, no hair loss was seen in the cyclophosphamide-treated p53 knockout mice up to 14 days after cyclophosphamide administration. In particular, 7 days after cyclophosphamide treatment (16 days postdepilation), all HF in p53-null mice were in anagen VI stage. These HF were characterized by a large volume of the proximal hair bulb and dermal papilla, by active keratinocyte proliferation in the hair matrix, and by single TUNEL-positive granules in the melanogenic area above dermal papilla (Fig. 2, A, C, and E). Therefore, anagen HF in p53 knockout mice kept a high rate of keratinocyte proliferation in the hair matrix and active hair shaft production, did not show intrafollicular apoptosis, and, therefore, were resistant to the cyclophosphamide treatment. Most importantly, at days 9–11 after cyclophosphamide administration (18–20 days postdepilation), all HF in the p53 knockout mice showed apparently normal patterns of physiological transformation from the active growth to the regression and then to the resting phase (not shown).

Hair Follicles in p53-deficient Mice Are Characterized by Down-Regulation of Fas and IGBP3, and by Increased Expression of Bcl-2. To identify factors that might contribute to p53-dependent cyclophosphamide-induced hair loss, we compared the expression of number of molecules (Fas, p55 kDa TNF receptor, p75 kDa neurotrophin receptor, Bax, Bcl-2, IGF-BP3) implicated in the control of cyclophosphamide-induced apoptotic cell death in the HF (5, 13). Several of the proteins analyzed (Fas, Bax, Bcl-2, IGF-BP3) are encoded by genes, the transcription of which is regulated by p53 during DNA damage induced by chemotherapeutic agents (10–12, 19).

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We found that, in contrast to the wild-type HF treated by cyclophosphamide, p53-null HF showed strongly reduced expression of Fas in the proximal outer and inner root sheaths (Fig. 3, A and B). This observation is consistent with the reported involvement of p53 in intracellular trafficking of Fas receptor (10). Interestingly, immunoreactivity for p55 kDa TNF receptor was also reduced in the proximal outer root sheath of the HF in p53 knockout mice, compared with wild-type animals (Fig. 3, C and D). However, the expression patterns of another member of “death domain” containing growth factor receptors, namely p75 neurotrophin receptor, showed no differences between wild-type and p53-null HF treated by cyclophosphamide (Fig. 3, E and F).

Furthermore, the immunoreactivity for IGF-BP3 was strongly reduced in the p53-deficient HF, compared with wild-type HF (Fig. 3, G and H). Bax expression was relatively unchanged in p53-null HF, whereas Bcl-2 was highly expressed in the HF dermal papilla of 53 knockout mice, compared with that of wild-type mice (Fig. 3, I–L).

Taken together, our data suggest that p53 is essential for triggering apoptotic cell death in the HF that is induced by cyclophosphamide in mice. This puts HF in the same category of other organs sensitive to genotoxic stress, such as the hematopoietic system or epithelia of the digestive tract, which respond to genotoxic stress by p53-dependent apoptosis, thus limiting the tolerable doses of anticancer drugs or radiation (2, 3, 8). Considering the similarity of chemotherapy-induced alterations in HF of humans and mice (6), we assume that p53 plays a similar role in hair loss occurring in cancer patients during chemotherapy.

As is true for other sensitive tissues, the exact mechanism of p53-dependent apoptosis in HF remains unclear. It may involve at least several p53-responsive genes acting through different mechanisms. We speculate that p53 mediates cyclophosphamide-induced apoptosis by: (a) up-regulation of IGF-BP3 and stimulation of the extracellular neutralization of the insulin-like growth factor-I (IGF-1), implicated in retarding physiological transition of the HF from active growth to apoptosis-driven regression (20); (b) up-regulation of the membrane “death domain” containing receptor Fas/Apo-1 implicated in mediating apoptosis in the HF (13); and (c) modulation (reduction) of the Bcl-2:Bax ratio in the HF triggering mitochondrial-mediated

![Fig. 3. Cyclophosphamide-treated HF of p53-null mice show down-regulation of Fas and IGBP3 and up-regulation of Bcl-2. Skin sections of wild-type (WT) and p53 knockout (−/−) mice were analyzed at day 5 after cyclophosphamide administration (day 14 postdepilation) for immunoreactivity of Fas, p55 kDa TNF receptor (p55 TNFR), p75 kDa neurotrophin receptor (p75NTR), IGBP3, Bax, and Bcl-2. A and B, Fas. Prominent expression of Fas in the HF outer root sheath (A, arrows) and inner root sheath (A, arrowhead) in wild-type skin, and weak expression of Fas in the HF outer root sheath in p53-null skin (B, arrows). C and D, p55 TNFR. Wild-type HF shows p55 TNF receptor immunoreactivity in the outer root sheath (C, arrow). Decrease of immunoreactivity in the p53 null HF (D, arrow). E and F, p75 NTR. Expression in the outer root sheath of wild-type (E, arrows) and p53 null HF (F, arrows). G and H, IGBP3. Expression in the proximal outer root sheath (G, arrows) and inner root sheath (G, arrowheads) of the wild-type HF. Down-regulation of IGBP3 in the HF of p53 knockout mice (H). I and J, Bax. Expression in the matrix and dermal papilla (arrows) of the wild-type (I) and p53-null HF (J). K and L, Bcl-2. Expression in the dermal papilla (K, arrow) and in the hair matrix (K, arrowhead) of the wild-type HF. Up-regulation of Bcl-2 in the dermal papilla (L, arrow) and hair matrix (L, arrowhead) of the p53-null HF. Bar, 100 μm.](image-url)
apoptosis (13). The relative impact of the above factors in HF regression after chemotherapy remains to be determined.

Recently, we showed that a small molecule acting as a p53 inhibitor efficiently reduces the side effects of ionizing radiation in mice, presumably by suppressing p53-mediated apoptosis (4). We thus presume that a local pharmacological blockade of p53 by synthetic antagonists may provide a new therapeutic strategy for the prevention of chemotherapy-induced hair loss, one of the most devastating side effects of cancer treatment.

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