Anticancer Drugs Induce Apoptosis in Mouse Hair Follicles

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Summary: Apoptosis represents an important mode of cell death induced by chemotherapeutic drugs. It is plausible that apoptosis may also play an important role in alopecia caused by anticancer drugs. C57 BL/6 mice were induced to enter into anagen by hair shaft depilation and a single intraperitoneal injection of cyclophosphamide (150 mg/kg) was given. Skin specimens were taken from the backs of mice at day 9, 10, 11, 12 and 13 postdepilation (p.d.). Specimens were stained with TUNEL method, and DNA was isolated from the plucked hair follicles and was electrophoresed. Many TUNEL-positive cells were observed in hair bulbs in the cyclophosphamide group at days 10-13. At day 13 p.d., most follicles were damaged and extensive hair loss was observed. Fragmented DNA, but not so distinct, were observed in a DNA ladder pattern corresponding to the in situ results. This study suggests that anticancer drugs can induce apoptosis-related damage to the hair follicles in mice. Control mice when treated with saline also showed apoptosis when they spontaneously entered into catagen, but they never showed hair loss. Consequently, hair loss induced by anticancer drugs may result from a wave of induced apoptotic death.

Key words TUNEL, mouse, chemotherapy, alopecia, DNA ladder

INTRODUCTION

Many drugs used in cancer chemotherapy affect growth and metabolism not only in malignant cells but also in certain normal tissues. In general, tissues with higher metabolic and mitotic rates, such as bone marrow, gastro-intestinal epithelium, and oral mucosa, are most noticeably affected. Damage to these tissues provides the most common measurable signs of "toxicity" observed during the administration of anticancer agents. The roots of growing human scalp hairs also have high metabolic and mitotic activity. It is not surprising, therefore, that the same anticancer agents may interfere markedly with the normal function of the hair root.

Apoptosis is a cascade of events within a cell which leads to death, and is believed to be the mechanism by which cells undergo programmed cell death [1-3]. Morphologically, apoptosis is characterized by condensation of nuclear chromatin, compaction of cytoplasmic organelles, and changes in the cell surface [1,2]. In many types of cells, during later stages of apoptosis, nuclear DNA is digested into oligonucleosomal-length fragments which are shown as distinctive DNA ladders on gel electrophoresis [4]. This degradation is also associated with the appearance of a hypodiploid peak in the DNA histogram [5]. Apoptosis occurs in a variety of physiological situations, where an organism has to remove unwanted cells [1,6-10]. However, apoptosis can also be induced by pathological stimuli, and most anticancer drugs have been shown to induce apoptotic features in normal and tumor cells [11-15].

It has recently been proposed that apoptosis might be an important mode of cell death for cells treated with chemotherapeutic drugs [11,14,16]. This assumption leads to the speculation that, even in alopecia caused by anticancer drugs, apoptosis may play an important role, although it had previously been regarded as the results of cytotoxicity [17]. In this study, we demonstrated that anticancer drugs can induce apoptosis in mouse hair follicles.
MATERIALS AND METHODS

Animals and induction of alopecia

Six- to eight-week-old female C57 BL/6 mice purchased from Charles River Japan (Yokohama, Japan) were used. Only mice with all hair follicles of the back skin in telogen phase (judged by their pink skin color) were induced to enter into anagen by hair shaft depilation under anesthesia as described by Paus et al. [18]. When all the back skin follicles entered into anagen VI at day 9 postdepilation (p.d.), a single intraperitoneal injection of cyclophosphamide (CYP) (150 mg/kg body weight, dissolved in saline, Sigma, St. Louis, MO) was given. Skin specimens were taken from the backs of mice, euthanized by intraperitoneal injection of nembutal at 9, 10, 11, 12, 13, 14 and 15 p.d.

As controls, mice were injected with saline instead of CYP, at 9 p.d., and skin specimens were taken at 17, 18 and 19 p.d., when depilation-induced anagen follicles spontaneously entered into catagen phase.

TUNEL staining

Formalin-fixed deparaffinized tissue sections were treated with proteinase K (10 μg/ml) for 30 min at room temperature to strip proteins away from the nuclei, and were rinsed with deionized distilled water. Sections were immersed in 2% hydrogen peroxide in phosphate-buffered saline (PBS) for 5 min at room temperature to inactivate endogenous peroxidase activity. After rinsing with PBS, TUNEL staining was performed with an Apop Tag apoptosis detection kit (Oncor, Gaithersburg, MD). Briefly, the specimens were incubated with equilibration buffer for 10 sec at room temperature. After excess liquid was removed, TdT enzyme diluted with reaction buffer was applied and the sections were incubated in a humidified chamber for 1 hr at 37 ºC. The specimens were then treated with a pre-warmed stop/wash buffer for 30 min at 37 ºC. Localization of the labeled nucleotides was achieved with anti-digoxigenin-peroxidase antibodies for 30 min at room temperature. The specimens were rinsed in 3 changes of PBS. Visualization of reaction product was achieved with a solution containing 20 mg of 3-amino-9-ethylcarbazol (Sigma) dissolved in 5 ml of N,N-dimethylformamide, 95 ml of 0.1 mol/l acetic buffer at pH 5.2, and 0.1 ml of 30% hydrogen peroxide for 1-5 min. Control slides were incubated without TdT enzyme. As a positive control, ventral prostate of Wister strain rat sampled at 4 days after castration was used [19,20].

DNA isolation and agarose gel electrophoresis

Hair follicles were plucked from the back of mice with forceps, and put into 750 μl of lysis buffer (50 mM Tris-HCl, pH 7.8, 10 mM EDTA-Na, 0.5% w/v sodium-N-lauroylsarcosinate (Wako, Osaka, Japan)). After vortexing, they were incubated with 0.5 mg/ml proteinase K in lysis buffer for 48 hrs at 50 ºC, thereafter further incubated with 0.5 mg/ml RNase for 30 min at 50 ºC. DNA was extracted with phenol-chloroform, precipitated with ethanol, and resuspended in TBE (89 mM Tris-base, 89 mM boric acid, and 2 mM EDTA). The DNA sample was quantitated spectrophotometrically. Three micrograms of DNA per lane was electrophoresed in a 1.5% agarose gel containing ethidium bromide (0.5 μg/ml) in TBE buffer at 100 V for 1 hr. At the end of electrophoresis, the gel was visualized by UV fluorescence, and then photographed.

RESULTS

Macroscopic findings and histology

As Paus et al. [18] previously reported, a single intraperitoneal injection of CYP to mice at day 9 p.d. induced a stepwise macroscopic decrease in skin pigmentation accompanied by a rapid development of alopecia, which reached its maximum by day 15/16 p.d. At day 12 p.d., hair shafts over the entire back could be easily pulled out merely by rubbing the skin gently. This was not observed in the control mice.

Histologically, all hair follicles entered into anagen VI by day 12 p.d. At day 13 p.d., hair follicles in the CYP group showed significant damage such as rapid termination of anagen (dystrophic catagen pathway) or by entry into an abnormally prolonged dystrophic anagen state (dystrophic anagen) [18]. The changes lasted until day 15 p.d. All of vehicle-injected control animals remained in anagen until day 15 p.d., and then entered into catagen phase around day 18 p.d. (not shown).

TUNEL staining

At day 9 p.d., there was no evidence of positive TUNEL staining in the follicles. At day 10 p.d., however, many TUNEL staining cells were observed in every follicle in outer and inner root sheaths and in the hair matrix (Fig. 1a). Thereafter, increasing
Fig. 1. DNA fragmentation assessed by TUNEL method in longitudinal sections.

a: TUNEL staining-positive cells were seen in hair bulb in skin sample of a CYP-treated mouse at day 10 p.d. (×100)
b: At day 11 p.d., TUNEL staining-positive cells were found in hair bulb, outer and inner root sheaths. (×100)
c: TUNEL staining-positive cells were present in regressing follicles at 12 p.d. (×100)
d: Only a few cells were positive for TUNEL method at day 13 p.d. because of massive degradation. (×100)

Fig. 2. Agarose gel electrophoresis showing ethidium-bromide-stained DNA fragmentation. In lane 1, a molecular marker Lambda Hind III was shown. Lane 2 showed very weakly stained, imperceptibly fragmented DNA at day 10 p.d. DNA extracts of at day 11 p.d. (lane 3) and at day 12 p.d. (lane 4) showed faintly visible DNA laddering. In lane 5, leaded with DNA at day 13 p.d., no DNA laddering was seen. In lanes 2, 3, 4 and 5, DNAs were extracted from hair follicles of CYP-treated mice. Lane 6 showed weakly stained, faintly fragmented DNA in control mice treated with saline at day 17 p.d. In lane 7 day 18 p.d. and lane 8 day 19 p.d., slightly visible stained DNA fragmentation was seen.
numbers of positive cells were noted in all hair follicles at 11 p.d. (Fig. 1b). By day 12, positive cells were present in the lower portions of the follicles (Fig. 1c). At day 13 p.d., only a few positive cells showed positive staining in the bulbs (Fig. 1d). Fibroblast of the dermal papilla were never seen to be TUNEL positive during any of the examined hair cycle stages.

In control mice injected with saline, at day 17, 18 and 19 p.d., TUNEL positive cells were seen in the follicles, as expected in the natural hair cycle [21].

**DNA isolation and agarose gel electrophoresis**

In the electrophoresis studies, fragmented DNA was observed as faintly visible DNA ladders in DNA samples obtained from CYP-treated mice at day 10, 11 and 12 p.d. and in those obtained from control mice 17, 18 and 19 p.d. These DNA samples were obtained not from full back skin but from plucked hair follicles (Fig. 2). At 13 p.d., sufficient DNA could not be extracted from the hair follicles of CYP-treated mice, since the hair follicles were much smaller.

**DISCUSSION**

The psychological impact of chemotherapy-induced alopecia represents one of the more devastating side effects of cancer chemotherapy, and, in some instances, leads patients to refuse potentially curative chemotherapy. The problem of alopecia is severe when patients are treated with the anthracycline antibiotic, doxorubicin (adriamycin), which is beneficial in many types of cancer. Accordingly, it is necessary to elucidate the mechanism of alopecia caused by anticancer drugs, which will hopefully shed new light on the patients suffering from the alopecia.

Goldberg et al. [22] examined with an in vivo nuclear aberration assay the toxic effect of CYP on the proliferative cell population of hair follicles plucked from the human scalps. Cells with nuclear aberrations (indicating apoptosis) and mitotic cells, in the hair matrix were counted for each patient before and after treatment. The mean percentages of cells with nuclear aberrations in patients treated with CYP were significantly higher than those for pre-treatment counts or from patients given a placebo injection. The authors insisted that the hair follicles were in the anagen phase from most patients, and, therefore, scores for the incidence of apoptosis counted were significant. However, their observation was based on a morphological study using the Feulgen method alone [23]. Consequently, a study based on a more acceptable confirmation of fragmented DNA is essential. In the present study, DNA fragmentation was observed both histologically in sections by a TUNEL method and in DNA electrophoresis as a DNA ladder.

Lindner et al. [24] also demonstrated DNA laddering when a single injection of CYP was given to mice. However, they extracted DNA from full back skin and observed the DNA ladder only at day 10 p.d. More recently, Schilli et al. [25] also performed the intraperitoneal injection of CYP and carried out DNA electrophoresis. Since there was no distinct fragmentation detectable on the gel, they originated an excellent idea, that is, the isolated DNA was incubated with biotin-16-dUTP and terminal deoxynucleotidyl transferase. After precipitating DNA by ethanol, a gel electrophoresis was performed. The DNA was then blotted on a GenescreenPlus membrane, and the membrane was incubated with alkaline-phosphate streptavidin, and finally visualized using BCIP/NBT as substrate. This method is superior when DNA electrophoresis is carried out from the relatively small number of apoptotic nuclei compared with the vast majority of nonapoptotic cells in full-thickness skin. However, they also used full back skin preparations containing hair follicles as the same as Lindner et al. [24] did. Full back skin contain not only hair follicles but also normal lymphocytes, which show apoptosis in the ordinary condition. In this study, we extracted DNA from plucked hair follicles, and showed that fragmented DNA, but faint, appeared at day 10, 11 and 12 p.d. although they were not so distinct as observed in camptothecin-pretreated, apoptotic HL-60 cells which were used as positive control in the study by Schilli et al. [25]. It is, in general, difficult to show distinct DNA ladder when apoptosis is induced in keratinocytes and follicular cells. Consequently, our study in which only plucked hair follicles were used seemed to be more accurate, and confirmed that CYP-induced apoptosis occurs in hair follicles.

Cece et al. [26] also investigated the mechanism of doxorubicin (DXR)-induced alopecia in newborn rats by light and electron microscopy and TUNEL method. A single DXR treatment induced apoptosis and a decrease in mitotically active cells, and they concluded that DXR treatment induces injury in a cell subset localized in the hair matrix. Most investigators used CYP as an agent in chemotherapy-
induced alopecia so far, but they showed DXR also induces apoptosis in murine hair follicular cells.

Previously, we have examined whether or not apoptosis occurs in hair follicles during catagen regression in the natural hair cycle in mice [21]. DNA fragmentation was observed in both sections histologically and by DNA electrophoresis. In the present study, the morphology of hair follicles responding to the CYP showed evidence of dystrophic catagen (rapid termination of anagen) and dystrophic anagen (abnormally prolonged dystrophic anagen state). No normal catagen changes were noticed, that were similar to those previously described by Paus et al. [18].

In the present study, hair loss was observed only in mice treated with CYP. Control mice treated with saline alone, which spontaneously entered into catagen never showed hair loss at the same time as the CYP group. Thus, the anticancer drug induces apoptosis-related damage in the hair follicles which was much more significant in that hair loss was observed only in mice treated with CYP.

ACKNOWLEDGMENTS: We thank Professor Geoffrey Rowden (Dalhousie University) for reviewing the manuscript.

REFERENCES