Topical Calcitriol Enhances Normal Hair Regrowth but Does Not Prevent Chemotherapy-induced Alopecia in Mice

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ABSTRACT

Using a murine model that mimics chemotherapy-induced alopecia (CIA) in humans particularly well, we show here that in contrast to previously reported CIA-protective effects in neonatal rats, topical calcitriol does not prevent CIA in adolescent mice but enhances the regrowth of normally pigmented hair shafts. When, prior to injecting 1 x 120 mg/kg cyclophosphamide i.p., 0.2 μg calcitriol or vehicle alone were administered topically to the back skin of C57BL/6 mice with all hair follicles in anagen, no significant macroscopic differences in the onset and severity of CIA were seen. However, hair shaft regrowth after CIA, which is often retarded and patchy, thus displaying severe and sometimes persistent pigment disorders, was significantly accelerated, enhanced, and qualitatively improved in test compared with control mice. Histomorphometrical analysis suggests that this is related to the fact that calcitriol-pretreated follicles favor the “dystrophic catagen pathway” of response to chemical injury, i.e., a follicular repair strategy allowing for the unusually fast reconstruction of a new, undamaged anagen hair bulb. Thus, it may be unrealistic to expect that topical calcitriol can prevent human CIA, but topical calcitriols may well enhance the regrowth of a normal hair coat.

INTRODUCTION

Sudden and often dramatic hair loss (alopecia) is a much-feared effect of many chemotherapy protocols and is widely experienced as one of the most distressing aspects of cancer therapy. Even long after chemotherapy and the occurrence of hair regrowth, lasting hair shaft discoloration and/or changes in hair morphology and pigmentation can add to the patient’s discomfort and stigmatization. Yet, no satisfactory remedy—namely, therapeutic regimens not risking diminishing of the effectiveness of chemotherapy on potential scalp metastases—is as yet available to suppress this ill-studied alopecia, the pathobiology of which is largely obscure (1, 2). Therefore, it is important to analyze CIA3 in appropriate models that allow examination of its pathobiology as well as to explore new strategies for its management.

Recently, two animal models for the study and pharmacological manipulation of CIA have been presented. One of them uses neonatal rats (3-5), and the other uses adolescent mice (6). The latter model strikingly mimics the characteristic follicle histopathology previously reported for alopecia in humans as induced by a widely used alkylating agent (CYP: Ref. 7). This model allows the study of the effects of chemotherapy on well-defined, homogeneous, and mature populations of precisely the type of hair follicles that are severely damaged by chemotherapy, resulting in alopecia and disturbances in hair regrowth (i.e., so-called anagen VI follicles). Also, the murine model allows as yet unparalleled insights into the basic patterns of the follicle response to and recovery from chemotherapy. These features of the murine model are based on the high degree of hair cycle synchrony displayed by the mouse strain used (for discussion, see Ref. 6).

Topical pretreatment with 1,25-dihydroxyvitamin D3 (calcitriol) before administration of cyclophosphamide, etoposide, or Adriamycin has been reported to prevent alopecia in a neonatal rat model (8). Here, we report that topical calcitriol does not prevent CIA in the murine model. We show, however, that calcitriol pretreatment substantially accelerates the regrowth of normally pigmented hair shafts after murine CIA, most likely by favoring a defined follicle response pattern to chemical injury.

MATERIALS AND METHODS

Resting (telogen) hair follicles over the entire back skin of 6–8-week-old female C57BL/6 mice were induced to enter active hair growth (anagen) by hair shaft depilation, as described in detail (6). When anagen VI had been reached, a single dose of 120 mg/kg CYP (ENDOXAN; Asta Medica; containing NaCl to yield a 0.9% NaCl solution after dissolving the compound), freshly dissolved in distilled water, or vehicle alone was injected i.p. on day 9 after anagen induction. Corresponding to the neonatal rat study (8), mice were topically treated during the 5 days before CYP administration once daily with 0.2 μg calcitriol dissolved in 100 μl 100% ethanol or with vehicle alone (calcitriol was from LEO Pharmaceutical Co., Ballerup, Denmark; stored and handled in accordance with the manufacturer’s instructions for preserving optimal compound activity).

Test and control mice were assessed macroscopically for the percentage of mice within an experimental group showing signs of alopecia, the average percentage of the back skin area displaying alopecia, or the color and nature of the regrowing fur coat. Because individual mice from test and control groups were sacrificed for histology at various time points, only the relative data were recorded, without further statistical analysis. The total number of animals assayed in four independent experiments with respect to the test parameters listed is indicated for each group in the figures that document the macroscopic effects of calcitriol pretreatment. Data from all four experiments were pooled, because all experiments showed similar trends (not counting data from extensive pilot experiments, all of which also were in line with the observations reported below). Notably, the calcitriol-induced hair growth effects in the murine model for CIA reported here were repeated in three different laboratories by different investigators (R. P., B. H., and M. B. S., Humboldt University; A. M., Schering Corp.; and P. P., Jagiellonian University) using different C57BL/6 mouse stocks (Berlin experiments, Charles River, Sulzfeld, Germany; Krakow experiments, Animal Breeding Facility, Silesian Medical Academy, Katowice, Poland).

Based on the previously determined patterns of the follicle response to and recovery from CYP-induced damage in C57BL/6 mice (6), macroscopic and microscopic analyses of test and control mice were performed between days 12 and 33 after anagen induction (i.e., 3–24 days after CYP injection). Histological analysis (histomorphometry) was performed during different time points after CYP and calcitriol administration, i.e., microscopic classification of the detectable hair follicle populations according to their hair cycle stage and the presence of signs of follicle dystrophy, as described (6). Shown below are selected histomorphometric results, which best reflect the initial follicle response to CYP damage (day 15) and the subsequent follicle recovery (day 33) and which were obtained from analyzing >150 hair follicles at a x100-400 magnification per mouse, analyzing at least five mice per time point and group.

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Received 1/29/96; accepted 8/1/96.

The abbreviations used are: CIA, chemotherapy-induced alopecia; CYP, cyclophosphamide; VDR, vitamin D3 receptor.
The histomorphometric data were pooled and statistically evaluated using the Mann-Whitney U test.

RESULTS

Twelve to 15 days after anagen induction by depilation (i.e., 3–6 days after CYP injection), test and control mice showed the same degree of hair loss (Fig. 1). No significant differences were noted between calcitriol- and vehicle-pretreated mice, when the total numbers of animals with any signs of alopecia (Fig. 2A) or the mean percentages of the back skin area displaying alopecia (Fig. 2B) at various relevant time points after CYP injection were compared. Histomorphometric analysis on day 15 revealed that both control and test mice exclusively showed hair follicles with signs of follicle dystrophy, such as the presence of characteristic disorders of follicular melanogenesis, distention of the follicular canal, and bulging of the dermal papilla (compare with Ref. 6).

However, histomorphometry revealed that calcitriol pretreatment significantly changed the immediate response of anagen VI hair follicles to CYP-induced damage; whereas dystrophic anagen follicles dominated in control mice, mice pretreated with calcitriol displayed mainly dystrophic catagen follicles (Fig. 3, A and B). In several mice, this could already be appreciated macroscopically (predominantly between days 16 and 20). The thickness of all murine skin compartments is strictly hair cycle dependent (i.e., the epidermis, dermis, and subcutis are significantly thicker in skin with all hair follicles in anagen than skin dominated by catagen and telogen follicles; Refs. 9 and 10). Therefore, the predominance of catagen follicles in some calcitriol-treated mice with a particularly high percentage of dystrophic catagen follicles corresponded to palpably thinner skin compared with control mice. In addition, the skin of these test mice was more pink than that of control mice. This reflects the catagen-associated termination of intrafollicular melanogenesis (11, 12) and the higher percentage of “dystrophic catagen” follicles in test mice, as opposed to the predominance of melanin-producing “dystrophic anagen” follicles in control mice.

When test and control mice were followed over an extended period that allowed assessment of hair follicle recovery and hair shaft regrowth patterns after CYP-induced follicle damage, substantial differences between calcitriol- and vehicle-treated mice became apparent. As documented in Fig. 4, control mice showed irregular regrowth of hair shafts with severe pigmentation disturbances (Fig. 4A), in contrast to test mice, which displayed earlier, denser, more homogeneous, and almost normally pigmented regrowth of the fur coat (Fig. 4B). A quantitation of the differences in alopecia (no hair shafts) and hair shaft pigmentation (light gray to black hair shaft color), recorded between 21 and 33 days after anagen induction is presented in Fig. 5.

These macroscopic differences in the velocity, quality, and quantity of hair regrowth were reflected by significant differences in the percentages of histomorphometrically classified and quantified hair follicle types during the follicular recovery phase. Less than one-fourth of the hair follicles in control skin had reentered into a normal, second anagen cycle without any signs of follicle dystrophy, as opposed to test mice, in which the back skin follicles of 85% had done so. No dystrophic follicles at all were seen in calcitriol-treated mice, compared with almost one-half of the hair follicles, which still were dystrophic, in control mouse skin (Fig. 6, A and B).

In additional, preliminary studies, we have most recently observed very similar macroscopic hair growth effects on CYP-induced hair loss and regrowth in this mouse model with two other topically applied synthetic vitamin D derivatives, calcipotriol (2.0 μg/100 μl ethanol) and KH 1060 (0.02 μg/100 μl ethanol; from LEO Pharmaceutical Co.); both failed to prevent CIA but signifi-
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Fig. 3. A, histomorphometric comparison of follicle damage in mice pretreated with vehicle (♀, n = 7) or calcitriol (♂, n = 10) on day 15 after anagen induction (i.e., 6 days after CYP injection = initial follicle response phase to chemical injury), corresponding to Figs. 1 and 2. Note that in both test and control mice, only dystrophic hair follicle forms were notable. *, P < 0.05 (comparing the respective follicle subtype between test and control. B and C, representative histology of the dominant hair follicle population in C57BL/6 back skin on day 15 in vehicle-pretreated mice (B) versus calcitriol-pretreated mice (C). Note that the vehicle treatment is associated with the development of dystrophic anagen follicles (B), whereas calcitriol treatment favors the development of dystrophic catagen follicles (C) (6). Follicle dystrophy is evident from the severe disruption of the normal pigmentation apparatus, namely by the presence of ectopic, abnormally large clumps of melanin (6, 14).

DISCUSSION

The results from this study, obtained with a murine model of CIA that resembles the clinical situation more closely than any other currently available animal model (6), shows calcitriol to be a potent enhancer and accelerator of normal hair regrowth after CIA in mice. This strongly encourages one to explore and develop calcitriols as drugs for accelerating and improving the clinically and psychologically important regrowth of a normally pigmented hair coat after human CIA.

Our data conflict with the apparently CIA-protective effect of topical calcitriol reported for neonatal rats (8) but are supported by our finding that two synthetic calcitriols, calcipotriol and KH 1060, also failed to prevent or retard the occurrence of CYP-induced alopecia in the same mouse model, whereas both drugs accelerated and enhanced the regrowth of a normally pigmented fur coat (13). Although our data question whether it is realistic to expect that calcitriols will be capable...
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Fig. 4. Representative example of hair regrowth pattern after CYP-induced alopecia in mice pretreated with vehicle (A) or calcitriol (B) (day 33 after anagen induction, i.e., 24 days after CYP injection). Note the substantially denser, darker and more homogeneously pigmented fur coat in B compared with A.

of preventing the occurrence of CIA in humans, the discrepant calcitriol results between the rat and mouse models of CIA may well reflect differences in the species and the age of the test animals and in the CYP dose used [one i.p. injection of 35 mg/kg CYP to 11-day-old Sprague-Dawley rats (8) versus one i.p. injection of 120 mg/kg CYP to adolescent C57BL/6 mice (6)]. More importantly, however, these differences between the rat and mouse models raise two basic questions: (a) which criteria should be met by a good animal model for CIA with reasonable predictive value for human CIA; and (b) how the problem of CIA may best be approached experimentally.

As we have reasoned previously (6), the murine model appears to have several advantages over the rat model in this respect. For example, the C57BL/6 mouse model displays an alopecia and hair regrowth pattern that reproduces the clinical phenomena associated with CYP-induced alopecia in humans strikingly well (e.g., it exactly mimics the characteristic follicle histopathology previously described for CYP-induced alopecia in humans; Ref. 7). In contrast to the rat model, the murine model studies the effects of chemotherapy on exactly defined, homogeneous, fully pigmented, and mature terminal hair follicles in that stage of the hair cycle, which is the main target of chemotherapy-induced damage (anagen VI). Furthermore, drugs long appreciated as potent manipulators of human hair growth also exert such effects in the murine model. This may give this model a greater predictive value for the human system than the neonatal rat model, in which agents not appreciated primarily as modulators of human hair growth reportedly exert alopecia-protective effects (e.g., Refs. 3–5). Finally, the murine model allows instructive insights into the basic patterns of follicle response to and recovery from chemotherapy. Therefore, the mouse model appears ideally suited as a preclinical testing ground for new management strategies of CIA in humans.

In most instances, human CIA mainly represents a so-called anagen effluvium, i.e., the shedding of hair shafts from severely damaged, “dystrophic” anagen hair follicles (1, 2). By light microscopy, CYP-induced follicle dystrophy is apparent by bulging of the dermal papilla, extension of the follicular canal, and a severe disruption of the melanogenic apparatus (Fig. 3B; compare with Refs. 6 and 7). The latter is characterized by the inhibition of normal melanosome formation, blockade of normal melanosome transfer into pre cortical matrix keratinocytes, pathological transfer of pigment granules to ectopic intrafollicular locations (e.g., outer and inner root sheath and proximal hair matrix), and melanin incontinence into the perifollicular dermis, proceeded and accompanied by a steep drop in tyrosinase activity (14). Depending on the level of damage inflicted on the follicle, chemotherapy-damaged anagen hair follicles may also enter a “dystrophic catagen” state (1, 2, 6, 7).

Previously, we have shown that CYP-damaged anagen follicles in mice follow two distinct pathways of response and recovery, which determine the characteristics of visible hair regrowth and which are likely of great clinical importance for the course of human CIA, as well (6). They may enter into the dystrophic anagen pathway, which actually extends the normal duration of anagen and yields, after the initial anagen effluvium, relatively early but very sparse regrowth of faultily pigmented hair shafts of inferior structural quality. Only then, dystrophic anagen is finally switched off, which enables the follicle to...
enter into a second, normal anagen cycle, which produces normally pigmented hair shafts. Alternatively, damaged anagen follicles may abort anagen abruptly, immediately enter into the dystrophic catagen pathway, and run through an extremely shortened resting period (telogen). Thus, these follicles enter into a second, normal anagen cycle (implying the construction of a new, undamaged anagen hair bulb with a restored pigmentation apparatus) significantly faster than dystrophic or normal anagen follicles would do. This latter pathway, therefore, allows for a greatly accelerated follicle regeneration and is associated with substantially earlier and qualitatively better regrowth of largely normally pigmented hair shafts (6).

In analyzing whether or not a test compound “protects” against CIA, it is, therefore, crucial to distinguish the initial degree and type of follicle dystrophy in the immediate response of a chemotherapy-damaged anagen follicle from its subsequent recovery and to check whether pretreatment of follicles before chemotherapy affects the ratio of dystrophic anagen:dystrophic catagen follicles. Against this background, our histomorphometric data provide a reasonable explanation for enhanced hair regrowth in calcitriol-pretreated mice; early after CYP injection, most of the follicles in test mice have entered into the dystrophic catagen pathway, whereas the control mice show most of their hair follicles to be in dystrophic anagen (Fig. 3). Thus, due to a much-accelerated switch off of their dystrophic anagen phase, the CYP-damaged follicles of calcitriol-pretreated mice have managed to reenter into a second, normal anagen cycle very soon after the initial shedding of hair shafts. This enables the rapid reconstruction of a normal anagen hair bulb and results in accelerated production of a new, largely normal fur coat. Control mice, in contrast, still display a substantial percentage of hair follicles in dystrophic anagen incapable of generating fully pigmented, normal hair shafts (Fig. 5).

Calcitriol appears to target key genes and/or signal transduction pathways responsible for determining the follicle response to and recovery from chemical damage. Although the nature of these molecular targets of cytostatic drug damage remains obscure from the current study, they certainly are a previously underappreciated, yet promising, area for future hair research. In this respect, it is important to note that calcitriol shows intriguing parallels to dexamethasone or cyclosporin A in its modulation of CYP-induced murine alopecia; these immunosuppressive drugs greatly alter the degree and onset of visible CIA as well as the hair regrowth pattern by favoring either the dystrophic anagen (cyclosporin) or dystrophic catagen (dexamethasone) pathway (6). Because both calcitriol and dexamethasone have catagen-promoting properties in our model, along with similar effects on skin thickness and pigmentation (compare with Refs. 6 and 15), related ligands of the steroid hormone receptor superfamily are reasonable candidates for the novel strategy of CIA management suggested by our observations: acceleration of follicle regeneration by therapeutic catagen induction, rather than the futile attempt to prevent CIA from occurring.

Although the underlying molecular mechanisms remain to be dissected, the striking effects of calcitriol on rodent hair follicles (see Refs. 8 and 13) lend further support to the concept that vitamin D receptor ligands are functionally important modulators of normal hair follicle growth and cycling (see Refs. 16 and 17). For example, normal murine hair follicles display prominent, hair cycle-dependent expression of vitamin D2 receptor (VDRs) by key regulatory cell populations of the follicle epithelium and mesenchyme, with maximal VDR expression in outer root sheath keratinocytes and dermal papilla fibroblasts during the later stages of anagen development (17). Because topical calcitriol treatment up-regulates cutaneous VDR expression (see Refs. 16 and 18), it is conceivable that increased VDR-mediated signaling in the hair follicle, rather than indirect effects on extrafollicular cell populations, is critically involved in the hair growth-modulatory effects of calcitriol reported here.

One promising approach to defining the molecular basis of the observed calcitriol effects may be to study how they relate to the modulation of programmed epithelial cell death (apoptosis) in situ by...
ligands of the VDR, which is abundantly expressed in key structures of the hair follicle (17–19). Interestingly, keratinocyte apoptosis in the hair follicle is a crucial component of normal hair follicle regression (catagen; see Refs. 15 and 20), represents one standard response of anagen follicles to damage (20, 21), and is much enhanced by CYP-induced apopotic hair bulb keratinocytes (23). Therefore, it is conceivable that calcitriol pretreatment reduces the likelihood that anagen hair bulb keratinocytes would respond to chemical damage with the initiation of apoptosis, although it remains uncertain whether this is functionally important for the enhanced hair regrowth pattern observed after calcitriol pretreatment.

Finally, novel strategies for the therapeutic management of CIA should more systematically take into account that, like the vast majority of patients with abnormal hair loss or gain seen in clinical practice, CIA and its subsequent hair regrowth disorders predominantly reflect defined alterations of the normal patterns of hair follicle cycling, rather than of hair shaft production (see Ref. 20). This implies that real progress in the prevention and treatment of CIA can only be accomplished by dissecting the molecular interactions of alopecia-inducing drugs with the as yet obscure, intracutaneous "biological clock" that dictates the normal cyclic growth and regression activity of the hair follicle (see Ref. 20).

Our data encourage one to test topical calcitriols and related ligands of the steroid hormone receptor superfamily as agents for promoting normal hair regrowth after CIA and suggest that, as much as the obvious limitations of any animal model allow, the C57BL/6 mouse offers an instructive and easily reproducible model system for exploring the clinical potential of new strategies for the management of CIA.

ACKNOWLEDGMENTS

The support and advice of Prof. W. Sterry, Prof. S. Lukiewicz, and Dr. J. Reichrath, the technical assistance of R. Plet and B. Plonka, and the help of Dr. S. Eichmüller with graph preparation and statistical analysis are most gratefully acknowledged.

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*Cancer Res* 1996;56:4438-4443.

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