BROAD-SPECTRUM PROTECTION AGAINST
CHEMOTHERAPY-INDUCED ALOPECIA
BY ACIDIC AND BASIC FIBROBLAST GROWTH FACTORS

DISSERTATION

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By

Jie Wang, M.S.

* * * * *

The Ohio State University
2005

Dissertation Committee:
Professor Jessie L.-S. Au, Advisor
Professor M. Guillaume Wientjes
Professor Thomas D. Schmittgen

Approved by
Advisor
College of Pharmacy
Our laboratory reported that acidic and basic fibroblast growth factors (aFGF/bFGF, FGFs) confer broad-spectrum chemoresistance in solid tumors. The four studies in this dissertation tested the hypothesis that the combination of these two growth factors offers broad-spectrum protection against chemotherapy-induced alopecia (CIA). In chapter 2, we established and characterized CIA animal models by different chemotherapeutic agents representing four major classes of anticancer drugs, i.e., alkylators (cyclophosphamide), topoisomerase 2 inhibitors (doxorubicin), antimicrotubules (paclitaxel), and antimetabolites (cytosine arabinoside, or ara-c). In chapter 3, we evaluated the pharmacodynamic endpoints in the two CIA animal models. The data showed that the chemotherapeutic agents induced dermal layer shrinkage, reduced hair bulb diameter, shortened hair follicle length, altered hair bulb matrix cell proliferative activity and induced hair follicular apoptosis. Paclitaxel induced disruptions of hair follicle melanogenesis in the black C57BL-6 mice resulting in relocation of melanin to ectopic hair bulb locations. The collective data indicated that hair bulb matrix and outer root sheath (ORS) were the two major targets of chemotherapy in hair follicles. In chapter 4, we evaluated the protective effect of subcutaneous FGFs against CIA in the two animal models. The results showed that FGFs offered broad-spectrum protection against CIA by all four chemotherapeutic
agents. In addition, FGFs accelerated the hair regrowth after CIA by high dose cyclophosphamide. Chapter 5 further investigated the protective effect of topical FGFs dissolved in 75% dimethyl sulfoxide (DMSO) against CIA. The data showed that topical FGFs reversed CIA by all four chemotherapeutic agents in the animal models. The data further showed that no or insignificant exposure of bFGF after subcutaneous or topical delivery of bFGF, suggesting that applying FGFs to the scalp will not compromise the efficacy of chemotherapy in tumor.
Dedicated to my parents,
my sisters and brothers,
and my beautiful wife, Jing Li,
for their love, understanding, and support
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VITA

April, 1974
Born – Shandong, China

1993-1997
B.S., College of Pharmacy, Beijing Medical University, Beijing, China

1997-2000
M.S., College of Pharmacy, Peking University, Beijing, China

2000-2005
Ph.D. Candidate, Division of Pharmaceutics, College of Pharmacy, The Ohio State University

PUBLICATIONS


**FIELDS OF STUDY**

Major Field: Pharmacy
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CHAPTER 1

REVIEW AND HYPOTHESIS:

PROTECTION AGAINST CHEMOTHERAPY-INDUCED ALOPECIA

1.1 INTRODUCTION

The hair follicle represents a unique hair-shaft-producing organ, which is characterized by postnatal life cyclic changes in its activity with three distinct phases of relative resting (telogen), active growth and hair shaft production (anagen), and apoptosis-driven regression (catagen). The hair follicle represents an un-intended target for cancer chemotherapeutic agents due to the rapid proliferation rate of hair matrix keratinocytes during anagen growing phase. Chemotherapy-induced alopecia (CIA) is one of the most psychologically devastating undesirable side effects in oncologic practice for which there is currently no effective prevention therapy in man at hand. Hence, protection or reversal of CIA is likely to improve the quality of life of cancer patients and the patient care.

This chapter reviews the recent advances in protection against CIA. We first present in this chapter a broad overview of the hair biology, i.e., hair follicle structure, hair growth cycle and hair follicle stem cells, for a confident starting point of
understanding of the terms and concepts, followed by the characteristics and mechanisms of CIA and the state-of-the-art animal models. Then we outline the previous findings in protection against CIA, including working hypotheses and strategies. The limitations of these previous findings are also discussed. Finally, the hypotheses and the overview of this dissertation are outlined.

1.2. HAIR FOLLICLE BIOLOGY

1.2.1. Hair follicle structure

Hair follicle structure is related to its stages of a growth cycle, in which the structural alterations occur repeatedly. Figure 1.1 shows the longitudinal view of the microscopic anatomy of a hair follicle. A fully mature anagen hair follicle can be divided into three segments, which are also the functional divisions of the follicle. These segments, from top to bottom with respect to the skin, are the infundibulum, the isthmus, and the inferior segment. Hair bulb is located in the inferior segment, which is sometimes called bulbar segment. Sebaceous gland, hair follicle and hair shaft is considered a pilosebaceous unit. The hair bulb contains the hair matrix and the dermal papilla. Matrix cells in the lower part of hair bulb have a high mitotic rate, while the matrix cells in the upper part have low mitotic rate and can differentiate into cells in the inner root sheath (IRS). The pigment-producing melanocytes are located in the hair bulb matrix. Dermal papilla consists of an oval mass of fibroblasts and is totally encapsulated by the matrix epithelium. The size of dermal papilla correlates with the size of the hair follicle and the size (diameter) of the produced hair shaft; e.g., larger
dermal papilla correlates with larger hair follicles and produces thicker hair shaft. The bulge in the outer root sheath near the insertion of arrector pili muscle is the location of epithelial stem cells that may serve as a reservoir for hair bulb matrix, epidermal and sebaceous gland cells. When the hair bulge is damaged during chemotherapy, irreversible permanent alopecia may occur.

From side to side, a hair follicle consists of several enclosed epithelial cylinders. The follicular sheath consisting of basement membrane and connective tissue surrounds the whole hair follicle. The basement membrane is known as a hyaline (or vitreous or glassy) membrane and shows strong periodic acid-Schiff-positive staining. Inside the basement membrane of the hair follicle, the innermost cylinder is the hair shaft and the outermost cylinder is the outer root sheath (ORS) that separates the follicle from the dermis. The middle cylinder, IRS, molds the shaft. IRS consists of three layers. The outermost layer of IRS, Henle’s layer, is tightly attached to ORS. The innermost cuticle layer of the IRS is interlocked with the cuticle of the hair shaft surface. This allows the IRS and hair shaft to move together during the period of growth. Distortion of IRS results in distorted hair shaft. At the level of or slightly below the sebaceous gland duct, IRS breaks down and the shaft and IRS separate. The cortex cells in the hair shaft are densely packed and are usually heavily melanized.

1.2.2 Hair growth cycle.

Under normal circumstance, hair growth in each hair follicle occurs in a cycle consisting of three main phases: anagen, catagen and telogen (figure 1.2). The process of hair shaft formation and release involves a complex mechanism of matrix cell
proliferation, keratinization and differentiation in IRS and ORS. Anagen is the period for the regeneration of the lower, cycling portion of the follicle, and production of a hair shaft. Only the upper third of the follicle remains stable through life and does not take part in the follicular adaptation to the growth cycle. In early anagen, the germinating epithelial cells at the base of the telogen follicle grow downward into the dermis as an epidermal finger, and ORS is established. Once the early anagen follicle reaches its destined depth, the cells in the central cylinder reverse their growth direction and progress upward, forming the IRS and the hair shaft. During the anagen growth phase, matrix cells in the hair bulb continuously divide and maintain a constant high mitotic rate. The proliferation of matrix cells provides a rapid stream of cells into the central portion of the hair follicle and move upward. The follicle and hair shaft are extended during anagen. Hair length is determined by the duration of anagen and not by the follicle length. On a normal human scalp, anagen is the longest phase with length of 6-10 years, and 90% of hair follicles are in anagen phase.

Catagen is a degenerative phase, when the isthmus and bulb regress. Like anagen, catagen is a highly controlled process, involving the separation of the papilla from the bulb, loss of the layered differentiation of the lower follicle, cessation of bulbar epithelial cell division and shrinkage of the lower follicle by means of apoptosis. In the early stage of catagen, the structure with positive eosinophilic staining in the basement membrane surrounding the hair bulb crumbles as the ORS collapses; and this phenomena serves as a marker of catagen stage of hair growth cycle. Catagen hair follicles can also be recognized by the cessation of pigment production by melanocytes located in the hair matrix. The proximal end of a hair shaft is usually not
pigmented when a hair follicle stops growing. During catagen, the hair follicle moves upward, and the IRS gets shorter until it forms only a collar around the telogen shaft base. The lower one-half to two-thirds of the hair follicle, from the hair bulb to hair bulge (around the arrector pili muscle insertion site), is lost during the catagen phase. At the end of catagen, ORS cells in the most superficial portion of the bulge, or keratogenous zone, undergo terminal differentiation or tricholemmal keratinization. This creates an irregular corona around the hair shaft that interlocks with the proximal part of ORS cells, thereby anchoring the hair shaft to the follicle during telogen.\(^8\) During telogen, the hair follicle is stable with no obvious structural changes. In humans, the telogen phase lasts for at least several months. The hair shaft is usually retained in the hair follicle during catagen and telogen. Shedding of hair shaft is associated with the formation of a new hair shaft from the next cycle in the hair follicle canal, and this process is called exogen. Anagen and exogen are likely independent events. Human hair follicles shed the club hairs at anagen onset when new hair grows. However, in mice, hair follicles tend to retain their club hairs and a single hair follicle may possess several club hairs with only one growing.\(^9\) After telogen ends, the follicle re-enters anagen and a new hair growth cycle starts.

### 1.2.3 Stem cells

The dynamic and cyclic changes of hair follicle in its growth cycle that involves well-controlled processes of degradation and regeneration suggest the presence of stem cells in this self-renewing tissue. Several strategies have been employed to search for the location of stem cells in hair follicles based on the expected properties of stem
cells. a) Stem cells are relatively undifferentiated. b) Stem cells have a large potential of proliferation. c) Stem cells are responsible for long-term tissue maintenance and self-renewal. d) Stem cells are slowly cycling. e) The location of stem cells should be well-protected and is close to a population of rapid dividing cells. That hair matrix cells in the hair bulb have high mitotic rates led to the hypothesis that hair follicle stem cells are located in or close to hair bulb. The hypothesis that hair follicle stem cells should be responsible for the hair follicle regeneration was tested by measuring the potential of hair follicle regeneration after removing predetermined parts of hair follicle surgically or by radiation followed. The results indicated that stem cells were not located in the hair bulb but in the upper part of ORS. The fact that stem cells are usually well protected also supports that the stem cells are located in upper part of ORS rather than the hair bulb, since the lower part of a hair follicle usually undergoes cyclic degeneration. The first evidence that hair follicle stem cells are located in hair bulge was provided by the chase-labeling experiment by Cotsarelis et al, which showed that label-retaining cells were located in hair bulge. Cotsarelis also hypothesized the “Bulge Activation Hypothesis” to explain how the hair growth cycle is controlled by the stem cells in the hair bulge. Briefly, stem cells in the hair bulge are activated by dermal papilla during the late telogen phase or the anagen initiation phase. The activated stem cells form a downward growth and divide into matrix cells, or transient amplifying (TA) cells. In the catagen phase, the dermal papilla moves upward through the contractile activities of the ORS to remain close to the hair bulge. In 2004, three groups independently proved the location of hair follicular stem cells was in hair bulge. The stem cells in hair follicles expressed β1-integrin and CD34,
typical markers for epithelial stem cells. Hair follicle stem cells share some common gene expression patterns with embryonic stem cells and stem cells from hematopoietic and neural system. These studies also indicated that stem cells in hair bulge are responsible for hair follicle regeneration and for tissue renewal in the whole skin epithelium including sebaceous gland and epidermis.

1.2.4. Hair growth pattern.

In most mammals, hair growth occurs in a wave pattern, or synchronous hair growth, during which hair follicles communicate with each other. For example, rodent hair follicles follow a wave pattern of hair growth in their first cycle after birth, which starts at the head and moves backwards to the tail and from abdomen to the back. Because of the wave pattern, a group of hair follicles in rodents at a specific area are usually in the same stage of hair growth cycle. Only around 10% of hair follicles in adult rodents are in anagen and the period of anagen only lasts one to two weeks. The normal hair growth cycle in human scalp, however, occurs in a mosaic pattern, or asynchronous hair growth, which means that each individual hair follicle determines its own growth cycle without communication with neighboring hair follicles. This difference in growth pattern has important implications for animal models using rodents to study hair loss. For example, the wave pattern of hair growth in 7-8-day-old neonatal rats may result in different sensitivity to chemotherapy of hair follicles at different parts of body. In this model, hair loss usually starts from the head and moves to the tail region. Guinea pig and Angora rabbit share the synchronous mosaic hair growth pattern as that in humans. However, they are not good models for study CIA because doxorubicin did not induce hair loss in the guinea pigs even at high dose, and
the hair loss in the angora rabbits was incomplete\textsuperscript{25}. The wave pattern of hair growth in animals may be affected by environmental temperatures, photoperiod (during of daylight), and other conditions. The hair growth pattern may also be regulated by the sexual cycle of the animals and the hormonal level.

1.3. CHEMOTHERAPY-INDUCED ALOPECIA

Chemotherapy induces three major side effects: a) bone marrow suppression including neutropenia, thrombocytopenia and anemia; b) gastrointestinal disturbances including oral mucositis, nausea, vomiting and diarrhea; and c) CIA. Alopecia affects nearly as many patients as gastrointestinal toxicities and is considered to be one of the most negative factors in cancer patients care\textsuperscript{2,26-28}. Chemotherapeutic agent related hair loss began to appear in the medical literature in the late 1950s\textsuperscript{8,29}. CIA is one of the most tangible, difficult and emotionally painful side effects that patients experience, which often causes patients to refuse chemotherapy. Alopecia affects perception of physical appearance and body image, which, however, goes beyond being a cosmetic problem, because it might affect the sexuality, self-esteem, and deprives patients of the privacy of having cancer. The national Coalition for Cancer Survivorship cites CIA as one of the most emotionally upsetting aspects of coping with cancer\textsuperscript{30}. The negative psychological impact of CIA indicates that this side effect is likely to have undesirable consequences; depression is known to lower immune function and is linked to cancer progression\textsuperscript{31}. CIA is becoming more prevalent as taxanes are becoming more frequently employed against breast and ovarian cancer of female patients. Female
patients are particularly affected by CIA; 47% consider alopecia the most traumatic side effect of chemotherapy, and 8% rejects chemotherapy as a result of fear of alopecia\textsuperscript{32,33}. Hence, protection against or reversal of CIA is likely to improve the compliance and quality of life of cancer patients. Quality of life is gaining importance as the emphasis of cancer treatment is shifting from cure to long-term maintenance, as noted in the lay press\textsuperscript{34}. Finally, it is noteworthy that substantial efforts have been expended and, consequently multiple drugs have been developed, to manage the other side effects of chemotherapy, including potentially life-threatening bone marrow suppression and toxicities that mainly cause discomforts to patients. In comparison, the development of CIA treatment is lagging and no effective treatments for CIA are available, which indicate the healthy market for treatment of CIA.

Similar to bone marrow, GI tract, and other rapidly proliferating cells in normal tissue, anagen hair follicles represent an un-intended target during cancer chemotherapy, because most chemotherapeutic agents, especially the traditional cytotoxic agents, are designed to attack rapid proliferating cells with no distinction between cancer and normal cells. Under chemotherapy, premature apoptosis-driven hair follicle regression, as well as the impaired metabolic and mitotic processes in anagen hair follicles, eventually result in hair loss. Chemotherapy disrupts the proliferation of hair matrix cells and possibly also IRS and ORS cells in anagen, thereby forces anagen follicles to enter a dystrophic stage in which the integrity and/or keratinization of the hair shaft is compromised, and the hair breaks and falls out\textsuperscript{35}. CIA may be related to apoptosis. Deletion of p53 can protect against CIA\textsuperscript{36,37}. 

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In humans, CIA usually begins at 1-3 weeks and is complete at 1-2 months after initiation of chemotherapy\textsuperscript{38}. To be noticeable, at least 50% of the hair must be lost. CIA is usually reversible, with regrowth starting after a delay of 3-6 months after the end of treatment \textsuperscript{39,40}. In 65% of cases, the new hair shows graying and/or changes in hair structure and texture. The hair growth rate can also be significantly reduced. Multiple classes of anticancer drugs induce alopecia \textsuperscript{38}. The frequencies of alopecia for the four major drug classes are: over 80% for antimicrotubule agents (e.g., paclitaxel), over 60% for alkylators (e.g., cyclophosphamide), 60-100% for topoisomerase inhibitors (e.g., doxorubicin), and 10-50% for antimetabolites (e.g., 5-fluorouracil plus leucovorin)\textsuperscript{41}. The severity of CIA depends on the drug, administration route, dose, and treatment schedule. Combination therapy produces higher incidence of and more severe CIA compared to single agent. With the trend toward combining two or more chemotherapeutic agents with different acting mechanisms, the likelihood of producing alopecia during chemotherapy is increasing. Radiation-induced alopecia occurs only when the head is included in the radiation port, and is more variable and less predictable.

1.4. ANIMAL MODELS.

Research in manipulating CIA has been hampered partially due to the lack of a reproducible animal model. Although the hair follicle cellular response to chemotherapeutic drugs can be studied in in vitro systems such as cell cultures and histocultures, the complexity of hair growth and cycling reduces the in vitro – in vivo
correlation in CIA\textsuperscript{38,42-45}. Transplantation of human scalp skin with hair follicles to mice offers an interesting model to study the biology of human hair follicles and to explore the effects of chemotherapy on human hair follicles\textsuperscript{46-49}. In this human hair follicle graft model, the transplanted hairs are shed within a month, and the regrowth of new hairs begins within several months after transplantation\textsuperscript{50}. However, the transplantation may affect the physiological situations of hair follicles, and the microenvironment in the skin such as growth factors and other cytokines may be altered during surgery and storage of skin sample.

CIA occurs when hair follicles are in anagen\textsuperscript{51}. About 90-95\% of hair follicles in human scalp are in the anagen phase lasting 2-6 years, <1\% are in the catagen phase lasting 2-3 weeks, and <10\% are in telogen phase lasting 3-4 months\textsuperscript{52}. Thus, the basic idea to establish CIA animal models is to induce hair follicles into the anagen growing phase. Seven to eight-day-old newborn rats show over 90\% hair follicles in anagen for about a week. Hair follicles on the back of adult C57BL-6 mice can be synchronized into the anagen growth phase by depilation. The following sections review the establishment and characteristics of these two CIA animal models.

1.4.1 Newborn rat model

The newborn rat CIA model was first introduced by Hussein\textsuperscript{39,53}. Using the 7-8-day-old rats, it was shown that several chemotherapeutic agents induced hair loss. Intraperitoneal injection of cytosine arabinoside, ara-c, a cell cycle S-phase specific antimetabolite, at 50–75 mg/kg/day for 5 consecutive days, induced whole body alopecia in one week after the first dose administration\textsuperscript{53-55}. Cyclophosphamide, a cell
cycle nonspecific alklator, induced whole body alopecia after a single dose of 50 mg/kg by intraperitoneal injection. Anthracycline antibiotic doxorubicin, a topoisomerase II inhibitor, induced hair loss confined to the head, proximal neck, and drug administration site after 7 consecutive dose of 2 mg/kg/day or 4 consecutive dose of 3 mg/kg/day by intraperitoneal injection. Doxorubicin induced severe apoptosis in hair bulb. Combination of cyclophosphamide and doxorubicin induced whole body alopecia. Etoposide induced whole body alopecia after 3 doses of 1.5mg/kg/day to 11 day-old rats.

Hair loss in neonatal rats usually starts from the head region and progresses to involve the entire body in approximately 2 days, which is visible and can be assessed objectively. The drawback of this model is that the hair follicles are in their first cycle of hair growth after birth and the hair shafts are not pigmented. The levels of growth factors, i.e., aFGF and bFGF, in hair follicles of neonatal rats might be different than that in developmentally mature animals, and may therefore alter the response of hair follicles to FGF treatments.

1.4.2 C57BL-6 mouse model

Significant progresses into the understanding of the follicular pathology of CIA were achieved using the C57BL-6 mice model developed by Paus. In this model, hair shafts are well pigmented and the hair follicles have traversed several postnatal hair growth cycles. In C57BL-6 mice, skin melanocytes are confined to the hair follicles and skin color is associated with the stage of hair growth cycle, with a pink skin color in telogen phase and a black color in anagen phase. Hair follicles are induced into
anagen phase by depilation\textsuperscript{42,57,59}. The depilation induced hair follicles to enter the anagen VI phase in about 8 to 9 days. A single intraperitoneal administration of cyclophosphamide, 150 mg/kg, induced premature catagen development, dystrophic follicles, and complete alopecia in 6 days\textsuperscript{42}. Hair follicles at different anagen stage (I-VI) may respond to cyclophosphamide in different ways\textsuperscript{60}. Cyclophosphamide induced massive apoptosis of keratinocytes in hair bulb of anagen hair follicles. Apoptotic cells were mainly found in hair bulb matrix, while dermal papilla fibroblasts appeared to be resistant to cyclophosphamide\textsuperscript{61}. Hair bulb melanocytes also undergo apoptosis after cyclophosphamide administration\textsuperscript{62}. Cyclophosphamide upregulated death receptors, Fas/Apo-1, and the mitochondrial pro-apoptotic protein Bax in hair follicles, and Fax and c-kit are involved in melanocyte apoptosis and migration during CIA\textsuperscript{63,64}. The recovery of cyclophosphamide-induced alopecia in C57BL-6 mice followed defined patterns, i.e., primary recovery and secondary recovery. Dystrophic anagen hair follicles recover quickly in the primary recovery, but the secondary recovery is delayed. In contrast, dystrophic catagen hair follicles show only secondary recovery\textsuperscript{42,65}. Normal pigmented hair shafts are produced during secondary recovery.

CIA model using C57BL-6 mice has only been established with cyclophosphamide, a cell cycle non-specific agent. As shown in the neonatal model, cell cycle specific agent, i.e., ara-c, might show different characteristics of CIA compared to cell cycle non-specific agent, i.e., cyclophosphamide\textsuperscript{55,66}. Hence, additional CIA models in C57BL-6 mice need be established for studying CIA by other chemotherapeutic agents, for studying CIA by combination of drug with different action mechanisms, as combination chemotherapy has become a common modality in cancer patients care.
1.5. TREATMENT OPTIONS FOR CIA

Interventions to prevent or minimize the hair loss during chemotherapy have been of three major types: scalp tourniquets, scalp hypothermia or cooling, and pharmacological agents. Scalp tourniquets occlude the superficial blood flow to the scalp and reduce the amount of drug delivered to the hair follicles. Scalp tourniquet is the first attempt to protect against CIA in literature\textsuperscript{67}, but it is no longer recommended due to the high pressure applied and uncomfortable feeling of patients. Scalp hypothermia or cooling use ice or temperature-controlled devices to decrease the drug uptake into the scalp and/or alter the metabolism of agents in the cell\textsuperscript{68}. Both scalp tourniquets and hypothermia are impractical during continuous drug infusions, which require a relatively long time for the device left in the place\textsuperscript{38,69}. Methods in achieving scalp hypothermia are still understudy in some clinical trials\textsuperscript{27,39,70-72} but will not be the focus in this review. At present, there are no proven treatments of pharmacological agents for CIA in humans. Different strategies and approaches have been tried in the animal models.

1.5.1. Drug-specific antibodies.

Antibody to doxorubicin incorporated in liposomes and applied topically reduced the severity of doxorubicin-induced alopecia in the neonatal rat model\textsuperscript{73}. The limitation of this strategy is obvious since that it is only specifically applicable for doxorubicin-induced alopecia and cancer is usually treated with combination of different chemotherapeutic agents\textsuperscript{74}. 
1.5.2. Hair growth cycle modifiers and hair growth enhancers.

Immunosuppressive immunophilin ligand such as cyclosporine A induces active hair growth (anagen), inhibits hair follicle regression (catagen) and promotes hair growth activity in several alopecia models. Topical application of cyclosporine A protected rats from local alopecia induced by various agents in the neonatal rats model, and its systemic administration retarded the cyclophosphamide-induced hair loss and prolonged the primary recovery in C57BL-6 mice. Topical application of cyclosporine A prevented the damaged hair follicles from moving into telogen after cyclophosphamide administration and thus induced rapid hair regrowth. Another immunomodulator, AS101, protected against CIA by ara-c in rats and reduced the severity of alopecia in patients treated with a combination of carboplatin and VP-16. The protection by AS101 was possibly mediated by macrophage-derived factors and could also be related to PGE2 secretion. Minoxidil, a drug known to be a hypertrichotic agent and to treat hypertension, shortens telogen phase and causes hair follicle entry into anagen phase, and thus stimulates hair growth. Minoxidil increases the duration of anagen and enlarges miniaturized follicles affected by various conditions, irrespective of the underlying cause. The responses of hair follicles to minoxidil and the related possible mechanisms have been reviewed by Messenger and Rundegren recently. Topical formulations of minoxidil have been developed for the treatment of androgenetic alopecia. In a rodent model, minoxidil protects against CIA by ara-C but not cyclophosphamide. Minoxidil also shortens the duration of CIA in some situations, but does not protect against CIA in humans.
1.5.3. Cytokines and growth factors.

Development and growth of hair follicles are influenced by a number of cytokines and growth factors including fibroblast growth factors, which might act though autocrine and/or paracrine mechanisms since their receptors are also found in hair follicles. No single growth factor, however, appears to exert ultimate control over the hair growth cycling. Imuvert, a biological response modifier from the bacterium Serratia marcescens, and interleukin 1 (IL-1) protected rats from CIA by cell cycle-specific agents, i.e., ara-C, but not cell cycle-nonspecific agent, i.e., cyclophosphamide. Both Imuvert and IL-1 can induce the release of multiple cytokines or growth factors with a wide range of biological effects. The protection by Imuvert is presumably mediated by IL-1. Further screening of various cytokines and growth factors found that aFGF and epidermal growth factor (EGF) protected CIA by cell cycle specific agent (i.e., ara-c) but had no effect in protecting against CIA by cell cycle non-specific agent (i.e., cyclophosphamide) in the neonatal rat model. The mechanisms aFGF or EGF protected ara-c-induced hair loss were not clear. EGF, at concentrations between 1 ng/ml and 25 ng/ml, significantly inhibits the uptake of thymidine by hair matrix cells in an organ culture. Antioxidant N-acetylcysteine, an analog and precursor of glutathione, when applied topically in liposome or delivered parentally, protected rats from hair loss by cyclophosphamide, and protected mice from alopecia by doxorubicin. The combination of N-acetylcysteine and Imuvert offered protection against CIA by a combination of cyclophosphamide and ara-c in the neonatal rat model. Fibroblast growth factor 2, FGF-2 or bFGF, had little influence on DNA synthesis in hair matrix cells, and delayed the first hair cycle initiation and...
development in mice by subcutaneous injection\textsuperscript{92}, but there is no report so far that bFGF could protect against CIA by any anti-cancer agent. bFGF has also been shown to be a natural mitogen for melanocyte and can sustain melanocyte growth and survival\textsuperscript{93}. Keratinocyte growth factor, FGF7 or KGF, stimulate the proliferation of keratinocytes within hair follicles, and partially protected neonatal rats against CIA by ara-c\textsuperscript{94,95}. KGF pretreatment also increased the hair follicle survival following lethal irradiation\textsuperscript{96}. FGF5, which is expressed just before the end of anagen phase, might trigger catagen onset\textsuperscript{97,98}. Mice that lack FGF5 have longer hair with extended anagen phase. There is no report using FGF5 to protect against CIA.

\textbf{1.5.4. Cell cycle or proliferation modifiers.}

The passage of a cell through cell cycle is controlled by cyclin-dependent kinases (Cdks) associated with cyclins in cytoplasm. Anagen hair follicles represent an unintended target of chemotherapy, which is in part due to the rapid cell proliferation in the hair follicles and the lack of cancer cell selectivity of anticancer agents. Inhibition of cell proliferation was thus assumed to decrease the hair follicular cells sensitivity to chemotherapy\textsuperscript{46,46,99}. Inhibitors of cyclin-dependent kinase 2 (CDK2), by blocking cell cycle progression, reduced the sensitivity of hair follicles to antitumor agents and protected neonatal rats against CIA by etoposide and a combination of cyclophosphamide and doxorubicin\textsuperscript{46}. The CDK2 inhibitors are small molecules that can possibly be absorbed into the systemic circulation after topical application, which may compromise the efficacy of chemotherapy.
Calcitriol, 1,25-Dihydroxyvitamin D₃, probably through stimulating of keratinocyte differentiation and inhibiting of DNA synthesis in keratinocytes, protects rats from alopecia by cyclophosphamide, etoposide and a combination of cyclophosphamide plus doxorubicin⁵⁸. In the C57BL-6 mouse model, this compound was shown to enhance the normal pigmented hair shafts regrowth, reduced apoptosis in hair bulb, but did not prevent or retard the hair loss after the administration of cyclophosphamide¹⁰⁰,¹⁰¹. In a phase I clinical trial in cancer patients, this agent proved to be ineffective and induce local dermatitis in the drug exposed areas¹⁰².

Parathyroid hormone (PTH) related peptide (PTHrP) is an antiproliferative factor that participates in the regulation of hair follicle cell growth¹⁰³. PTH agonist enhanced the intrafollicular apoptosis, forced hair follicles into dystrophic catagen and thus enhanced the hair loss but accelerated the hair follicle recovery after the CIA by cyclophosphamide. Antagonists of PTH and PTHrP, however, reduced the apoptosis in hair follicles and forced hair follicles into dystrophic anagen and thus retard the onset of hair loss after the administration of cyclophosphamide in C57BL-6 mice¹⁰⁴. Although these compounds may alter the responses of hair follicles to chemotherapy, agonist of PTH and antagonists of PTH and PTHrP did not reverse CIA.

1.5.5. Apoptosis Signaling Inhibitors.

The transcription factor and tumor suppressor protein p53 induces apoptosis in response to a variety of cellular stress signals. Both p53 and its target genes are involved in chemotherapy-induced apoptosis in hair follicles. Thus, pharmacological inhibition of p53 signaling or function was hypothesized to protect against CIA¹⁰⁵.
P53 deficient mice showed neither hair loss nor apoptosis of keratinocytes after cyclophosphamide administration. M50054, 2,2’-methylenebis, an inhibitor of apoptosis by inhibition of caspase-3 activation, inhibited apoptosis induced by etoposide in U937, a human monocytic leukemic cell line. In the neonatal rat model, topical application of M50054 in acetone showed reduction of hair loss induced by etoposide. However, a lack of functional p53 may be accompanied by high rates of genomic instability and rapid tumor progression and this approach would be applicable only for tumors that lack functional p53. On the other hand, p53-independent pathways can also mediate apoptosis. Apoptosis may not be the only cause of CIA, since many chemotherapeutic agents function through anti-proliferation or other pathways. Thus, p53 inhibitors are not sufficient to provide broad-spectrum protections against CIA.

1.5.6. Summary.

Major compounds or strategies that have been tried to protect against CIA are summarized in table 1.1. The data shows that all the previous CIA-protective agents fall into two categories, one that protects CIA by cell-cycle-specific agent, i.e., ara-c, and one that protects against CIA by cell cycle-nonspecific agents, i.e., cyclophosphamide. Hence, the above earlier agents typically protected against CIA by one drug or one drug class in animal models, did not show broad-spectrum protection against all classes of anticancer drugs or combination of several drug classes, some do not offer protection to 100% of the hosts, and some are not effective against CIA in humans. None of these agents is known to protect against CIA by paclitaxel, the major agent used against breast and ovarian cancer in female patients.
These concerns, together with the fact that cancer is usually treated with combinations of drugs with different action mechanisms, suggest limited application for these earlier agents. Hence, one prerequisite for therapeutic progress in manipulating CIA is that promising compounds to protect against CIA should be effective for different drugs with different action mechanisms, or the protection should be selective to hair follicles. The data also suggest that the problem of protection against CIA is much more complex than previously appreciated, and combinations of different strategies mentioned above may be helpful to protect against this distressing side effect. In designing pharmacological compounds for manipulating CIA, one should take into consideration that the compound should be topically applicable and not systemically available so that the efficacy of chemotherapy to tumors will not be compromised.
We hypothesize that combination of acidic and basic fibroblast growth factors (aFGF and bFGF, or FGF1 and FGF2, FGFs) offer broad-spectrum protection against CIA. This hypothesis is based on the following findings. a) Previous studies in our laboratory have shown that aFGF and bFGF, at clinically relevant concentrations, induce up to 10-fold resistance for multiple chemotherapeutic agents with diverse chemical structures and action mechanisms in tumor cells. We further found that bFGF is required to induce resistance, whereas aFGF amplifies the chemoprotective effect of bFGF.\textsuperscript{109} FGFs-induced resistance applies to both anti-proliferative and apoptotic effects of drugs and the extent of resistance is dependent on FGFs levels. Hence, FGFs can be used to protect cells against drugs that act mainly by inhibiting cell growth and drugs that act mainly by inducing apoptosis. b) FGFs and their receptors (FGFRs) are found throughout the hair follicle that are important for hair growth, retention and keratinization\textsuperscript{1} (Figure 1.3). bFGF is found in the suprabasal layer of the epidermis, ORS and the basement membrane adjacent to the proliferating zone of hair bulb. aFGF is found in the epidermis, cells in the keratogenous zone, and IRS\textsuperscript{110}. FGFR1 is found in dermal papilla, FGFR2 in hair bulb matrix, FGFR3 in precuticle cells of hair bulb, and FGFR4 in IRS and ORS bulb periphery. The two growth factors, thus, have different and maybe exclusive localization in hair follicles, which suggest their related and perhaps complementary differentiation and proliferation functions, which might act through an autocrine and/or paracrine mechanism, in controlling normal hair growth and cycling. Development and growth of hair follicles are influenced by a number of cytokines and growth factors, however, no single growth factor appears to
exert ultimate control over the entire hair growth cycle, suggesting that combinations of these growth factors may be useful for treatment of hair growth disorders. The basement membrane of hair follicles is enriched in heparan sulfate proteoglycan, which binds FGFs andfacilitate the binding of FGFs to FGFRs. It is interesting to mention that bFGF is also required for melanocyte growth and survival, with the consideration that hair graying is often associated with CIA. c) Previous studies have shown that growth factors protected against CIA by ara-c in neonatal rats (section 1.5.3). The remaining chapters in this dissertation are organized according to the purposes and experimental designs to test the hypothesis addressed.

Chapter 2 investigates the animal models to study CIA. The purpose of this chapter is to establish CIA models by different chemotherapeutic agents with different chemical structures and anticancer mechanisms, i.e., alkylators (cyclophosphamide), topoisomerase 2 inhibitors (doxorubicin), antimicrotubules (paclitaxel), and antimetabolites (cytosine arabinoside, or ara-c). These chemotherapeutic agents are known to cause alopecia in humans and represent 4 major classes of anticancer drugs. Doxorubicin and paclitaxel are among the most commonly used and most alopecigenic anticancer drugs for cancers in women (e.g., breast, ovarian). We established CIA models in neonatal rats by doxorubicin, cyclophosphamide, ara-c and paclitaxel, and CIA models in C57BL-6 mice by cyclophosphamide and paclitaxel. The characteristics of these CIA models were studied and described.

Chapter 3 evaluates the pharmacodynamic endpoints of CIA in the two animal models. We established hair follicle morphology-based pharmacodynamic endpoints, i.e.,
dermal layer thickness, hair bulb diameter, hair follicle length, and hair follicle density. These pharmacodynamic endpoints showed similar time-dependent changes during CIA. Dermal layer thickness was chosen as the endpoint to evaluate CIA and FGFs protective effect in the subsequent studies. Apoptosis studies revealed that hair bulb matrix and ORS were the major targets of chemotherapy in hair follicles.

Chapter 4 evaluates the protective effect of FGFs against CIA by subcutaneous injection as an initial proof-of-principle. The results showed that FGFs offer broad-spectrum protection against CIA by all four chemotherapeutic agents applied in the animal models. FGFs also accelerated the hair regrowth.

Chapter 5 investigates the protective effect of FGFs against CIA by topical application. The results showed that topical FGFs dissolved in 75% dimethyl sulfoxide offered broad-spectrum protection against CIA by all four chemotherapeutic agents in the animal models. The data further show that no or insignificant systemic exposure of bFGF after subcutaneous or topical delivery of bFGF, indicating that FGFs will not compromise the chemotherapy in tumors.

Chapter 6 summarizes the contributions of this dissertation research and discusses future investigations.
Table 1.1. Pharmacological compounds for protection against CIA. The agents fall into two categories. One group (a) that protects against CIA by cell-cycle-specific agent, i.e., ara-c. The other group (b) that protects against CIA by cell cycle-non-specific agent, i.e., cyclophosphamide. None of the agents shows protection against CIA by paclitaxel, the major compound for breast and ovarian cancer in female patients. “+” represents successful protection against CIA, and “–” represents ineffective in protection against CIA. Anti-dox, antibody to doxorubicin; CDK In, CDK inhibitors; Csp A, cyclosporine A; n-Ace, n-acetylcysteine; 1,25 OH-VD, 1,25-dihydroxyvitamin D3 or calcitriol; PTHrP, Parathroid hormone related peptide; Apop in, apoptosis inhibitors; CTX, cyclophosphamide.
Figure.1.1. Hair follicle structure. The microscopic anatomy of a hair follicle in its mature anagen phase. From top to bottom, a hair follicle can be divided into three segments, infundibulum, isthmus and inferior segment (hair bulb). From central to peripheral, the basic units of the follicle are hair shaft, hair shaft cuticle, IRS (including inner root sheath cuticle, Huxley’s layer and Henle’s layer), ORS and the connective tissue sheath.

Picture modified from website:

http://www.derm.ubc.ca/hairnailsmucousmembranes/structure.htm
Figure 1.2. Hair growth cycle. Each hair growth cycle consists of anagen, catagen and telogen. In the anagen VI (i), matrix cells in hair bulb have high mitotic activity and hair shaft is produced. Catagen is the apoptosis-driven process with the shrinkage of the lower two-thirds of the hair follicle (ii). Telogen is a resting stage (iii). The onset of a new anagen phase is controlled by the communication between the stem cells in hair bulge and dermal papilla (iv). E, Epidermis; HS, Hair Shaft; DP, Dermal Papilla; M, Matrix; CTX, Connective Tissue Sheath; B, Bulge; S, Sebaceous Gland.

(Sarah E. Millar, the Journal of Investigative Dermatology, 2002, 118: 216-224)
Figure 1.3. Distribution of aFGF and bFGF in hair follicle. aFGF (yellow) is found in the epidermis, keratogenous zone, and IRS. bFGF (blue) is found in the suprabasal layer of the epidermis, ORS and the basement membrane adjacent to the proliferating zone of the hair bulb. The indicated structures are: bl, basal layer; dp, dermal papilla; e, epidermis; f, fiber; fb, follicle bulb; kz, keratogenous zone; sg, sebaceous gland; sl, suprabasal layer.

CHAPTER 2

ESTABLISHMENT OF ANIMAL MODELS FOR STUDY OF
BROAD-SPECTRUM PROTECTION AGAINST
CHEMOTHERAPY-INDUCED ALOPECIA

2.1 INTRODUCTION

The hair follicle represents a unique hair-shaft-producing organ, which is characterized by postnatal life cyclic changes in its activity with three distinct phases of relative resting (telogen), active growth and hair shaft production (anagen), and apoptosis-driven regression (catagen)\(^1\). The hair follicle represents an un-intended target for chemotherapeutic agents due to the rapid proliferation rate of hair matrix keratinocytes during anagen growing phase. Chemotherapy-induced alopecia (CIA) is one of the most psychologically devastating undesirable side effects in oncologic practice for which there is currently no effective therapy.

Research in manipulating CIA has been at least partially hampered due to the lack of a reproducible animal model. The complexity of hair growth and cycling prevents utilization of cell culture and organ culture systems to study CIA. Progress in protection against CIA was achieved using the neonatal rat model first introduced by Hussein\(^54\). It was found that cytosine arabinoside (ara-c) induced whole body complete...
alopecia in 8-day-old rats with treatment of 50 mg/kg/day via intraperitoneal injection daily for 5-7 consecutive days. Cyclophosphamide was also shown to induce whole body complete alopecia in the neonatal rat model.

Significant progress into understanding the follicular pathology of CIA was achieved using the adult C57BL-6 mice model developed by Paus. In this adult mouse model, a single dose of cyclophosphamide, 150 mg/kg, administered by intraperitoneal injection was sufficient to induce complete alopecia. However, only cyclophosphamide, a cell cycle non-specific agent, has been employed to induce hair loss in this model. We chose paclitaxel to establish another CIA model in C57BL-6 mice, based on the following considerations. a) It has been shown in neonatal rats that cell cycle-specific agents might behave differently than cell cycle non-specific agent in the CIA model. Paclitaxel is a cell cycle-specific agent targeting G2/M phase of the cell cycle. b) Paclitaxel and docetaxel are the two commercially available taxanes that are among the most useful antitumor agents. The action mechanisms of taxanes are different than that of other compounds mentioned above, which is to enhance tubulin polymerization, promote microtubule assembly, bind to microtubules, stabilize microtubule dynamics, induce mitotic block at the metaphase and anaphase transition, and induce apoptosis. Paclitaxel and docetaxel show similar side effect profiles, and both of them have high potential to induce severe alopecia in humans. c) Paclitaxel failed to induce complete hair loss in neonatal rats after systemic administration. As far as we know, there is no reported suitable model to evaluate the protection against paclitaxel-induced alopecia. CIA is becoming more prevalent as
taxanes are becoming more frequently employed against breast and ovarian cancer of female patients.

Several pharmacological compounds have been shown to protect against the CIA by above chemotherapeutic agents in the models (chapter 1, table 1.1). However, the translation of these compounds to protect against CIA in humans has been hampered by the fact that cancer is usually treated with combinations of anti-cancer drugs with different action mechanisms. Hence, one prerequisite for therapeutic progress in manipulating CIA is that a promising compound to protect CIA should be tested in different models of different drugs with different action mechanisms. Thus, in order to test our hypothesis that aFGF and bFGF can broadly protect CIA, the first step is to establish CIA animal models by different chemotherapeutic agents representing different drug classes with different action mechanisms.

In this chapter we studied 4 drugs that are known to cause alopecia in humans and represent 4 major classes of anticancer drugs, i.e., alkylators (cyclophosphamide), topoisomerase 2 inhibitors (doxorubicin), antimicrotubules (paclitaxel), and antimetabolites (cytosine arabinoside, or ara-c). These four agents also show different chemical structures, pharmacokinetic and pharmacodynamic properties, and mechanisms of antitumor activity (Figure 2.1 and table 2.1). Two CIA models were established, i.e., neonatal rat model and C57BL-6 mouse model.
2.2. MATERIALS AND METHODS

2.2.1 Materials and chemotherapeutic agents

Clinically formulated cyclophosphamide (USP) and ara-c (USP) were purchase from the James Cancer Hospital Pharmacy of the Ohio State University. Doxorubicin hydrochloride and Cremophor EL were purchased from Sigma Co. (St. Louis, MO). Paclitaxel was obtained from the Britol Myers Squibb Co. (Wallingford, CT) and the National Cancer Institute (Bethesda, MD). All chemotherapeutic agents were > 99% pure and fresh solutions of each agent were prepared just before use. Paclitaxel was first dissolved in 1:1 ethanol and Cremophor and then diluted to different concentrations in saline upon use. Doxorubicin, cyclophosphamide and ara-c were dissolved in normal saline.

2.2.2. Animals

Female pregnant Sprague-Dawley rats and female C57BL-6 mice (6-8 week old, weighing 18-22 g, with normal black fur) were purchased from Charles River Laboratories, Wilmington, MA. The animal care was provided by the Laboratory Animal Resources at the Ohio State University. Neonatal rats were housed together with the mother rat for at least 3 weeks after birth. Neonatal rats delivered from the same mother rat (10-14 newborns per mother rat) were randomly divided into different groups according to their body weight at the experimental time. The animals were housed in community cages with 12-hour light periods.
2.2.3. Hair follicle synchronization in adult C57BL-6 mice

Hair follicles in the neonatal rats are spontaneously in the early anagen phase immediately after birth and no experimental induction is required. Hair follicles on the back of adult C57BL-6 mice were synchronized into anagen phase by depilation as described before. Briefly, hair follicles of the back skin in 6-week-old C57Bl-6 mice in telogen phase are recognized reliably by the homogeneous pink color of the skin. The hair shafts on the back were first covered by a warm melted resin-wax mixture (45-60 °C, 1:1 weight ratio) and then plucked out by peeling off the mixture after hardening. The hair follicles spontaneously entered into the anagen phase (stage I) with corresponding skin pigmentation immediately after depilation (Figure 2.2).

2.2.4. Induction of alopecia in neonatal rats

Seven-day-old rats were used for treatments with doxorubicin and eight-day-old rats were used for treatments with the other chemotherapeutic agents. Rats were given intraperitoneal injection of doxorubicin (1.5 mg/kg/day for 7 consecutive days), cyclophosphamide (50 mg/kg single dose for cyclophosphamide-1, or 30 mg/kg/day for 2 consecutive days for cyclophosphamide-2), or ara-c (50 mg/kg/day for 5 consecutive days).

Intraperitoneal injection of paclitaxel failed to induce complete alopecia in neonatal rats due to drug toxicity resulting in early animal death before the complete hair loss occurred. With doses lower than 0.6 mg/kg, up to 7 multiple daily injections starting on day 7 after birth did not cause significant body weight loss and did not induce apparent hair loss. With dose levels ranging from 0.8 mg/kg to 2.0 mg/kg, a single
dose did not induce significant hair loss, while multiple doses (3 to 7 daily doses) caused partial hair loss but with significant body loss (> 20%, compared to control animals on day 4 after the first dose). When doses were between 3.0 mg/kg and 6.0 mg/kg, a single dose was sufficient to induce partial hair loss on the head. In all the experiments with observable hair loss, all the animals died before complete hair loss. When dose was higher than 6.0 mg/kg, a single dose of paclitaxel caused 100% animal death in 5 days after paclitaxel injection. Hence, it was necessary to use local drug injection to induce CIA by paclitaxel. Briefly, two doses of paclitaxel, 2.5 µg each in 50 µl saline, were injected directly on the head between two ears subcutaneously on day 8 and day 11 after the birth of animals.

In each experiment, control animals received normal saline injections. The dosing schedule of each chemotherapeutic agent is shown in Figure 2.3. Hair loss occurred naturally without any physical forces applied. Body weight of each animal was measured every 24 hours to evaluate the toxicity of the treatments.

2.2.5. Induction of alopecia in C57BL-6 mice

When early anagen VI had been reached after depilation, i.e., on day 9 post-depilation, a single dose of cyclophosphamide, 150 mg/kg, was given by intraperitoneal injection as reported before\textsuperscript{42}. For paclitaxel, the adult mice were able to tolerate higher dose of systemic administration, compared to neonatal rats. The higher systemic doses resulted in CIA. A single dose of paclitaxel, ranging from 20 mg/kg to 100 mg/kg, was given on day 9 post-depilation via intraperitoneal injection. For multiple injections, mice were given paclitaxel, 10 mg/kg/day for 6 days, 20 mg/kg/day for 6 days, 30
mg/kg/day for 5 days or 40 mg/kg for 3 days, starting on day 9 post-depilation. The injection dosing volume for both cyclophosphamide and paclitaxel were 0.5 ml for a 20-gram mouse. The dosing schedule of each treatment is show in figure 2.8. Hair loss occurred naturally without any physical forces applied. Body weight of each animal was measured every 24 hours to evaluate the systemic toxicity of drug treatments.
2.3 RESULTS

2.3.1. Induction of alopecia in neonatal rats

The treatment schedules of the chemotherapeutic agents are listed in figure 2.3. All 4 drugs caused alopecia in 100% of the hosts. The extents and characteristics of CIA are drug and dosing schedule-specific (Figure 2.4). Doxorubicin caused severe to complete alopecia only on the head between the ears on day 13 after the first dose of doxorubicin. Ara-C and high dose cyclophosphamide (cyclophosphamide-1, 50 mg/kg) induced whole body alopecia starting on day 6 after the first dose administration. Ara-c-induced hair loss in neonatal rats appeared only after repeated treatments. Intraperitoneal injection of paclitaxel failed to induce complete alopecia in neonatal rats due to the toxicity and early animal death. Subcutaneous injection of paclitaxel induced complete hair loss around the location of injection site. Hair loss was observed starting on day 8 and was completed on day 10 after the first dose of paclitaxel. Low dose cyclophosphamide (cyclophosphamide-2, 30 mg/kg/day for 2 days) induced complete alopecia confined to the head and proximal neck region.

Treatments with doxorubicin induced severe body weight loss, i.e., >25% after the last dose administration and most animals died within 10 days after the last dose administration. Treatments with ara-c only resulted in moderate toxicity with less than 10% body weight loss. Cyclophosphamide and paclitaxel did not cause significant body weight loss. The data indicated that hair loss was a relatively independent toxicity, since the characteristics and severity of hair loss were not correlated with the systemic toxicity of the different chemotherapeutic agents.
2.3.2. Wave pattern of hair loss and hair regrowth in neonatal rats

In the neonatal CIA models with ara-c and high dose cyclophosphamide, which induced whole body complete alopecia, hair loss started from the head and proximal neck region and then progressed to the tail region involved the whole body in about 2 days (Figure 2.5). The wave direction of hair regrowth was opposite to the direction of hair loss. There were two waves of hair follicle recovery after whole body hair loss in neonatal rats (Figure 2.6). One wave was from abdomen to back and the other wave was from the tail region to the head region. The hair regrowth in general followed the combination of these two waves. But in some cases only one wave with a tail-to-head direction was observed. For example, Figure 2.7 shows the regrowth wave, from tail to head, after ara-c-induced whole body hair loss. These opposite wave patterns of hair loss and growth in rats and mice resulted in the observation that the head was the first to lose the hair during CIA and the last to recover from CIA.

2.3.3. Induction of alopecia in adult C57-BL-6 mice

CIA in adult C57BL-6 mice was confined to the area where the hair follicles were induced into anagen phase by depilation. A single dose of cyclophosphamide (150 mg/kg) induced complete hair loss on day 5-6 after drug administration. Intraperitoneal delivery of paclitaxel has been applied for ovarian cancer patients\textsuperscript{118}. Our laboratory has shown that paclitaxel has a bioavailability of 34% after a single dose intraperitoneal injection in mice. Intraperitoneal paclitaxel-induced alopecia showed a dose dependent manner. When the dose of paclitaxel was lower than 50 mg/kg, a single dose injection was not sufficient to induce complete hair loss. When
the dose of paclitaxel was higher than 60 mg/kg, a single injection was sufficient to induce complete alopecia on day 6 after drug administration. When the dose of paclitaxel was higher than 80 mg/kg, severe body weight loss (>20%) was observed on the second day after drug administration, but no animal death was found when dose was lower than 100 mg/kg. When the dose of paclitaxel was lower than 60 mg/kg, repeated injections, i.e., 40 mg/kg/day, induced complete hair loss. When doses were lower than 40 mg/kg even repeated treatments failed to induce complete hair loss (Figure 2.9). The data indicated: a) a threshold paclitaxel level for induction of CIA, b) a single high paclitaxel induced more severe CIA, compared to a higher dose (120 vs 60 mg/kg) administered as 3 divided daily doses. It is important to mention that our laboratory has shown that fractionation of paclitaxel dose resulted in higher drug penetration and accumulation in solid tumors in a rat model\textsuperscript{119}. 
2.4. DISCUSSION

We established two animal models to study CIA by four different drugs, i.e., doxorubicin (topoisomerase 2 inhibitor), paclitaxel (antimicrotubule), Ara-C (antimetabolite) and cyclophosphamide (alkylator), with different action mechanisms and chemical structures. These drugs are known to cause alopecia in humans. Ara-c and paclitaxel are cell cycle-specific agents, with target cells in S phase and G2/M phase, respectively. Doxorubicin and cyclophosphamide are considered cell cycle-nonspecific agents. Doxorubicin and paclitaxel are among the most commonly used and most alopecigenic anticancer drugs, especially in women cancer patients (e.g., breast, ovarian).

The time course of CIA in the neonatal rat models was drug dependent. Complete alopecia was induced in 6 to 7 days after the first dose of ara-c and cyclophosphamide, in 10 days after the first dose of paclitaxel and in 13 days after the first dose of doxorubicin. Alopecia by ara-c or cyclophosphamide occurred when hair follicles in the normal control rats were in the anagen phase, alopecia by paclitaxel occurred when hair follicles in control rats were in the anagen/catagen transition phase and alopecia induced by doxorubicin occurred when hair follicles in control rats were in the telogen phase. In the C57BL-6 mouse model, complete alopecia occurred in 6-7 days after the first dose administration, when the hair follicles in normal control mice were in anagen phase. Thus, the established CIA models by the four drugs covered all stages of a hair growth cycle.
In neonatal rats, hair growth followed a wave pattern in the first hair growth cycle after birth, and the wave started at the head, moved backward to the tail, and from abdomen to back. Because of the wave pattern, a group of hair follicles in a rat at a specific area were usually in the same stage of hair growth. This wave pattern of hair growth resulted in different sensitivity to chemotherapeutic agents of hair follicles at different parts of body with more severe hair loss on the head region and less severe hair loss on the tail region. Thus, when localized hair loss was induced by doxorubicin or cyclophosphamide-2, it was more likely that the hair loss occurred on the head; since hair follicles at this region were in the mature anagen phase in the 8-day-old rats used in the model and were more susceptible to chemotherapy. Due to the wave pattern, the sensitivity of hair follicles to chemotherapy changed with time, and it is possible the same treatment when given at different time, the hair loss will occur at different location. It is not clear which factor, such as a signal protein, is responsible for or controlling the wave pattern of hair growth in neonatal rats. Hence, when using neonatal rats model to evaluate CIA, it is important to maintain the same dosing schedule in animals of the same age.

The CIA models in neonatal rats were highly reproducible and the hair loss was visible and can be easily assessed objectively. The disadvantages of this model are: a) Hair follicles in the neonatal rats are in their first growth cycle after birth, and thus the formation of hair shaft in this cycle is actually an integral part of follicular morphogenesis, in which the whole structure of hair follicles is generated from a primitive epidermis, while in postnatal hair growth cycle only lower part of hair follicle structure is regenerated. b) Whether the levels of growth factors in hair
follicles of neonatal rats are comparable to the levels in developmentally mature animals is another considerable limitation of this model. c) Hair shafts in neonatal rats are not pigmented, which limits its application in study the effect of chemotherapy on hair color, since graying of hair occurs in over 60% of patients. In comparison with neonatal rats, hair follicles in C57BL-6 mice are mature, pigmented and have already traversed some postnatal hair growth cycles.

In summary, we established animal models for the pharmacological manipulation of CIA in both neonatal rats and C57BL-6 mice. These models together enabled the studies on CIA by different major classes of chemotherapeutic agents.
Figure 2.1. Chemical structures of the chemotherapeutic agents.
<table>
<thead>
<tr>
<th>Drug</th>
<th>M.W.</th>
<th>Action Mechanism</th>
<th>Cell Cycle</th>
<th>Half-life</th>
</tr>
</thead>
<tbody>
<tr>
<td>Doxorubicin</td>
<td>539</td>
<td>TopoII inhibition Free radicals DNA damage</td>
<td>Non-specific</td>
<td>α=12 min, β=3.3 hr, γ=29.6 hr</td>
</tr>
<tr>
<td>Ara-C</td>
<td>243</td>
<td>DNA incorporation DNA polymerase inhibition S</td>
<td>S</td>
<td>10-20 min</td>
</tr>
<tr>
<td>Cyclophosphamide</td>
<td>279</td>
<td>Cross-linking DNA strands</td>
<td>Non-specific</td>
<td>4-10 hr</td>
</tr>
<tr>
<td>Paclitaxel</td>
<td>853</td>
<td>Antimicrotubule</td>
<td>G2/M</td>
<td>α=16-29 min, β=6-13 hr</td>
</tr>
</tbody>
</table>

Table 2.1. Pharmacological and pharmacokinetic properties of the chemotherapeutic agents.

Data were obtained from [http://www.bccancer.bc.ca/default.htm](http://www.bccancer.bc.ca/default.htm).
Figure 2.2. Hair follicle synchronization in C57BL-6 mice by depilation. The melted mixture of wax and rosin (1:1) was applied on the back skin and peeled off after hardening. The depilated hair follicles were in telogen phase, indicated by the pink color of the back skin (day 0, A). The depilation resulted in skin pigmentation and synchronized anagen induction. Hair follicles developed into anagen VI within 9 days, indicated by the gray to black color of back skin (day 9, B). On day 10 to day 12, the black hair shaft could be observed on the surface of skin (day 12, C). The anagen phase and associated black hair shaft production were maintained to day 16 after depilation (day 16, D).
Figure 2.3. Dosing schedule of the chemotherapeutic agents in neonatal rat CIA model. Seven to eight-day-old rats were given intraperitoneal injections of doxorubicin (1.5 mg/kg/day for 7 days), ara-c (50 mg/kg/day for 5 days), or cyclophosphamide (cyclophosphamide-1, a single dose of 50 mg/kg; cyclophosphamide-2, 30 mg/kg/day for 2 days). Subcutaneous injections of paclitaxel were given on day 0 and day 3 of experiment (2.5 µg each in 50 µl normal saline). All the treatments induced alopecia in 100% of the animals. The time-course of alopecia was drug and dosing schedule dependent.
Figure 2.4. Characteristics of CIA by different chemotherapeutic agents in neonatal rats. Neonatal rats were treated with different drugs or different dosing schedules as shown in figure 2.3. The extents and characteristics of CIA are drug-dependent. Doxorubicin-induced complete alopecia was confined to the head and cyclophosphamide-2-induced complete alopecia was confined to the head and proximal neck. Both cyclophosphamide-1 and Ara-c induced whole body complete alopecia. Paclitaxel-induced complete alopecia was confined only to the subcutaneous injection site. Dox, doxorubicin; CTX-1, cyclophosphamide-1 (50 mg/kg); CTX-2, cyclophosphamide-2 (30 mg/kg/day for 2 days). (n>6)
Figure 2.5. Wave pattern of ara-c-induced hair loss in neonatal rats. Eight-day-old rats were treated with ara-c, 50 mg/kg/day, for 5 consecutive days. Hair loss started from the head region and progressed to involve the entire body in approximately 2 days. It is important to mention that the normal hair growth in control animals also showed a wave pattern in their first growth cycle after birth, and the wave was from head to tail and from abdomen to back. The wave pattern of hair loss indicated different sensitivity of hair follicles to chemotherapy. Similar results were found in cyclophosphamide-induced alopecia. (n>6)
Figure 2.6. Wave pattern of hair regrowth after cyclophosphamide-induced whole body alopecia. Eight-day-old rats were treated with cyclophosphamide, 50 mg/kg, by intraperitoneal injection. Whole body hair loss was observed on day 7 after drug administration. Two waves were observed during hair regrowth, one from abdomen to back, and the other from tail to head. The pattern of the hair regrowth in general was the combination of these two waves with an overall growing direction from the tail region to the head region. The entire animal body was covered by hair shaft again in 23-25 days. (n>6)
Figure 2.7. Wave pattern of hair regrowth after ara-c-induced whole body alopecia. Eight-day-old rats were treated with ara-c, 50 mg/kg/day, for 5 consecutive days by intraperitoneal injection. Complete whole body alopecia was observed on day 7 after the first dose of ara-c. The overall direction of hair regrowth was from tail region to head region. The data indicated different sensitivity of hair follicles to chemotherapy in their first growth cycle after birth. The data also indicate the direction of wave of hair regrowth is not drug dependent. (n>6)
Figure 2.8. Dosing schedule of cyclophosphamide or paclitaxel in adult C57BL-6 mice. Mice were given a single intraperitoneal injection of cyclophosphamide (150 mg/kg) or paclitaxel (20 mg/kg/day for 6 days, 30 mg/kg/day for 5 days, 40 mg/kg/day for 3 days, single dose of 60 mg/kg or single dose of 80 mg/kg) on day 9 post-depilation. A single dose of cyclophosphamide induced complete alopecia on day 15 after depilation. For paclitaxel, when the dose was lower than 40 mg/kg, multiple doses did not induce complete hair loss. When the single dose was higher than 60 mg/kg, paclitaxel induced complete hair loss on day 15 post-depilation. When dose was between 40 mg/kg and 60 mg/kg, multiple doses of paclitaxel induced complete hair loss on day 15 post-depilation.
Figure 2.9. Characteristics of chemotherapy-induced alopecia in C57BL-6 mice by cyclophosphamide or paclitaxel. Animals were treated with cyclophosphamide (CTX) or paclitaxel (pac) with different dosing schedules as mentioned in figure 2.8. Chemotherapy-induced complete alopecia in C57BL-6 mice was confined to the area where hair follicles were induced into anagen phase by depilation. Paclitaxel-induced alopecia showed a dose dependent manner. A minimum single dose of 60 mg/kg or accumulative multiple doses of 120 mg/kg over 3 days are required to induce complete hair loss by paclitaxel. (n>3 per group)
CHAPTER 3

EVALUATION OF PHARMACODYNAMIC ENDPOINTS OF CHEMOTHERAPY-INDUCED ALOPECIA IN ANIMAL MODELS

3.1 INTRODUCTION

Chemotherapy-induced alopecia (CIA) is widely experienced as one of the most psychologically distressing side-effects of cancer patients, for which there is currently no satisfactory remedy available in clinic practice. During the last decade, progress in understanding molecular mechanisms and pathobiology of CIA was achieved using the newborn rat model and the adult C57BL-6 mouse model. A variety of pharmacological compounds have been found relatively effective in protecting against CIA in the animal models. However, these compounds or attempts either do not offer broad-spectrum protection in the animal models or are not effective in humans, which suggest that the major pathways of CIA are not targeted by these substances partially due to the lack of the full spectrum of the pharmacodynamic responses of hair follicles to chemotherapy.

Chemotherapeutic agents impair metabolic and mitotic processes in anagen hair follicles, as well as stimulate premature apoptosis-driven catagen induction, which eventually result in hair loss. Apoptosis has been suggested as a mechanism
of both catagen development and CIA\textsuperscript{57,100,123,124}. Most studies on drug-induced apoptosis used CIA by cyclophosphamide. Cyclophosphamide upregulated death receptors, Fas/Apo-1, and the mitochondrial pro-apoptosis protein Bax in hair follicles, and Fax and c-kit are involved in melanocyte apoptosis and migration\textsuperscript{63,123}. Detailed information on general targets of chemotherapy in hair follicles, detailed characterization of the kinetics of hair follicular damages, as well as the mechanisms hair follicles shedding the hair shafts during CIA, are not available. The established CIA animal models (Chapter 2) allowed us to study the responses of hair follicles to different chemotherapeutic agents.

In both the newborn rat model and the adult C57BL-6 mouse model, hair loss is visible, and both CIA and its protection are usually assessed visually. For example, some research groups scored CIA into 4 grades: grade 1, no detectable alopecia; grade 2, mild alopecia with less than 50% hair loss; grade 3, moderately severe alopecia with more than 50% hair loss; and grade 4, total or virtually hair loss\textsuperscript{53,55,122}. Some other research groups, however, assessed CIA into 3 grade: negative, mild and severe\textsuperscript{56}. These grading systems were mainly based on the experience of the researchers and the CIA was assessed visually. However, hair loss represents a delayed manifestation of drug effects on hair follicles. To measure CIA, the following criteria should be required\textsuperscript{125}. a) The pharmacodynamic endpoints should be able to describe the CIA precisely in terms of onset, severity, duration and recovery. b) The pharmacodynamic endpoints can be quantitatively measured so that the evaluation of CIA by different descriptors and information from multiple studies can be compared and contrasted. c) The pharmacodynamic endpoints should be able to evaluate the responsiveness to
therapeutic measures. Thus, the pharmacodynamic endpoints can guide the establishment of the best treatment schedule of a pharmacologically effective compound in protection against CIA. Early onset pharmacodynamic endpoints need to be defined for direct measures of drug effect on hair follicles. On the other hand, the pharmacodynamic study of CIA could also give insight into the targets of chemotherapeutic agents in hair follicles, the responses of hair follicles to chemotherapeutic agents, the mechanisms of hair loss, and new treatment strategies.

The first goal of this chapter was to identify appropriate pharmacodynamic endpoints that fulfilled these requirements. Because doxorubicin-induced alopecia in neonatal rats occurred over a time period corresponding to all the major phases of a hair growth cycle, i.e., anagen, catagen and telogen (Chapter 2, figure 2.3), it was used to evaluate four hair follicle morphology-based pharmacodynamic endpoints, i.e., dermal layer thickness, hair follicle length, hair bulb diameter and hair follicle density. The results showed the same time-dependent changes for all four pharmacodynamic endpoints. In addition, the dermal layer thickness showed the lowest variability and was the easiest to measure. This endpoint was therefore selected for subsequent studies in CIA by three other chemotherapeutic agents, i.e., cyclophosphamide, ara-c and paclitaxel.

The second goal was to examine two major drug effects, i.e., antiproliferation and cell kill (apoptosis), in hair follicles. We evaluated the effect of ara-c on hair bulb matrix cell proliferation and studied the hair follicular apoptosis induced by cyclophosphamide, paclitaxel and ara-c in the two animal models.
3.2. MATERIALS AND METHODS

3.2.1 Chemicals and reagents

Bromodeoxyuridine (BrdU) and monoclonal antibodies to BrdU were purchased from Sigma Co. (St. Louis, MO). Ki67 antigen kit (NCL-Ki67-Paraffin) was purchased from Novocastra Laboratories Ltd (United Kingdom). ApopTag apoptosis detection kit was purchased from Serologicals (Norcross, GA). RNase It Ribonuclease Cocktail was purchased from Stratagene (La Jolla, CA). Labeled streptavidin-biotin (LSAB) horseradish peroxidase kit was purchased from Dako (Carpinteria, CA). Other tissue culture supplies were purchased from GIBCO (Grand Island, NY). Chemotherapeutic agents were the same as show in Chapter 2 (section 2.2.1). Fresh solutions of each agent were prepared just before use.

3.2.2. Animals

Female pregnant Sprague-Dawley rats and female C57BL-6 mice (6-8 week old, weight 18-22 g) were purchase from Charles River Laboratories (Wilmington, MA). Animal care was provided by the Laboratory Animal Resources at the Ohio State University. Neonatal rats were housed together with the mother rat for at least 3 weeks after birth. Seven or eight-day-old neonatal rats delivered from the same mother rat (10-14 newborns per mother rat) were randomly divided into different groups according to their body weight (14-16 gram) at the time of experiment. Hair follicles in the neonatal rats are spontaneously in the early anagen phase immediately after birth and no experimental induction was required. Hair follicles on the back of adult
C57BL-6 mice were synchronized into anagen growing phase by depilation as described in Chapter 2 (section 2.2.3).

3.2.3. Determination of the duration of anagen phase

In neonatal rat model, starting from day 3 after birth, one group of animals was sacrificed and the skin samples from head between two ears were removed, covered with foil to keep the skin flat and then flash frozen in liquid nitrogen. Skin samples were stored at –70 °C until cut into 6 µm thick parallel to the longitudinal axis of hair follicles, fixed in 10% formaldehyde and stained with hematoxylin. Cryo-sections were used to measure the dermal layer thickness and observe the hair follicle morphology. Dermal layer thickness was measured as the distance from the underlayer of stratum granulosum to the top of muscle layer under the guidance of microscope at a 100× magnification (figure 3.1). The proliferating cells in hair follicles were detected by BrdU labeling and Ki67 staining (Figure 3.2). Anagen phase in C57BL-6 mice can be recognized by the darkened skin color\textsuperscript{42,59,126,127} (Chapter 2, Figure 2.2). Anagen VI hair follicles can also be recognized by the appearance of hair shaft through the skin epidermis.

3.2.4. Drug treatment and tissue samples collection

Seven-day-old rats were used for treatments with doxorubicin and eight-day-old rats were used for treatments with other chemotherapeutic agents, i.e., ara-c, cyclophosphamide, and paclitaxel. Rats were given intraperitoneal injection of doxorubicin (0.5, 1.0, 1.5, 2.0 or 2.5 mg/kg/day for 7 consecutive days), cyclophosphamide (single dose of 50 mg/kg), or ara-c (50 mg/kg/day for 5
consecutive days). Two subcutaneous doses of paclitaxel, 2.5 µg each in 50 µl saline, were injected directly on the head between two ears on day 8 and day 11 after the birth of animals. In each experiment, control animals received normal saline injections. In the C57BL-6 mouse model, when hair follicles reached anagen VI after depilation, i.e., on day 9 post-depilation, a single dose of paclitaxel, 60 mg/kg or 80 mg/kg, was given by intraperitoneal injection. The time of the first dose drug administration was recorded as day 0 of experiment.

Skin and hair samples from different locations of animals were harvested at different predetermined time in each treatment. Skin samples were either flash frozen in liquid nitrogen and kept at –70 ºC or fixed in 4% buffered formaldehyde at 4 ºC for 24 hrs. For measurement of the hair mass density, hair shafts in each group of animals were carefully shaved off from a predetermined square area (0.5 cm × 0.5 cm) on the head. The shaved area and the weight of shaved hair shafts were measured.

For observation of the whole hair shaft structure and measurement of hair shaft length, hair shafts were removed by hair pluck. Briefly, a hemostat with rubber tips was used to grasp about 10 hair shafts at the base of the hair shaft and a quick and forceful pull was then applied to pluck the hair from the skin. Care was executed to maintain the tension as constant as possible and to complete the whole process quickly (<1 sec) to minimize the distortion. The removed hair shafts were then mounted on a slide for later microscopic evaluation.
3.2.5. Morphology-based pharmacodynamic endpoints measurements

Frozen sections were used to measure the dermal layer thickness and observe the hair follicle morphology. Skin samples were cut into 8 μm thick parallel to the longitudinal axis of hair follicles. Hair follicles at different stages of hair growth cycle were recognized as described before\textsuperscript{127}. Dermal layer thickness was measured as the distance from the underlayer of stratum granulosum to the top of muscle layer under microscope at 100× magnification. Hair follicles with recognizable hair matrix, dermal papilla and sebaceous gland were used for the measurements of hair follicle length, hair bulb diameter and hair follicle number density. Hair follicle length was measured from the proximal tip of the dermal papilla to the distal orifice of the hair follicle at the epidermis of the skin under the guidance of a microscope at a 100× magnification. For measurement of hair bulb diameter, hair follicles were first stained with Ki67 to facilitate the identification of hair matrix cells, the diameter was then measured at the widest part of the dermal papilla (critical level of Auber\textsuperscript{128}) under microscope at 400× magnification. Shaving or grooming has been a standard method to evaluate alopecia when the hair loss is not complete and cannot be assessed visually\textsuperscript{129}. We used two parameters, i.e., hair shaft weight density and hair follicle number density to evaluate partial alopecia. The hair shaft weight density was measured as the weight of hair shafts per shaving area. The hair follicle density was measured as number of hair follicles per mm of skin sections. The hair shaft length was measured as the distance from the distal tip of the hair shaft to the proximal hair follicular keratinization zone.
3.2.6. BrdU labeling of hair follicles

Neonatal rats or C57BL-6 mice were administered with BrdU, 100 mg/kg, in normal saline by intraperitoneal injection. One hour later, the animals were sacrificed. Skin samples were obtained and fixed in 4% buffered formaldehyde at 4 ºC for 48 hrs and then processed into paraffin. The skin samples were sectioned into 6 µm thick parallel to the longitudinal axis of hair follicles and stained with monoclonal antibodies to BrdU. Skin sections were lightly counter stained with hematoxylin.

3.2.7. Immunohistochemical staining of Ki67 in hair follicles

Skin samples were removed and fixed in 4% buffered formaldehyde at 4 ºC for 24 hrs, processed, embedded into paraffin, and cut into 6 µm thick sections. For the cryo-sections, the skin samples were fixed in 10% buffered formaldehyde for 5 min, placed in 1000 ml 0.01 M citrate buffer (pH 6.0) and boiled for 15 min. After washing with Tris buffered saline (TBS, pH 7.4) for 5 min, the skin sections were incubated with normal rabbit serum for 10 min, followed by primary antibodies for 60 min, at room temperature. After washing the samples with TBS for 10 min, secondary antibody (biotinylated rabbit anti-mouse, diluted 1:1000 in TBS) was applied and incubated for 30 min at room temperature. ABC reagent was then applied after washing the sections with TBS for 10 min. The chromogen was diaminobenzidine tetrahydrochloride (DAB). The sections were then lightly counter stained with hematoxylin.
3.2.8. Apoptosis detection by TUNEL

TUNEL immunohistochemistry staining for apoptosis in hair follicles was performed using the ApopTag in situ Apoptosis Detection Kit as previously described with minor modifications. Skin samples were fixed in 4% buffered formaldehyde at 4 °C for 48 hrs, processed, and embedded into paraffin and cut into 6 µm thick sections. Skin sections were deparaffinized in xylene, rehydrated, and rinsed in PBS. Sections were then treated with 20 µg/ml proteinase K for 15 min at room temperature, rinsed with PBS (pH 7.4) and incubated with 2% H₂O₂ to inactivate endogenous peroxidase for 15 min, and followed by RNAse-digestion (2 µl/ml in Tris-buffered saline) for 15 min at 37 °C. The sections were then rinsed with PBS and incubated with terminal deoxynucleotidyl transferase (TdT) and biotin-deoxyuridine triphosphate in TdT buffer for 90 min at 37 °C. After incubation with stop buffer for 15 min and rinsing with PBS, the sections were incubated with 10 mg/ml BSA in PBS for 5 min at room temperature for non-specific binding block. The sections were then incubated with avidin-biotin reagent containing horseradish-peroxidase for 60 min. The color was developed with DAB and the sections were lightly counter stained with hematoxylin.
3.3. RESULTS

3.3.1 Anagen phase determination in neonatal rats

On day 3 after birth, proliferating cells in hair bulb matrix were observed, indicating the early anagen phase of hair follicles. Hair follicles showed a downward growth of ORS shortly after the birth and the length of hair follicles increased and reached the maximum value on day 7-8 after birth. Starting on day 5, the IRS containing the hair shaft grew upward. On day 7-8, the hair shaft tips emerged through the epidermis, indicating the anagen VI was accomplished. The downward growth of ORS was associated with the thickening of dermal layer, which also reached the maximum value on day 7-8 and was maintained for another 7 days. Thus, the duration of anagen phase in neonatal rats began on day 7 and ended on day 14 after birth (Figure 3.1). The proliferating cells in hair follicles were located in hair matrix and ORS, indicated by BrdU and Ki67 staining (Figure 3.2).

3.3.2 Anagen phase determination in C57BL-6 mice

After depilation, telogen hair follicles on the back of C57BL-6 mice were immediately transformed into anagen hair follicles accompanied by melanogenesis, which resulted in skin pigmentation and thickening for the next 5-6 days\textsuperscript{42}. A gray to black skin color was developed in 8-9 days after depilation, and black uniform hair shaft tips began to protrude from the surface of epidermis, indicating the accomplishment of anagen VI phase\textsuperscript{42} (Chapter 2, Figure 2.3). The anagen phase was maintained for additional 7 days. Similar to that in neonatal rats, proliferating cells were located in bulb matrix and ORS of anagen hair follicles, indicated by BrdU and Ki67 staining (Figure 3.2).
3.3.3. Chemotherapy-induced dermal layer shrinkage

Chemotherapy caused shrinkage of dermal layer. The kinetics of dermal layer shrinkage was drug dependent. In neonatal rats, after the first dose administration, cyclophosphamide, paclitaxel, doxorubicin and ara-c caused significant decrease of dermal layer thickness starting at 72, 48, 120 and 72 hrs, respectively, (Figure 3.3). In 2-3 days, the dermal layer thickness reached the minimum value. In C57BL-6 mice, paclitaxel caused shrinkage of dermal layer at 24 hrs after drug administration. Skin sections obtained from the head and tail regions showed similar kinetics of dermal layer shrinkage (Figure 3.4).

Doxorubicin-induced shrinkage of dermal layer thickness in neonatal rats was accompanied by shortened hair follicle length and reduced hair bulb diameter. Changes of these parameters showed similar kinetik as for dermal layer thickness. For example, doxorubicin-induced shrinkage of dermal layer thickness, shortened hair follicles length and reduced hair bulb diameter all occurred at 120 hrs after the first dose administration (Figure 3.5).

3.3.4. Kinetics of hair density changes during CIA

In the control group, the hair shaft weight increased with time after birth, by 5-10% from day 17 to day 21 after birth, and reached the maximum value on day 21. Doxorubicin-induced hair loss showed a time and dose-dependent manner. With doses lower than 1.0 mg/kg, no significant change of hair shaft weight was observed on day 14 after the first dose administration. With doses higher than 1.5 mg/kg, significant
hair loss, over 50%, was observed on day 10, and over 90% of the hair shafts were lost on day 14 (Figure 3.6).

The kinetics of changes of hair follicle density and hair shaft weight showed similar patterns. For example, both hair follicle density and hair shaft weight decreased on day 11 after doxorubicin (1.5 mg/kg) administration. Because hair shaft weight was more convenient to measure in comparison with counting the hair follicle number, we chose hair weight density as the endpoint to evaluate partial alopecia and FGF protective effect in subsequent studies.

3.3.5. Effect of chemotherapy on matrix cell proliferation and hair growth rate

In control animals, hair matrix cells maintained a high mitotic activity from day 6 to day 13 with a BrdU labeling index of 56–60%, followed by a quick drop to 0% on day 15, indicating the end of the anagen phase. The total number of BrdU-positive cells showed a similar kinetic pattern as that of BrdU labeling index (Figure 3.7, solid lines). The hair shaft growth rate during day 10 to day 12 was 0.67 mm/day.

Compared with control, ara-c decreased the number of BrdU-positive cells and total cell number in hair bulb after the second dose administration, while the labeling index remained at the same high level as in control during the treatments from day 9 to day 11 (Figure 3.7, dashed line). Ara-c also slightly reduced the hair shaft growth rate to 0.63 mm/day (0.1>p>0.05, compared to control animals). These ara-c-induced changes were accompanied by the abnormal hair shaft structure (Figure 3.8). On day 5 after ara-c treatment, ara-c treated animals showed significant thinner hair shaft root diameter (6.7±1.1 µm), compared to control (33.2±3.6 µm).
3.3.6. Chemotherapy-induced apoptosis in hair follicles

All chemotherapeutic agents, i.e., ara-c, cyclophosphamide and paclitaxel, induced apoptosis in hair matrix cells at 24 or 48 hrs after drug administration. Cyclophosphamide and paclitaxel also induced apoptosis in ORS. Ara-c occasionally induced apoptosis in the lower part of IRS. No apoptosis was detected in the dermal papilla (Figure 3.9).

3.3.7. Paclitaxel-induced melanin relocation in C57BL-6 mouse hair follicles

In control animals, the distribution of melanin was confined to the hair bulb surrounding the upper half of the dermal papilla and no melanin were observed in the lower half of the hair bulb and ORS. Paclitaxel induced abnormal deposition of melanin in hair follicles. Shortly after paclitaxel administration (i.e., 24 and 48 hrs), irregularly shaped melanin clumps were detected in hair bulb matrix. At later time, i.e., 72 and 96 hrs, melanin clumps were found in hair bulb matrix and ORS (Figure 3.10).
3.4. DISCUSSION

Skin thickness has been applied to evaluate hair follicle development and growth cycle\textsuperscript{130}. The present studies evaluated four hair follicle morphology-based pharmacodynamic endpoints of CIA, i.e., dermal layer thickness, hair follicle length, hair bulb diameter, and hair follicle density. Dermal layer thickness is related to the stage of hair growth cycle due to the degeneration or regression of isthmus and hair bulb during catagen phase. Compared to control animals, all four chemotherapeutic agents, i.e., cyclophosphamide, doxorubicin, ara-c and paclitaxel, induced shrinkage of dermal layer thickness, shortened hair follicle length and reduced hair bulb diameter. As such changes are usually observed during catagen, we propose that chemotherapy induces premature onset of catagen. The data suggested an association between these hair follicle morphology-based pharmacodynamic endpoints. Because dermal layer thickness was more convenient to measure and was less variable (cv % < 8%), compared to hair follicle length (ranging from 0.3 mm to 1.3 mm) and hair bulb diameter (cv % > 15%), we chose dermal layer thickness as the endpoint to evaluate CIA and related studies.

Ara-c is a cell cycle S-phase specific chemotherapeutic agent that inhibits cell growth accompanied by a rapid inhibition of DNA synthesis\textsuperscript{131-133}. Ara-c-induced complete alopecia in neonatal rats occurred only after repeated treatments. Interestingly, the BrdU labeling index in hair matrix cells remained at high level as in control animals even after three ara-c treatments. The continuous high state of cell proliferation may render the hair follicles continually susceptible to ara-c until the hair follicles are severely damaged and hair loss occurs.
Cyclophosphamide, paclitaxel and ara-c induced apoptosis in hair bulb matrix. Cyclophosphamide and paclitaxel also induced apoptosis in ORS. It has been reported that doxorubicin-induced apoptosis was mainly confined to hair matrix and ORS\textsuperscript{57}. These data indicated hair matrix and ORS as the major targets of chemotherapy in hair follicles. These components are also the locations of BrdU and Ki67-positive cells. No apoptosis was detected in the dermal papilla, which may explain the ability of hair follicle regeneration and the reversibility of CIA.

In C57BL-6 mice, the skin melanocytes are mostly confined to the hair follicles and their melanogenic activity is strictly coupled to the anagen growth stage of the hair growth cycle\textsuperscript{134}. Chemotherapy induced apoptosis of melanocytes and alterations of hair and skin pigmentation\textsuperscript{63,135}. At the end of anagen or beginning of catagen, retraction of melanocyte dendrites and suppression of melanogenesis are the earliest signs of hair follicle regression, occurring prior to the structural changes\textsuperscript{136}. It has been shown that cyclophosphamide induced disruption of hair follicle melanogenesis\textsuperscript{137}. The present study is the first to show that paclitaxel also induced disruptions of hair follicle melanogenesis. The disordered hair pigmentation may also occur in the subsequent hair growth cycle during hair follicle recovery\textsuperscript{137}. The abnormal locations of melanin in hair follicles after chemotherapy are also the locations of BrdU-positive cells and apoptotic cells. The mechanisms and cellular signals that cause the melanin migration and presence of melanin in ectopic hair bulb locations during chemotherapy are poorly understood.
In summary, we have established four hair follicle morphology-based pharmacodynamic endpoints, i.e., dermal layer thickness, hair follicle length, hair bulb diameter, and hair follicle density to evaluate CIA. Chemotherapy impaired the mitotic activity in hair follicles and induced cell death. The collective data showed that the hair bulb matrix and ORS were the major targets of chemotherapy during CIA. The data suggest that successful prevention of CIA will depend on the selective protection of these cells in the hair follicle.
Figure 3.1. Anagen phase of hair growth cycle in neonatal rats after birth. Dermal layer thickness, measured as the distance from the underlayer of stratum granulosum to the top of muscle layer, was used to indicate the hair growth stage (n=3-5 animals per data point, mean ± SD). Anagen VI was achieved on day 7-8 after the birth was maintained for additional 7 days.
Figure 3.2. Anagen hair follicles in neonatal rats and C57BL-6 mice. Skin samples were taken from 8-day-old neonatal rats or from C57BL-6 mice on day 10 after depilation and then stained with anti-BrdU, anti-Ki67 or hematoxylin (H), respectively. BrdU, 100 mg/kg, was given by intraperitoneal injection 1 hr before skin sample collection. The BrdU-positive cells and Ki67-positive cells were located in hair bulb matrix and outer root sheath (ORS).
Figure 3.3. Chemotherapy-induced dermal layer shrinkage in neonatal rats.

Arrows indicate the onset time of dermal layer shrinkage. Dox, doxorubicin; pac, paclitaxel; CTX, cyclophosphamide. (n=3-5 animals per data point)
Figure 3.4. Paclitaxel-induced dermal layer shrinkage in C57BL-6 mice. A single dose of paclitaxel, 60 mg/kg or 80 mg/kg, was administered to C57BL-6 mice by intraperitoneal injection on day 9 after depilation. At 0, 24, 48 and 72 hours after paclitaxel administration, skin samples from the head region and the tail region were obtained for dermal layer thickness measurement. The arrow indicates the onset of dermal layer shrinkage (n=2 animals per data point)
Figure 3.5. Doxorubicin-induced shortened hair follicle length and reduced hair bulb diameter in neonatal rats. Seven-day-old rats were given intraperitoneal doxorubicin, 1.5 mg/kg/day, for 7 consecutive days. Skin samples were harvested from the head between the two ears after doxorubicin administration. Hair follicles were first stained with Ki67 to facilitate the identification of hair follicle structure. Hair follicle length was measured from the proximal tip of the dermal papilla to the distal orifice of the hair follicle at the epidermis of the skin. The hair bulb diameter was measured at the widest part of the dermal papilla. Dox, doxorubicin. (n=3-5 animals per data point, 10-20 hair follicles per animal)
Figure 3.6. Doxorubicin-induced changes of hair density in neonatal rats. Seven-day-old rats were given different doses of doxorubicin daily for 7 consecutive days by intraperitoneal injection. Dox, doxorubicin. (n=3 animals per data point)
**Figure 3.7. BrdU labeling in hair follicles w/o ara-c treatment.** Eight-day-old rats were given intraperitoneal injection of ara-c, 50 mg/kg/day, for 7 consecutive days. Control animals received normal saline injections. BrdU, 100 mg/kg, was administered by intraperitoneal injection 1 hr before skin sample collection. Ctl, control; #BrdU/100, number of BrdU-positive cells divided by 100; DL, dermal layer, measured from the under layer of stratum granulosum to the top of muscle layer; BrdU Ind, BrdU labeling index, calculated by the percentage of BrdU-positive cells in a hair bulb. (n=2-5 animals, 10-20 hair follicles per animal)
Figure 3.8. Effect of ara-c on hair growth rate and hair root structure in neonatal rats. Eight-day-old rats were given ara-c, 50 mg/kg/day, by intraperitoneal injection for 5 consecutive days. Control animals received normal saline. The hair growth rate in saline group was 0.67 mm/day, and 0.63 mm/day in ara-c treated group (n>10 animals for measurement of hair growth rate, 0.1>p>0.05). The lower photograph shows the hair shaft structures on day 5 in control and ara-c treated group.
Figure 3.9. Chemotherapy-induced apoptosis in hair follicles. Eight-day-old rats were treated with one single dose of Ara-c (50 mg/kg) or cyclophosphamide (50 mg/kg) by intraperitoneal injection. One single dose of paclitaxel (60 mg/kg) was administered to C57BL-6 mice on day 9 after depilation. Apoptotic cells in hair follicles were detected by the TUNEL method. All the chemotherapeutic agents induced apoptotic cells hair matrix. Cyclophosphamide and paclitaxel also induced apoptosis in ORS. Ara-c occasionally induced apoptosis in IRS. No apoptotic cells were found in dermal papilla. CTX, cyclophosphamide; Pac, paclitaxel; IRS, inner root sheath; ORS, outer root sheath.
Figure 3.10. Paclitaxel-induced melanin relocation in hair follicles in C57BL-6 mice. One single dose of paclitaxel, 60 mg/kg or 80 mg/kg, was administered to C57BL-6 mice by intraperitoneal injection on day 9 after depilation. Skin samples were stained with hematoxylin. Arrows indicate abnormal locations of melanin in hair follicles. Ctl, Control.
CHAPTER 4

BROAD-SPECTRUM PROTECTION AGAINST CHEMOTHERAPY-INDUCED ALOPECIA BY SUBCUTANEOUS INJECTION OF ACIDIC AND BASIC FIBROBLAST GROWTH FACTORS

4.1. INTRODUCTION

A variety of different strategies have been attempted to protect against CIA: a) drug-specific antibodies\textsuperscript{56}; b) hair growth cycle modifiers\textsuperscript{78,79}; c) cytokines and growth factors\textsuperscript{53,55}; d) cell cycle modifiers\textsuperscript{46}; and e) apoptosis signaling inhibitors\textsuperscript{106,107} (see Chapter 1). The above earlier agents do not broadly protect against CIA by all classes of anticancer drugs but typically protect against one drug or one drug class in animal models and some are not effective against CIA in humans. None of these agents is known to protect against CIA by paclitaxel, the major agent used against breast and ovarian cancer in female patients. These concerns, together with the fact that cancer is usually treated with combinations of different drugs with different anticancer mechanisms, suggest the limited application of these earlier agents in clinic.

One prerequisite for therapeutic progress in manipulating CIA is that the protection should be selectively targeted to hair follicles with a broad-spectrum protection against different anticancer agents. We hypothesize that combination of acidic and basic
fibroblast growth factors (aFGF and bFGF, FGFs) offer broad protection against CIA. This hypothesis is based on the following findings: (a) aFGF and bFGF induce up to 10-fold resistance for multiple chemotherapeutic agents with diverse chemical structures and action mechanisms in tumor cells. We further found that bFGF is required to induce resistance, whereas aFGF amplifies the chemo-protective effect of bFGF\textsuperscript{138}; (b) FGFs-induced resistance applies to both anti-proliferative and apoptotic effects of drugs. Hence, FGFs can be used to protect cells against drugs that act mainly by inhibiting cell growth and drugs that act mainly by inducing apoptosis; (c) FGFs and their receptors are found throughout the hair follicle. bFGF is found in the suprabasal layer of the epidermis, ORS and the basement membrane adjacent to the proliferating zone of hair bulb. aFGF is found in the epidermis, cells in the keratogenous zone, and IRS\textsuperscript{92}. Thus, the two growth factors have different localizations in hair follicles, suggesting their possible related and perhaps complementary functions in hair follicles. The basement membrane of hair follicles is enriched in heparan sulfate proteoglycan, which binds FGFs and facilitates the binding of FGFs to FGFRs\textsuperscript{139}. Hence, FGFs and their receptors are located in the regions of hair follicles that are important for hair growth, retention and keratinization. bFGF is also required for melanocyte growth and survival\textsuperscript{140}; (d) Growth factors have been shown to protect CIA by ara-c in neonatal rats\textsuperscript{141}.

In this chapter, we tested the protective effect of subcutaneous injections of FGFs against CIA in animal models, as an initial proof-of-principle.
4.2 MATERIALS AND METHODS

4.2.1 Chemicals and Reagents

Recombinant human FGF acidic (aFGF, or FGF1) and recombinant human FGF basic (bFGF, or FGF2) were purchased from R&D Systems, Inc (Minneapolis, MN). aFGF or bFGF (25 µg) was dissolved in 125 µl normal saline (0.5% BSA). Other chemicals or tissue culture supplies were the same as shown in Chapter 2 (section 2.2.1) and Chapter 3 (section 3.2.1).

4.2.2. Animals

Same as Chapter 3, section 3.2.2.

4.2.3. Induction of alopecia in neonatal rats

See Chapter 2, section 2.2.4. The time when the first dose of chemotherapeutic agent was administered was recorded as day 0. Hair loss occurred naturally without any physical forces applied. Body weight of each animal was measured every 24 hrs to evaluate the systemic toxicity of the treatments.

4.2.4. Induction of alopecia in C57BL-6 mice

Hair follicles were synchronized into anagen phase by depilation (see Chapter 2, section 2.2.3). C57BL-6 mice were given paclitaxel, 40 mg/kg/day for 3 consecutive days, starting on day 9 after depilation, by intraperitoneal injection. Hair loss occurred naturally without any physical forces applied.
4.2.5. FGFs treatments

aFGF (2 µg), bFGF (2 µg) or their combination (2 µg aFGF + 2 µg bFGF) was dissolved in 0.05 ml normal saline and applied by subcutaneous injection to animals in the middle between the two ears on the head. Pilot studies were conducted to establish appropriate FGFs treatment schedules for different chemotherapeutic agents. The following schedules were used. For ara-c and doxorubicin, animals received aFGF, bFGF or their combination 3 hrs before each chemotherapy dose. For low dose cyclophosphamide, animals received aFGF, bFGF or their combination 24 hrs and 3 hrs before the first chemotherapy dose, 3 hrs before the second chemotherapy dose and 24 hrs after the second chemotherapy dose. For paclitaxel and high dose cyclophosphamide, animals received aFGF, bFGF or their combination daily for 5 days and 4 days, respectively, starting on day 0 of experiment. For paclitaxel in C57BL-6 mice, FGFs were given 3 hrs before each dose of paclitaxel. Three control groups were included. Saline control animals received intraperitoneal and subcutaneous injections of normal saline. Drug control animals received intraperitoneal injections of chemotherapeutic agent and subcutaneous injection of normal saline. FGFs control group received intraperitoneal injections of normal saline and subcutaneous injections of FGFs. As a measurement of the efficacy of different FGF treatments, compared to control animals, less than 10% of hair loss was scored as total reversal, less than 50% hair loss (clinically significant) was scored as significant protection, and over 50% hair loss was scored as partial protection. The evaluation was performed based on a 0.5×0.5 cm² area at the FGF injection site.
4.3 RESULTS

4.3.1. Protective effect of subcutaneous FGFs

In the neonatal rat model, FGFs showed protection against CIA by all 4 drugs in all animals (n> 4 per group, Figure 4.1-4.4). As indicated by the presence of hair, the protected area exceeded but well confined to the FGF injection site. The extent of protection varied for different drugs. Subcutaneous injection of 2 µg aFGF totally reversed CIA by ara-c, significantly protected against CIA by low dose of cyclophosphamide and showed partial protection against CIA by doxorubicin and paclitaxel. Subcutaneous injection of 2 µg bFGF reversed CIA by doxorubicin, showed partial protection against CIA by ara-c and paclitaxel, but did not show protective effect against CIA by low dose of cyclophosphamide. The combination of 2 µg aFGF and 2 µg bFGF, totally reversed CIA by doxorubicin, ara-c and low dose cyclophosphamide and showed significant protection against CIA by paclitaxel. For all four drugs, the combination of aFGF and bFGF produced the greatest protection. aFGF, bFGF or their combination did not reverse the alopecia by high dose cyclophosphamide, but delayed the onset (as discussed in section 4.3.2).

In the adult C57BL-6 mouse model, only the combination of 2 µg aFGF and 2 µg bFGF showed significantly protective effect against paclitaxel-induced alopecia (Figure 4.5). Higher dose of 4 µg aFGF alone or 4 µg bFGF alone did not show significant protection (data not shown).
4.3.2. Acceleration of hair regrowth by subcutaneous FGFs in neonatal rats

In the high dose cyclophosphamide CIA model in neonatal rats, subcutaneous injection of 2 µg aFGF, 2 µg bFGF or their combination showed partial protection on day 6 after drug administration but the protection was lost on day 8. Animals without FGFs treatment showed alopecia on day 6 (Figure 4.6). Thus, FGFs treatments did not reverse the hair loss, but retarded the onset. Regrowth of hair shaft after whole body alopecia by high dose cyclophosphamide followed a wave pattern with a direction from tail to head and abdomen to back (Chapter 2). On day 19 after cyclophosphamide injection, hair follicles with FGFs treatment showed hair regrowth with hair shafts appearing on the skin surface. No hair shaft was found at the same location in the cyclophosphamide control group (Figure 4.6). Skin sections showed that hair follicles in the FGFs treated group were in the anagen VI growth stage, while hair follicles in the drug control group were in the early stage of anagen initiation (Figure 4.7). In the control rats without chemotherapy, hair follicles were in telogen phase. The FGFs treated group also showed a thicker dermal layer, in comparison with drug control group, which indicated an earlier onset of anagen after CIA. The length of hair shaft was measured on day 21 after cyclophosphamide administration. Compared to the drug control group, animals treated with aFGF, bFGF or their combination showed significant longer hair shaft (p<0.01) (Figure 4.8). FGFs delayed the onset of hair loss, and shortened the duration of telogen phase after CIA, which resulted in an earlier onset of hair regrowth, compared to cyclophosphamide treated control group (Figure 4.9). In summary, FGFs delayed the hair loss, as well as enhanced the hair follicle recovery.
4.3.3. FGFs delayed onset of chemotherapy-induced dermal layer shrinkage

Chemotherapy caused dermal layer shrinkage at 72, 48, 120 and 72 hrs after the first chemotherapy dose of cyclophosphamide, paclitaxel, doxorubicin and ara-c, respectively. FGFs delayed dermal layer shrinkage during CIA with the onset at 96, 72, 192 and 120 hrs after the first chemotherapy dose of cyclophosphamide, paclitaxel, doxorubicin and ara-c, respectively (Figure 4.10).
4.4. DISCUSSION

The data represented here showed that subcutaneous injections of aFGF and bFGF offered broad-spectrum protection against CIA by chemotherapeutic agents with different chemical structures and anticancer mechanisms. As far as we know, this is the first evidence of the protection against alopecia induced by paclitaxel, the major agent used for breast and ovarian cancer in female cancer patients. Additional interesting findings are as following.

The neonatal rats and adult C57BL-6 mice showed different responses to the protective effect of FGFs. Either aFGF or bFGF showed protective effect against CIA in neonatal rats, whereas only the combination of aFGF and bFGF showed protective effect against CIA in C57BL-6 mice. A possible reason for this difference may be the presence of endogenous aFGF and bFGF in hair follicles in neonatal rats which would have enhanced the effect of the exogenous aFGF or bFGF, whereas the endogenous aFGF or bFGF in C57BL-6 mice would be expected to be low. The data generally indicated the requirement of both growth factors for the broad-spectrum protective effect against CIA.

In the neonatal rat model, the FGFs protective effect was dependent on the dose intensity of cyclophosphamide. The FGFs protective effect reversed the hair loss by low dose of cyclophosphamide, but did not reverse the hair loss by high dose of cyclophosphamide. As shown in Chapter 2, high dose cyclophosphamide induced more severe whole body alopecia, compared to localized alopecia by low dose of cyclophosphamide. The limited protective effect of FGFs against CIA by high dose
cyclophosphamide may be due to the amount and/or distribution of FGFs applied. As shown in the next chapter (Chapter 5), topical application of FGFs reversed the CIA by high dose cyclophosphamide.

In the studies of CIA by ara-c, paclitaxel, doxorubicin, or low dose of cyclophosphamide, the protected hair shaft by FGFs were maintained till the hair regrowth from the next hair growth cycle, i.e., on day 19-21 of experiment. In the study of CIA by high dose cyclophosphamide, both drug control group and FGFs-treated group showed hair loss on day 8 of experiment, which allowed us to study the FGFs effect on hair follicle recovery (or hair regrowth) after CIA.

Hair follicles in both cyclophosphamide control group and FGFs treated group showed shrinkage of dermal layer after chemotherapy, suggesting the onset of catagen phase (Chapter 3), followed by the telogen phase and a new hair growth cycle. The hair regrowth after CIA by high dose cyclophosphamide occurred during the subsequent new hair growth cycle. FGFs treatments accelerated the hair regrowth after CIA, partly due to the shortened telogen phase after CIA (Figure 4.9) or an earlier anagen initiation of the following hair growth cycle. The anagen initiation during a new hair growth cycle is dependent on the communication between the dermal papilla and stem cells in the hair bulge\textsuperscript{10,142-144}. Dermal papilla and stems cells are usually not affected by chemotherapy, since CIA is reversible and apoptosis was not found in those two locations after chemotherapy (Chapter 3). The development and growth of hair follicles are affected by a number of cytokines and growth factors including the fibroblast growth factors\textsuperscript{145,146}. The functions of FGFs on dermal papilla and/or stem cells during chemotherapy need to be further studied.
In summary, the protection effect of FGFs is dramatically different from all previous reported agents (Chapter 1, section 1.5), in that FGFs offer broad-spectrum protection against CIA by multiple classes of drugs with diverse chemical structures and action mechanisms, and that FGFs accelerate hair regrowth after CIA. Finally, aFGF and bFGF treatments did not show significant effect in reversing the body weight loss by chemotherapy (Figure 4.11), and did not offer advantages of animal survival in the studies with doxorubicin (data not shown), suggesting no or insignificant systemic chemotherapy protection.
Figure 4.1. Protection against doxorubicin-induced alopecia in neonatal rats by subcutaneous aFGF/bFGF. Seven-day-old rats were given doxorubicin (Dox, 1.5 mg/kg/day) for 7 consecutive days by intraperitoneal injections. For studies of FGFs effect, rats in addition received 2 µg aFGF and/or 2 µg bFGF in 50 µl saline daily for 7 days by s.c. injection on the head between two ears, 3 hrs before each administration of doxorubicin. Control animals received normal saline injections. Protection was evaluated on day 13 after the first dose of doxorubicin. Arrows indicate the injection site of FGFs or saline. The rank order of protection effectiveness was aFGF+bFGF~bFGF > aFGF (n>6 per group).
Figure 4.2. Protection against ara-c-induced alopecia in neonatal rats by subcutaneous aFGF/bFGF. Eight-day-old rats were given intraperitoneal injections of ara-c (50 mg/kg/day) for 5 consecutive days. For studies of FGFs effect, rats in addition received subcutaneous injection of 2 µg aFGF and/or 2 µg bFGF in 50 µl saline for 5 days, 3 hrs before each administration of ara-c. Protection was evaluated on day 8 after the first dose of ara-c. Arrows indicate the injection site of FGFs or saline. The rank order of protection effectiveness was aFGF+bFGF~aFGF > bFGF (n>8 per group).
Figure 4.3. Protection against paclitaxel-induced alopecia in neonatal rats by subcutaneous aFGF/bFGF. Eight-day-old rats were given two doses of paclitaxel (25µg/50µl/site) on day 0 and day 3 of experiment by subcutaneous injection on the head between the ears. For studies of FGFs effect, rats in addition received daily subcutaneous injections of 2 µg aFGF and/or 2 µg bFGF in 50 µl saline for 5 days. Arrows indicate the paclitaxel and FGFs injection sites. Protection of alopecia was evaluated on day 10 after the first dose of paclitaxel. The rank order of protection effectiveness was aFGF+bFGF~bFGF~aFGF (n>4 per group).
Figure 4.4. Protection against low dose cyclophosphamide-induced alopecia in neonatal rats by subcutaneous aFGF/bFGF. Eight-day-old rats were given two doses of cyclophosphamide (CTX, 30 mg/kg/day) on day 0 and day 1 of experiment. For studies of FGFs effect, rats in addition received subcutaneous injection of 2 µg aFGF and/or 2 µg bFGF in 50 µl saline 24 hrs before the first dose of cyclophosphamide, 3 hrs before each cyclophosphamide administration, and 24 hrs after the second dose of cyclophosphamide. Protection was evaluated on day 10 after the first dose of cyclophosphamide. bFGF alone did not show significant protection to low dose cyclophosphamide-induced alopecia. (n>4 per group).
Figure 4.5. Protection against paclitaxel-induced alopecia in C57BL-6 mice by subcutaneous FGFs. Paclitaxel (Pac, 40 mg/kg/day) was administered by intraperitoneal injection starting on day 9 after depilation for three consecutive days. For study of FGFs effect, mice in addition received aFGF (2 µg) and bFGF (2 µg) by subcutaneous injection 3 hrs before each administration of paclitaxel. Arrows indicate the injection sites of FGFs or normal saline. Protection effect was evaluated on day 8 after the first dose of paclitaxel. aFGF alone or bFGF alone did not show significant protection. (n>3 per group)
Figure 4.6. Acceleration of hair regrowth after high dose cyclophosphamide-induced alopecia in neonatal rats by subcutaneous aFGF/bFGF. Eight-day-old rats were given a single dose of cyclophosphamide (CTX, 50 mg/kg) by intraperitoneal injection. For studies of FGFs effect, rats in addition received daily subcutaneous injection of 2 µg aFGF and/or 2 µg bFGF in 50 µl saline for 4 days. FGFs treatments showed protection effect on day 6 after cyclophosphamide administration. The protection was lost on day 8. On day 14, hair regrowth started with a tail-head wave direction. On day 19 after cyclophosphamide injection, hair regrowth on the FGFs treated site was observable, while no hair shaft could be observed in the cyclophosphamide control group. No FGFs treatment was applied after hair loss occurred. FGFs treatments not only delayed the hair loss, but also enhanced the hair regrowth (n>6 per group).
Figure 4.7. Acceleration of anagen initiation by aFGF/bFGF after high dose cyclophosphamide-induced alopecia in neonatal rats. Eight-day-old rats were given a single dose of cyclophosphamide (50 mg/kg) by intraperitoneal injection. In FGFs treated group, rats in addition received 2 µg aFGF (B), 2 µg bFGF (C), or a combination of aFGF and bFGF (D) by subcutaneous injection for 4 consecutive days. On day 19 after cyclophosphamide injection, skin samples from the FGFs injection site was cryo-sectioned and stained with hematoxylin. Compared with cyclophosphamide control group (A), aFGF, bFGF or their combination showed an earlier onset of anagen with significant thicker dermal layer (200 × magnification, n>6 per group).
Figure 4.8. Acceleration of hair regrowth by subcutaneous aFGF/bFGF after CIA by high dose cyclophosphamide in neonatal rats. A single dose of cyclophosphamide, (CTX, 50 mg/kg) was given to eight-day-old rats. FGFs-treated rats in addition received 2 µg aFGF, 2 µg bFGF, or their combination by subcutaneous injection for 4 consecutive days. On day 19, FGFs treated animals showed significant thicker dermal layer compared to the cyclophosphamide control group (*p<0.01). Hair follicles in the normal saline control animals (without chemotherapy) were in telogen phase, and was not comparable to drug treated animals. On day 21, FGFs-treated animals showed significant longer hair shaft, compared with cyclophosphamide control animals (*p<0.01 compared to CTX control, n>6 animals per group).
Figure 4.9. Protection against hair loss and acceleration of hair regrowth by aFGF/bFGF in CIA by high dose cyclophosphamide in neonatal rats. Eight-day-old rats were treated with a single dose of cyclophosphamide, 50 mg/kg, by intraperitoneal injection. FGFs-treated animals receive in addition of 2 µg aFGF, 2 µg bFGF or their combination by subcutaneous injection for 4 days. Cyclophosphamide injection resulted in premature catagen induction and hair loss. FGFs treatment delayed the onset of catagen development and hair loss. FGFs treatments shortened the duration of telogen phase after CIA and thus enhanced onset of the anagen initiation of the next hair growth cycle after CIA.
Figure 4.10. FGFs delayed chemotherapy-induced dermal layer shrinkage onset in neonatal rats. Seven to eight-day-old neonatal rats were treated with the chemotherapeutic agents and FGFs as described before. FGFs did not totally reverse but delayed the onset of dermal layer shrinkage (n>4 animals per group).
Figure 4.11. Body weight changes during CIA w/o FGFs treatment in neonatal rats. Chemotherapeutic agents and FGFs were given as mentioned before. FGFs treatment did not reverse the body weight loss by the chemotherapeutic agents. Paclitaxel did not induce body weight loss in the CIA model. FGFs treatment did not offer advantages of animal survival in the studies with doxorubicin (data not shown).
CHAPTER 5

BROAD-SPECTRUM PROTECTION AGAINST CHEMOTHERAPY-INDUCED ALOPECIA BY TOPICAL APPLICATION OF ACIDIC AND BASIC FIBROBLAST GROWTH FACTORS

5.1 INTRODUCTION

We have shown that subcutaneous injection of the combination of aFGF and bFGF (FGFs) offered broad-spectrum protection against chemotherapy-induced alopecia (CIA) by four anticancer agents, i.e., cyclophosphamide, doxorubicin, ara-c and paclitaxel, in the established animal models. Because FGFs confer broad-spectrum chemoresistance in solid tumors\textsuperscript{109}, it is important to minimize the systemic exposure of FGFs during the treatment of CIA. Thus, we hypothesize to use topical FGFs application in protecting against CIA, so that FGFs will not compromise the effectiveness of chemotherapy in cancer cells.

The stratum corneum is the major barrier to drug penetration in the skin. Recent studies indicate that hair follicles play an important role in the percutaneous absorption of large polar molecules that cannot readily penetrate the stratum corneum, and when formulated into drug carriers, i.e., liposome and microsphere, hair follicles can be privileged pathways for drug penetrate into the skin\textsuperscript{147-151}. Topical application of EGF with 75% dimethyl sulfoxide (DMSO) offered significant protection against ara-c-
induced alopecia limited to the treated area in neonatal rats\textsuperscript{55}. Topical application of aFGF with 75\% DMSO, however, did not show any protection effect against ara-c-induced alopecia in neonatal rats\textsuperscript{55}. Doxorubicin antibody incorporated in liposomes reduced the severity of doxorubicin-induced alopecia in neonatal rats\textsuperscript{73}. The penetration of minoxidil in the skin has been shown to have significant influence on its protection effectiveness against CIA in neonatal rats\textsuperscript{66}. In a phase I trial, topical application of 1, 25-Dihydroxyvitamin D3 induced local dermatitis, indicating the importance of proper formulations in treatment of CIA\textsuperscript{102}.

In this chapter, we tested the protective effect of FGFs against CIA by topical application. The distribution of topically applied bFGF in hair follicles was studied using immunochemical staining of the protein.
5.2 MATERIALS AND METHODS

5.2.1 Chemicals and Reagents

Dimethyl sulfoxide (DMSO) and mouse anti-human bFGF (clone FB-8) monoclonal antibodies were purchased from Sigma Co. (St. Louis, MO). bFGF ELISA Kit was purchased from Oncogene (Boston, MA). Other chemicals and tissue culture supplies were the same as described in Chapter 2 (section 2.2.1) and Chapter 3 (section 3.2.1).

5.2.2. Animals

See Chapter 3, section 3.2.2

5.2.3. Induction of alopecia in neonatal rats

Seven-day-old rats were used for treatments with doxorubicin and eight-day-old rats were used for treatments with other chemotherapeutic agents, i.e., cyclophosphamide, paclitaxel and ara-c. Neonatal rats were given intraperitoneal injections of doxorubicin (1.5 mg/kg/day for 7 days), Ara-C (50 mg/kg/day for 5 days), or cyclophosphamide (50 mg/kg, single dose). Two subcutaneous doses of paclitaxel, 2.5 µg each in 50 µl saline, were injected directly on the head between two ears on day 0 and day 3, respectively. The time when the first dose of chemotherapeutic agent administration was recorded as day 0. Hair loss occurred naturally without any physical forces applied. Body weight of each animal was measured every 24 hours to evaluate the systemic toxicity of the treatments.
5.2.4. Treatment of FGFs

A combination of aFGF (2 µg) and bFGF (2 µg) were first dissolved in 50 µl 75% DMSO in normal saline and then topically applied over a 0.5 cm × 0.5 cm area on the head between two ears. Control animals were treated with 75% DMSO without FGFs. 3 hrs after the FGFs treatment, the remaining solution on the skin was swiped and washed using normal saline to avoid the contamination to untreated area. FGFs were given at the same site when multiple applications were required in the experiment. For the effects of FGFs on ara-c or doxorubicin-induced alopecia, FGFs treated group in addition received topical FGFs 3 hrs before each drug administration. For studies with cyclophosphamide, FGF treated group received FGFs treatment for 4 consecutive days, starting on day 0 of experiment. For studies with paclitaxel, FGFs treated group received FGFs treatment for 5 consecutive days, starting on day 0 of experiment.

5.2.5. Distribution of bFGF in hair follicles after topical application

Eight-day-old rats were treated with 2 µg of bFGF by topical application in 50 µl of 75% DMSO as described before. Control animals received same volume of 75% DMSO. At 3 hrs and 24 hrs, skin samples were collected, flash frozen in liquid nitrogen, kept at −70 °C until cut into 6 µm thick. The skin samples were then fixed in 10% buffered formaldehyde without agitation for 5 min. Immunohistochemical staining of bFGF was performed to observe the location of bFGF in hair follicles.
5.2.6. Systemic exposure of bFGF after subcutaneous or topical application

Experiments were performed to evaluate whether subcutaneous or topical delivery of bFGF resulted in appropriate systemic exposure. Eight-day-old neonatal rats were treated with 2 µg bFGF by subcutaneous injection or topical application with 75% DMSO as described before. Control animals received same volume of normal saline or 75% DMSO. At 1 hr, 3 hrs, 6 hrs and 24 hrs, one group of animals were sacrificed and plasma samples were collected for bFGF concentration measurement. bFGF levels were measured using the bFGF ELISA kit. Briefly, to each well of the 96-well plate, 50 µl of the assay diluents RD1-43 was added followed by the addition of 100 µl of plasma samples or bFGF standard solutions. After 2 hrs of incubation at room temperature, the wells were washed with the washing buffer, followed by adding 200 µl of murine monoclonal anti-bFGF antibodies conjugated to horseradish peroxidase. After incubation for 2 hrs at room temperature, the wells were washed with the washing buffer and 200 µl of the substrate solution was added. The samples were incubated at room temperature for 30 min and 50 µl of the stop solution was added. The color intensity was read using a microplate reader at 405 nm. The detection limit of bFGF was 5.0 pg/ml.
5.3 RESULTS

5.3.1. Topical FGFs protected against CIA in neonatal rats

Topical application of the combination of aFGF and bFGF dissolved in 75% DMSO in normal saline showed broad-spectrum protection against CIA by all four chemotherapeutic agents in the neonatal rat model. The protection by topical FGFs was comparable to the protection by subcutaneous FGFs. The protection was confined to the topical application area (Figure 5.1-5.4). Topical application of FGFs also protected against the hair loss induced by high dose of cyclophosphamide. FGFs treatment did not show significant effect on body weight loss and animal survival during drug treatment, compared to the drug control group (data not shown).

5.3.2 Distribution of bFGF in hair follicles after topical application

In neonatal rats, topical application of 2 µg bFGF dissolved in 75% DMSO increased the bFGF level in epidermis, dermis, ORS and hair bulb (Figure 5.5). Nearly all animals showed irritation and rash of skin at the application site.

5.3.3 Plasma bFGF concentration after subcutaneous or topical application

The plasma bFGF level in control animals without bFGF treatment showed a wide range, 30.0-217.5 pg/ml (92.5±70.7 pg/ml, n=6 animals). After subcutaneous injection of 2 µg bFGF, the plasma bFGF level ranged from 67.5 to 142.5 pg/ml (108.1±31.2 pg/ml, n=3 animals, p>0.5 compared to control). After topical application of 2 µg bFGF, the plasma bFGF level ranged from 42.5 to 130.0 pg/ml (92.5 ± 45.1 pg/ml, n=3 animals, p>0.5 compared to control) (Figure 5.6).
5.4. DISCUSSION

Topical application of FGFs showed broad-spectrum protection against CIA by all four chemotherapeutic agents in the animal models. Topical application of FGFs showed significant protection against high dose of cyclophosphamide-induced alopecia, which could not be protected by subcutaneous delivery of the same amount of FGFs (Chapter 4).

DMSO has been shown to enhance the transfollicular pathway in topical delivery.\textsuperscript{152,153} Formulations of 60\% DMSO has been used in humans for topical application.\textsuperscript{154} Topical application of bFGF with DMSO has been studied in humans for skin-cartilage composite grafts.\textsuperscript{154,155} The present study showed that topical application of bFGF significantly increased the bFGF level in the skin, especially ORS and hair bulb, the two major targets of chemotherapy in hair follicles (Chapter 3). Besides their high affinity receptors in hair follicles, FGFs have strong binding affinities for heparan sulfate proteoglycans that are localized in hair follicle basement membrane. The half-life of bFGF in plasma was less than an hour.\textsuperscript{156} The localization due to binding and the short half-life might have contributed to the low or insignificant systemic exposure to bFGF after subcutaneous injection or topical application.

CDK inhibitors, P53 inhibitors, 1,25-Dihydroxyvitamin D\textsubscript{3}, minoxidil, and cyclosporine A, which are either ineffective to offer broad-spectrum protection or induce side effects in patients (Chapter 1, section 1.5), are exogenous small molecules that can be readily absorbed into the systemic circulation after topical application, which may compromise the efficacy of chemotherapy to tumors. FGFs, however, are
endogenous proteins with their receptors richly distributed in hair follicles. Multiple growth factors, i.e., epidermal growth factor (EGF), platelet-derived growth factor (PDGF), aFGF, bFGF, FGF4, keratinocyte growth factor-2 (KGF-2), transforming growth factor-β (TGF-β), and vascular endothelial growth factor (VEGF), have been formulated and studied in clinical trials for wound healing. PDGF has become FDA-approved as a topical agent\textsuperscript{157,158}. Considering the damaged skin structure in the treatment of wound healing, application of FGFs for treatment of CIA on normal skin should be safe. In fact, intravenous injection of aFGF (6 µg) or bFGF (6 µg) in tumor-bearing mice did not increase the tumor growth rate, enhance the cell proliferation, or induce metastasis\textsuperscript{159}, but increased the tumor uptake of chemotherapy\textsuperscript{160}. Thus, the protection against CIA by FGFs is unlikely to compromise the effectiveness of chemotherapy in tumor. Multiple formulations have been developed recently for hair follicular drug delivery especially for big molecules\textsuperscript{73,150,151,161-164}. The development of CIA treatment is lagging and no effective treatment is available. These suggest the development of FGFs formulation to treat CIA in cancer patient is an attractive option.

In summary, subcutaneous injection or topical application of aFGF and bFGF offered broad-spectrum protection against CIA by chemotherapeutic agents with different anticancer mechanisms and chemical structures. These two growth factors also showed significant effect in enhancing the hair regrowth after CIA and in maintaining the hair follicular integrity during CIA. Topical application of FGFs may produce selective protection against CIA without compromising the effectiveness of chemotherapy in tumor.
Figure 5.1. Protection against doxorubicin-induced alopecia in neonatal rats by topical application of FGFs. Seven-day-old rats were given intraperitoneal injections of doxorubicin (Dox, 1.5 mg/kg/day) for 7 consecutive days. For studies of FGFs effect, rats in addition received daily topical application of 2 µg aFGF and 2 µg bFGF in 50 µl 75% DMSO in saline on the head between two ears 3 hrs before each administration of doxorubicin. Protection was evaluated on day 13 after the first dose of doxorubicin. Arrows indicate the topical application site of FGFs or saline (n=6 per group).
Figure 5.2. Protection against ara-c-induced alopecia in neonatal rats by topical application of FGFs. Eight-day-old rats were given intraperitoneal injections of ara-c (50 mg/kg/day) for 5 consecutive days. For studies of FGFs effect, rats in addition received topical application of 2 µg aFGF and 2 µg bFGF in 50 µl 75% DMSO in saline for 5 days 3 hrs before each administration of ara-c. Arrows indicate the topical application site of FGFs or saline. Protection was evaluated on day 8 after the first dose of ara-c (n=6 per group).
Figure 5.3. Protection against paclitaxel-induced alopecia in neonatal rats by topical application of FGFs. Eight-day-old rats were given two doses of paclitaxel (Pac, 25 µg/50µl/site) on day 0 and day 3 of experiment by subcutaneous injection. For studies of FGFs effect, rats in addition received daily topical application of 2 µg aFGF and 2 µg bFGF in 50 µl 75% DMSO in saline for 5 days. Arrows indicate the injection or application site of paclitaxel, saline and/or FGFs. Protection was evaluated on day 10 after the first dose of paclitaxel (n=4 per group).
Figure 5.4. Protection against cyclophosphamide-induced alopecia in neonatal rats by topical application of FGFs. Eight-day-old rats were given a single intraperitoneal injection of cyclophosphamide (CTX, 50 mg/kg). For studies of FGFs effect, rats in addition received daily topical application of 2 µg aFGF and 2 µg bFGF in 50 µl 75% DMSO in saline for 4 days. Arrows indicate the application sites of saline or FGFs. Protection was evaluated on day 8 of experiment (n=6 per group).
Figure 5.5. Distribution of topical bFGF in the skin of neonatal rats. A solution of bFGF in 75% DMSO (2 µg in 50 µl) was applied topically to the head skin between the ears (0.5 cm × 0.5 cm). Skin samples from bFGF treated area were removed at 3 and 24 hrs, cryo-sectioned, fixed in 10% buffered formaldehyde and processed for bFGF immunohistochemical staining (brown). Compared to the control, topical bFGF administration increased the bFGF level in the epidermis, dermis, ORS and hair bulb.
Figure 5.6. Plasma bFGF concentration after single dose of 2 µg bFGF by subcutaneous injection or topical application. Eight-day-old rats were given 2 µg bFGF by subcutaneous injection or topical application with 75% DMSO. Control animals received normal saline injections or topical application of 75% DMSO without bFGF. At each time point, one group of animals was sacrificed and the plasma bFGF level was measured by ELISA. The average concentration did not show significant difference between control and bFGF treated group (p>0.5). One animal per data point.
The major finding of this dissertation research was that subcutaneous injection of aFGF/bFGF offered broad-spectrum protection against CIA by multiple anticancer drugs representing different major classes of anticancer agents, i.e., doxorubicin, paclitaxel, cyclophosphamide, and ara-c. The broad-spectrum protection against CIA by FGFs is dramatically different from all previously described agents. The protective effect of FGFs against CIA by paclitaxel is novel. The results showed that the broad-spectrum protective effect against CIA required both growth factors, suggesting the possible synergistic and complementary roles of aFGF and bFGF in protecting against CIA. Further studies using specific antibodies to aFGF or bFGF are needed to confirm this hypothesis. Whether the requirement of the combination of aFGF and bFGF, i.e., the dose and ratio of aFGF and bFGF, is different for different chemotherapeutic agents need be studied to identify the treatment schedule of FGFs.

In addition to the broad-spectrum protection against CIA, FGFs showed acceleration of hair regrowth by shortening the telogen phase after CIA by high dose of cyclophosphamide. The initiation of anagen phase depends on the communication between dermal papilla and stem cells in hair bulge. These two locations are generally
not affected by chemotherapy, since CIA is usually reversible. The effects of FGFs on dermal papilla and stem cells during CIA need be further studied to explain the mechanisms by which FGFs enhance the hair regrowth after CIA.

The second major finding of this dissertation research was that topical application of FGFs dissolved in 75% DMSO showed broad-spectrum protection against CIA by the four representative anticancer drugs. The data further showed that little or no systemic absorption of topically applied bFGF, even in the presence of DMSO that is known to diminish the barrier function of the stratum corneum and enhance drug penetration into skin. These results support our hypothesis that topical FGFs will selectively protect the hair follicles without compromising the efficacy of chemotherapy. Additional studies to develop hair follicle-targeting carriers to deliver FGFs will support this hypothesis. For example, liposome and microspheres have been applied for hair follicle-targeted delivery of big molecules such as protein.


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