Cytokines and Signal Transduction Pathways Mediated by Anthralin in Alopecia Areata-Affected Dundee Experimental Balding Rats

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Although many therapeutic modalities have been tested on alopecia areata, patient outcomes have been disappointing. Use of animal models would help to develop more efficient therapies as well as understanding therapeutic mechanisms. We have demonstrated that 0.1% topical anthralin ointment is 100% effective in restoring follicular activity in Dundee experimental balding rats. This is the most promising topical treatment for Dundee experimental balding rats among the therapeutic agents tested on this model. Various cytokines have been shown to be associated with the pathogenesis of alopecia areata. To test whether any of these cytokines might be modulated by anthralin, an RNase protection assay and the real-time polymerase chain reaction were performed to compare their expression between anthralin-treated and control skins. These experiments showed that expression of tumor necrosis factor-α and interferon-γ was inhibited by anthralin, whereas expression of interleukin-1α/β and their receptor antagonist, interleukin-1Ra, and interleukin-10 was stimulated by anthralin. In addition, using an antibody-based multi-immunoblotting technique, we found that certain signaling regulatory proteins were modulated by anthralin. Their potential roles in reversing the autoimmune-arrested follicular activity in Dundee experimental balding rats are discussed. Key words: autoimmunity/hair follicles/protein kinases. JID Symposium Proceedings 8:87–90, 2003

A wide range of treatments has been tried on alopecia areata (AA) patients, including contact sensitizers, immunomodulators, and biologic response modifiers (Rokhsar et al, 1998; Shapiro and Price, 1998). Unfortunately, none of the therapeutic modalities produces consistently successful results, and many have untoward side-effects. Topical anthralin treatment of AA was found to be effective on patients by some investigators (Fiedler-Weiss and Buys, 1987), but not by others (Nelson and Spielvogel, 1985). Animal models provide a means whereby new forms of treatment can be tested and the underlying mechanisms can be studied more extensively. One of two rodent models for AA is the Dundee experimental bald rat (DEBR), a subpopulation of the inbred rat strain, which exhibits hair loss lesions similar to that which is observed in human AA (Michie et al, 1991; Zhang and Oliver, 1994). All DEBR rats develop a normal coat of hair from birth up to 4 mo. Then, up to 30% of males and 80% of females start to lose hair on their heads and then typically progress to the flanks and the entire body (Michie et al, 1991). Several reports have demonstrated that the DEBR rat is a useful model to study drug effects on AA, including cyclosporine A, FK506, and leflunomide (Cao et al, submitted; Oliver and Lowe, 1995; McElwee et al, 1997, unpublished data). In this paper, we discuss our recent findings using DEBR rats and topical anthralin therapy as a model system to study anthralin’s therapeutic effects on restoring hair growth in AA-affected rats and the underlying molecular mechanisms that may be responsible for its therapeutic action.

THERAPEUTIC EFFECTS OF ANTHRALIN ON RESTORING FOLLICULAR ACTIVITY IN DEBR RATS

Topical anthralin treatment was found to be effective by other investigators for AA patients (Fiedler-Weiss and Buys, 1987). The underlying molecular mechanisms are unknown. Recently, we have found that topical treatment with 0.1% anthralin ointment is 100% effective on restoring the autoimmune-induced hair loss in AA-affected DEBR rats (Cao et al, submitted). Hair loss before treatment ranged from small patches to entire body surfaces. After 6–10 wk of anthralin treatment, follicular activity was restored and hairs covered nearly all treated sides in all of the rats tested, whereas the control sides remained bald (Cao et al, submitted). Topical anthralin appears to be the most promising therapeutic agent to date tested on AA-affected DEBR rats. Our findings demonstrate that the anthralin-treated DEBR rat is an excellent model system to explore molecular mechanisms for therapeutic actions on restoring follicular activity in AA.

MECHANISMS OF ANTHRALIN’S THERAPEUTIC ACTIONS

Anthralin remains one of the most effective and most widely used therapeutic agents for psoriasis (Griffiths, 1998; Van de

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Abbreviations: RPA, RNase protection assay; AA alopecia areata; DEBR, Dundee experimental balding rat.
Kerkof et al., 1998; Smith and Lefwohl, 2000). Its anti-psoriatic efficacy may be related to the induction of cutaneous inflammation via the expression of cytokines and cell adhesion molecules, including granulocyte-macrophage colony-stimulating factor, interferon (IFN)-γ, interleukin (IL)-6, IL-8, and tumor necrosis factor (TNF)-α (Richter and Davies, 1995; Mrowietz et al., 1997; Müller, 1997; Chodorowska, 1998; Lange et al., 1998). Whether any of these cytokines is directly associated with anthralin’s anti-psoriatic actions is unclear. Little progress has been made in identifying the molecular mechanisms of anthralin’s antipsoriatic actions owing to a lack of relevant animal models for psoriasis.

The mechanism of anthralin’s therapeutic effects in AA can only be speculated. Anthralin is unstable when exposed to air and the oxygen radicals generated from it are central to its anti-inflammatory and anti-proliferative effects in psoriasis. In AA therapy, free radicals generated from anthralin might be the mechanism of anti-inflammatory action for clearing the infiltrated lymphocytes. In this case, free radicals generated from anthralin within the skin should selectively suppress infiltrated lymphocytes and leave the follicular cells unhindered in affected skin; however, our preliminary studies showed that lymphocytes and different follicular cells were equally affected by anthralin in vitro (Fig 1), indicating that the mechanism is not simply a selective cytotoxicity against lymphocytes. This was further supported by the data of immunostaining using CD4 and CD8 antibodies (Cao et al., submitted). Histologic examination revealed no reduction in the total number of infiltrated lymphocytes; however, there was a general shift in the location of the CD8+ cells from perifollicular and intrafollicular regions in untreated skin to a uniform presence in the dermis upon successful anthralin treatment. This pattern of CD8+ redistribution was consistent between all the rats tested. It would be interesting to study what drives relocation of the CD8+ cells.

**CYTOKINES MODULATED BY ANTHRALIN IN DEBR RATS**

In skin, a large number of cytokines is produced under both normal and pathologic conditions, and these play important parts in the pathogenesis of inflammatory skin diseases. AA is believed to be an autoimmune disease and a number of cytokines have been indicated in its pathogenesis, including IL-1β, TNF-α, IFN-γ, and IL-1Ra (Hoffmann et al., 1994; Cork et al., 1996; Philpott et al., 1996; Carroll et al., 1997). As follicular activity in Ig AA-affected DEBR rats returned to near normal on all anthralin-treated sides, whereas control sides remained unchanged, the underlying cellular and molecular changes mediated by anthralin must occur locally within the treated skin. As anthralin does not exhibit a selective cytotoxicity against lymphocytes, the modulatory effects of anthralin on the production of the various cytokines might be the mechanism responsible for restoring follicular activity. Using a RNase protection assay (RPA) and quantitative real-time polymerase chain reaction, we compared the expression of selected cytokines in anthralin-treated and control DEBR rat skin. Among the dozen cytokines tested, the expression of IL-1α/β, IL-1Ra, and IL-10 were all consistently increased by anthralin treatment using RPA, whereas IL-18 showed no significant difference (Table I). Other cytokines, including IL-2, IL-12, TNF-α, granulocyte-macrophage colony-stimulating factor, and IFN-γ, were not detected consistently in the rat skin using RPA assay. As TNF-α and IFN-γ are two important cytokines that might be associated with AA (Philpott et al., 1996; Carroll et al., 1997), a more sensitive assay, real-time polymerase chain reaction, was used to test whether these two cytokines might be altered by anthralin in AA-affected rats. As shown in Table I, both IFN-γ and TNF-α demonstrated decreased expression after anthralin treatment. Using the real-time polymerase chain reaction assay, stimulation of IL-10, IL-1α, and IL-1Ra expression was confirmed. The expression of IL-1β and IL-1Ra modulation by anthralin was also confirmed at a protein level by immunohistochemistry and western blotting (unpublished data).

It is not clear at present whether any of these cytokines play a direct role in anthralin-induced hair regrowth in DEBR rats. The correlation of TNF-α and IFN-γ expression with anthralin’s efficacy is intriguing, as suppression of these cytokines has been associated with improvement of various autoimmune conditions (Cope, 1998; Feldmann and Maini, 2001). IL-10 and IL-1Ra have been generally considered to be anti-inflammatory cytokines and their increased expression by anthralin might be responsible for anthralin’s therapeutic action. The expression of IL-10 has also been shown to be induced by diphenylcyclopentenone (DPCP) therapy in AA patients (Hoffmann et al., 1994). A similar experiment was performed in our laboratory on another rodent model, AA-affected C3H/HeJ mice, and it was found that TNF-α expression was also reduced after anthralin treatment, using RPA (Tang et al., submitted).

The increased expression of IL-1α/β by anthralin was a surprise, as IL-1 has been shown to be associated with the pathogenesis of AA both in vivo and in vitro (Hoffmann and Happle, 1995; Philpott et al., 1996); however, there is also evidence indicating that IL-1 can be a positive regulator of hair growth1 (Chedid et al., 1994). IL-1 is one of the primary cytokines induced by injury and it is a primary stimulator of keratinocyte proliferation and differentiation during the early stages of wound healing (Singer and Clark, 1999). More directly, IL-1 has been shown to protect against chemotheraphy-induced alopecia (Jimenez et al., 1991). It is not clear how upregulation of IL-1 correlates with anthralin-induced hair regrowth in the AA-affected DEBR rats. Overexpression of IL-1 might be related to side-effects of

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1 Cairns JI, Cumpstone MB, Kennedy AH, Harmon CS: Murine IL-1α and IL-1β stimulates proliferation and collagenase secretion by cultured hair follicles from neonatal mice. *J Invest Dermatol* 92:410, 1989 (Abstr)

### Table I. Cytokines modulated by anthralin in DEBR rat skin

<table>
<thead>
<tr>
<th>Cytokines</th>
<th>Mean ratio (treated/control)</th>
<th>P value</th>
<th>Assay</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-1α/β</td>
<td>2.68</td>
<td>0.0288</td>
<td>PCR</td>
</tr>
<tr>
<td>IL-1Ra</td>
<td>4.8</td>
<td>0.0015</td>
<td>PCR</td>
</tr>
<tr>
<td>IL-10</td>
<td>1.94</td>
<td>0.033</td>
<td>PCR</td>
</tr>
<tr>
<td>TNF-α</td>
<td>0.40</td>
<td>0.026</td>
<td>PCR</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>0.35</td>
<td>0.020</td>
<td>PCR</td>
</tr>
<tr>
<td>IL-1α</td>
<td>2.34</td>
<td>0.0004</td>
<td>RPA</td>
</tr>
<tr>
<td>IL-1β</td>
<td>5.53</td>
<td>&lt;0.001</td>
<td>RPA</td>
</tr>
<tr>
<td>IL-1Ra</td>
<td>3.86</td>
<td>&lt;0.001</td>
<td>RPA</td>
</tr>
<tr>
<td>IL-10</td>
<td>3.57</td>
<td>0.0012</td>
<td>RPA</td>
</tr>
<tr>
<td>IL-18</td>
<td>1.18</td>
<td>&gt;0.1</td>
<td>RPA</td>
</tr>
</tbody>
</table>
inflammation (dermatitis) caused by anthralin. At the same time, its receptor antagonist, IL-1Ra, was also consistently induced by anthralin. The end result might be determined by the balance between IL-1α/β and IL-1Ra. The direct roles of IL-1 families in anthralin-induced hair growth in DEBR rats should be investigated further.

SIGNALING PROTEINS MODULATED BY ANTHRALIN IN DEBR RATS

Many diseases result from malfunctions of intercellular and intracellular signaling pathways, and small molecules blocking or activating specific pathways have been used to treat disease (Le-vitzki, 1994, 1996). Although the effects of various anti-inflammatory drugs have been well studied in both animals and patients, little is known about the signaling pathways involved in the therapeutic responses. Inflammation and autoimmune conditions trigger the activation of many transcriptional factors, such as c-Jun and nuclear factor κB, via mitogen-activated protein kinase pathways (extracellular signal-regulated kinase pathway, c-Jun N-terminal kinase pathway, and p38 pathway), and the IKB Kinase (IKK) complex (Hunter, 1995; Karin, 1999; Sampath et al., 1999). Certain signal transduction pathways may represent crucial molecular events in rat skin upon successful anthralin therapy, and this has never been explored. Using an antibody-based multiprotein kinase expression analysis system called Kinetworks™ Protein Kinase Screen (KPKS) 1.0 developed by Kinexus Bioinformatics Corporation (www.kinexus.ca), we examined protein expression for 77 protein kinases in total cell lysates pooled from 10 anthralin-treated and control skins. The level of protein expression was analyzed using Bio-Rad’s Fluor-S Max Imager and quantitated using Bio-Rad’s software QuantityOne™ (Bio-Rad, Hercules CA). A total of 53 different protein kinases were detected in the DEBR rat skin. Those protein kinases with more than a 1.5-fold difference between control and anthralin-treated sites are listed in Table II. Several protein kinases in the ERK pathway were downregulated at protein levels. This was further confirmed using phospho-specific antibodies against the active forms of ERK1/2, which showed a consistent reduction of ERK1/2 phosphorylation in anthralin-treated sites (unpublished data). Upregulation of this pathway might presumably be due to the free radicals generated by anthralin within the skin. Several isoforms of PKC were stimulated by anthralin (Table II). Whereas this is contradictory to the in vitro findings of Takahashi and Kamimura (2001), it agrees with the in vivo findings of Li et al. (1999). In addition, GSK-3 kinase expression and activity were decreased by anthralin. Its downstream substrate, β-catenin, and transcriptional factor, TCF1 (Lef1), have been tightly linked to follicular activity (Gat et al., 1998; Dasgupa and Fuchs, 1999). It would be important to determine whether these downstream regulators are also modulated in anthralin-treated DEBR rat skin. Other kinases that are modulated by anthralin in DEBR rat skin include PKG, LYN, JAK1, and CDC2 (Table II). These kinases are either important regulators of cell proliferation and differentiation (CDC2, PKG) or tightly linked to immune functions (LYN, JAK1). Potentially, they can be highly relevant to follicular activity and cutaneous immune responses to anthralin. We are continuing to study signaling pathways modulated by anthralin in rat skin and how they relate to the reversion of autoimmune-induced arrest of follicular activity in AA-affected rats.

CONCLUSIONS

Anthralin has been demonstrated to be the most effective drug in reversing autoimmune-induced arrest of follicles in AA-affected DEBR rats. There are many genes that can be modulated by anthralin in the skin. We have shown that downregulation of TNF-α and IFN-γ and upregulation of IL-10 and IL-1Ra may be responsible for anthralin’s therapeutic effects. We are currently investigating cytokine expression at different time points after anthralin treatment and will use more advanced “postgenomic” technologies to search for other genes associated with anthralin’s therapeutic effects. At the protein level, it is clear that certain signaling pathways involving various protein kinases and their regulators and effectors are modulated by anthralin. Further experiments will be performed to clarify which particular signaling pathways are directly linked to anthralin’s therapeutic actions.

Data obtained from classical single gene-based techniques is intriguing, but obtaining truly relevant in vivo genetic data regarding therapeutic actions using more exploratory approaches is difficult because gene expression varies between individuals. Our unilaterally treated DEBR rats with topical anthralin are excellent model systems to study the in vivo therapeutic mechanisms at molecular levels. We have demonstrated that the changes mediated by anthralin between control and treated sides in the same animal are quite consistent from animal to animal at both mRNA and protein levels. These topically and unilaterally treated animal systems eliminate the potential problems associated with variability between individuals when performing in vivo gene expression analysis. In addition, the accessibility and visibility of skin and hair follicles will provide the convenience and accuracy in correlating therapeutic efficacy with molecular events. Hopefully, in the near future, the newly developed exploratory molecular approaches will bring us to the molecular “fingerprints” of anthralin’s action. Identification of one or more molecular pathways responsible for the anthralin-induced hair regrowth in AA-affected rats will offer new insights and new potential treatment approaches to AA patients. The molecular events mediated by anthralin might be common to other autoimmune diseases and thus can potentially help in the design of more rational therapies for other autoimmune disorders as well.

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REFERENCES


Table II. Signal transduction proteins differentially expressed (1.5-fold or greater) between anthralin-treated and control DEBR rat skin

<table>
<thead>
<tr>
<th>Protein</th>
<th>Control (C)a</th>
<th>Treated (T)b</th>
<th>Ratio (T/C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ERK2</td>
<td>16.0</td>
<td>98</td>
<td>0.61</td>
</tr>
<tr>
<td>ERK3</td>
<td>64.6</td>
<td>39.0</td>
<td>0.60</td>
</tr>
<tr>
<td>LYN</td>
<td>76.7</td>
<td>37.7</td>
<td>0.49</td>
</tr>
<tr>
<td>PKC-β</td>
<td>ND</td>
<td>2.6</td>
<td>0.72</td>
</tr>
<tr>
<td>PKC-δ</td>
<td>2.8</td>
<td>4.3</td>
<td>1.50</td>
</tr>
<tr>
<td>PKC-ε</td>
<td>12.1</td>
<td>21.0</td>
<td>1.73</td>
</tr>
<tr>
<td>GSK-3p</td>
<td>7.1</td>
<td>3.3</td>
<td>0.46</td>
</tr>
<tr>
<td>JAK1</td>
<td>14.2</td>
<td>10.2</td>
<td>0.72</td>
</tr>
<tr>
<td>PKG-1</td>
<td>11.9</td>
<td>6.3</td>
<td>0.54</td>
</tr>
<tr>
<td>CDC2</td>
<td>38.3</td>
<td>2.2</td>
<td>0.59</td>
</tr>
</tbody>
</table>

aND, not detectable.
bRelative levels of expression are shown with arbitrary units.


