Table I. Apoptosis of Cultured Dermal Papilla Cells and Keratinocyte Cell Lines

Trigger of Apoptosis	Percent Apotosis[a] Dermal Papilla	KB	HaCaT
Control	2	0	7
IFN-γ	2	1	5
TNF-α	2	6	6
TNF-α plus IFN-γ	2	4	23
IL-1	0	4	9
Anti-FAS	0	4	9
IFN-γ + anti-FAS	0	58	16
Beauvericin	62	52	ND

[a] Percent apoptosis determined by Hoechst staining of cell cultures exposed to different triggers for apoptosis for 4 h. ND, not done.

induced by nuclear Hoechst staining or of DNA nicking as measured by TUNEL staining. We observed apoptotic cells in the dermal papilla with both Hoechst and TUNEL techniques. Staining for the anti-apoptotic protein Bcl-2 showed detectable levels of Bcl-2 in the dermal papilla of AA patients.

These studies are preliminary indication that dermal papilla damage may be a critical component of alopecia areata, and that apoptosis is one endpoint observed. The presence of Bcl-2 in the same papilla indicates that an apoptosis may be induced in the presence of intact anti-apoptotic defenses.

It must be noted that in hematopoietic and non-hematopoietic tumors, expression of FAS and Bcl-2 are not necessarily predictors of induction of or protection against apoptosis, respectively [7]. However, it is our experience that in human keratinocytes and melanocytes, Bcl-2 expression is an effective if imperfect protection against induction of apoptosis.

We propose that apoptosis is one of the cytotoxic effects seen in dermal papillae in alopecia areata as a result of immunologic cytotoxicity. Further studies are necessary to determine whether cell death or inhibition in the dermal papillae is a central component of alopecia areata.

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Cytokines and Dermal Papilla Function in Alopecia Areata

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The identity of the primary disease target in alopecia areata remains in doubt. Histologic and ultrastructural evidence of cellular damage and increased expression of class I and class II HLA antigens led to the suggestion that cells undergoing early cortical differentiation are the principal focus for immune-mediated attack [1]. However, increased HLA expression by hair-matrix keratinocytes appears to be a relatively late event and may result from cytokine release by infiltrating immune cells. We have previously shown that the cytokines interferon-gamma and tumor necrosis factor-alpha are insufficient stimuli to induce *in vitro* the patterns of increased human leukocyte antigen and intercellular adhesion molecule-1 expression seen in the hair follicle *in vivo* in alopecia areata, suggesting other factors are crucial in the pathogeneticsequence [2]. Alopecia areatais known to involve disturbance of immune function but there is no compelling evidence for a primary autoimmune mechanism. To unify theories on the pathogenesis, we proposed a new hypothesis that pro-inflammatory and chemo-attractant cytokine production due to dermal papilla dysfunction leads to hair-matrix keratinocyte damage with an inflammatory response and consequent disturbance of hair growth. To investigate dermal papilla function in alopecia areata, we studied the ability of cultured dermal papilla cells from perilesional sites to promote lymphocyte proliferation *in vitro*. We have also studied indirectly the role of locally produced antiproliferative cytokines of the type I interferon family by immunohistochemical assessment of the expression of Mx protein, a sensitive marker for interferon alpha/beta activity [3].

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MATERIALS AND METHODS

Perilesional and non-lesional scalp biopsies were obtained from alopecia areata patients (n = 8); normal control scalp tissue from patients having removal of benign tumors/nevi (n = 5). Cultured cells obtained as previously described [4] were used between passages 2 and 4 and cell-free supernatants prepared by filtration. Cultured dermal papilla cells from normal scalp and non-lesional and perilesional alopecia areata tissues were used to produce test supernatants with peripheral blood mononuclear cells from both normal individuals and patients with active alopecia areata as the responder population in a microculture lymphocyte proliferation assay using ^{3}H-thymidine incorporation to assess proliferation. Interleukin-6 (IL-6) was measured by the B9 hybridoma bioassay and an enzyme-linked immunosorbent assay technique. Mx protein expression was assessed by avidin-biotin immunoperoxidase staining of tissue sections using three monoclonal antibodies kindly supplied by Drs. H. Towbin and M.A. Horisberger, CIBA Geigy, Basel, Switzerland.

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RESULTS

A lymphocyte proliferative response was observed in cultures incorporating supernatants from cultured cells derived from perilesional tissues (median stimulation index 17.5, range 9.8–36), in contrast to normal controls (median stimulation index 1.3, range 0.97–1.8), p = 0.012, Mann-Whitney U test. Similar responses were observed with supernatants derived from both dermal papilla cells and interfollicular fibroblasts. This occurred irrespective of the source of responder cells and whether autologous or allogeneic combinations of dermal papilla cells and responder cells were used. No significant proliferative effect occurred in experiments using media from cultures derived from non-lesional sites in alopecia areata patients, or from normal control subjects.

Significantly higher levels of IL-6 were detected in supernatants derived from perilesional dermal papilla cells (median level 55 ng/ml, range 6.7–440) and in one cell line from a non-lesional site than in those from normal dermal papilla cells (median level 3.0 ng/ml, range 2.6–8.5), p = 0.037, Mann-Whitney U test. Biologic activity of the IL-6 in these supernatants was confirmed by neutralization with polyclonal goat anti-human IL-6 antiserum prior to performing the B9 assay. When the lymphocyte proliferation assay was repeated using supernatant samples pre-incubated for 1 h with neutralizing IL-6 antiserum, there was only a small reduction in the proliferative response that was not significant (analysis of variance), suggesting IL-6 was of only modest importance in inducing the mitogenic effect observed. In normal hair follicles, staining with the Mx antibodies was confined to the inner root sheath. Increased staining for Mx protein was observed within the hair follicles of perilesional alopecia areata tissues only. The distribution of increased Mx expression was mainly in the keratinocytes of the outer root sheath and matrix of lesional anagen follicles but was mildly increased also in the dermal papilla compared to normal scalp tissue.

DISCUSSION

These experiments showed that cultured dermal papilla cells and interfollicular dermal fibroblasts from perilesional sites in alopecia areata release soluble factors that stimulate lymphocyte proliferation. Biologically significant levels of IL-6 were identified in dermal papilla cell culture supernatants derived from cells up to passage 9 showing this property can be maintained for long periods *in vitro*. The observation of Mx protein expression signifies local production of type I interferons in the hair follicle in alopecia areata. The antiproliferative activity of these cytokines could well be relevant to the known pathodynamic disturbances of the hair-growth cycle including precipitation of catagen in newly involved follicles and failure to advance beyond the anagen III–IV stage in bald areas.

The likely role of cytokines in the pathogenesis of alopecia areata can be summarized in two hypotheses. First, cytokine gene dysregulation is a possible mechanism for the primary disease abnormality in alopecia areata. Increased cytokine production by the dermal papilla of the hair follicle could explain the observed inhibition of hair growth, disturbance of the hair-growth cycle, and the inflammatory response. In a patient with the appropriate genotype, the disease process could be triggered by diverse stimuli including infection, trauma, and stress, all of which have been implicated clinically. Alternatively, production of pro-inflammatory cytokines and chemoattractant factors by the dermal papilla may be central to the pathogenesis of alopecia areata, although not itself explaining the basic cause of the disease. The increased cytokine production found in alopecia areata may be a manifestation of a final common pathway of disturbances found in a variety of diseases.

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Autoimmune Diseases: Promising Emerging Therapies

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Alopecia Areata (AA), a condition involving patchy to complete hair loss, is marked by mononuclear cell infiltration in and around the hair follicles, as well as HLA associations, cytokine patterns, and abnormalities in follicular cells. These features, coupled with some clinical improvement after treatment with anti-inflammatory agents, suggest an autoimmune component in AA. This presentation will describe pathogenic mechanisms and therapeutic strategies under development in other probable autoimmune diseases that may be instructive in the understanding and possible treatment of AA. Two excellent review articles on this subject have been published recently by Steinman [1] and Miller and Karpus [2].

PATHOGENIC MECHANISMS

In neurologic diseases such as experimental autoimmune encephalomyelitis (EAE) and multiple sclerosis (MS), the pathogenic agent

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