Autoimmune Aspects of Depigmentation in Vitiligo

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Autoimmune depigmentation of the skin, vitiligo, afflicts a considerable number of people, yet no effective therapeutic modalities have been developed to treat it. In part, this can be attributed to the obscure etiology of the disease, which has begun to reveal itself only recently. It is known that pigment is lost as a function of reduced melanocyte numbers in the epidermis, and that depigmentation is accompanied by T cell influx to the skin in the vast majority of patients. Characterizing such infiltrating T cells as type 1 proinflammatory cytokine-secreting cells reactive with melanocyte-specific antigen is a major step toward effective therapy. Melanoma research has shown that differentiation antigens, also expressed by normal melanocytes, can be immunogenic when expressed in the melanosomal compartment of the cell. Similar reactivity to melanosomal antigens is apparent for T cells infiltrating vitiligo skin. It may eventually be possible to treat patients with decoy antigens that anergize such T cells, or to prevent recruitment of the T cells to the skin altogether. In this respect, it is important that T cells are recruited to the skin as a function of dendritic cell activation and that dendritic cells are likely activated at sites of epidermal trauma as a consequence of stress proteins that spill over into the microenvironment. Stress proteins chaperoning antigens representative of the cells from which they were derived are then processed by dendritic cells and contribute to their activation. Activated dendritic cells not only migrate to draining lymph nodes to recruit T cells but may execute cytotoxic effector functions as well. The contribution of the effector functions to actual depigmentation of the skin remains to be investigated.

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Vitiligo presents with gradual skin depigmentation (Das *et al*, 2001). Its etiopathology is plausibly explained by the autoimmune theory (Das *et al*, 2001). Research supporting autoimmunity is highlighted here, following an overview of general aspects of this disorder. Genes associated with vitiligo are subsequently explored, as they appear to support a role for the immune system in gradual depigmentation. Selective elimination of melanocytes from vitiligo skin has led many to believe that these melanocytes are more prone to undergo cell death, and research findings along this line are discussed. Melanocyte death can induce a humoral immune response through the release of previously sequestered intracellular antigens; data are reviewed relating to humoral responses, as are novel findings suggesting that cytotoxic T cells can eliminate melanocytes from the basal layer of the epidermis. The question of how an autoimmune response is initiated remains. Ongoing research is presented suggesting that stress-induced dendritic cell activation may contribute to this process. Finally, a parallel is drawn between autoimmunity to melanocytes and tumor immunity to melanoma cells, in keeping with the emerging view that knowledge regarding immune responses to melanocytic cells can support the development of effective immunotherapy for both vitiligo and melanoma.

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Abbreviations: DAF, decay accelerating factor; MCH, melanin-concentrating hormone; MCP, membrane cofactor protein; UV, Ultraviolet.

GRADUAL SKIN DEPIGMENTATION

Approximately 1% of the world population is subject to gradual depigmentation of the skin (Das *et al*, 2001). The associated skin disorder vitiligo strikes both genders and all races and can initiate at any age (Das *et al*, 2001). Depigmentation is more apparent in individuals of darker skin tones, yet vitiligo can be equally traumatizing for light- skinned individuals (**Fig 1**). The spotted appearance of patients resembles tuberculoid leprosy; thus, vitiligo is especially stigmatizing in countries where leprosy prevails (Ortonne *et al*, 1983). Surprisingly, there is little evidence that loss of melanocytes leaves depigmented skin more susceptible to UV-mediated skin damage and the development of skin cancer (Schallreuter *et al*, 2002).

It is imperative that we gain an understanding of the immunopathogenesis of vitiligo to enable development of effective therapy aimed at halting depigmentation and inducing repigmentation. Starting with evidence in support of the centrifugal removal of melanocytes from the basal layer of the epidermis during active disease, research has revealed that progressive, generalized vitiligo involves skin infiltration by immunocytes, including cytotoxic CD8 T cells (Le Poole *et al*, 1993; Le Poole *et al*, 1996; van den Wijngaard *et al*, 2000). New lesions typically develop following a traumatic event to the skin—for example,

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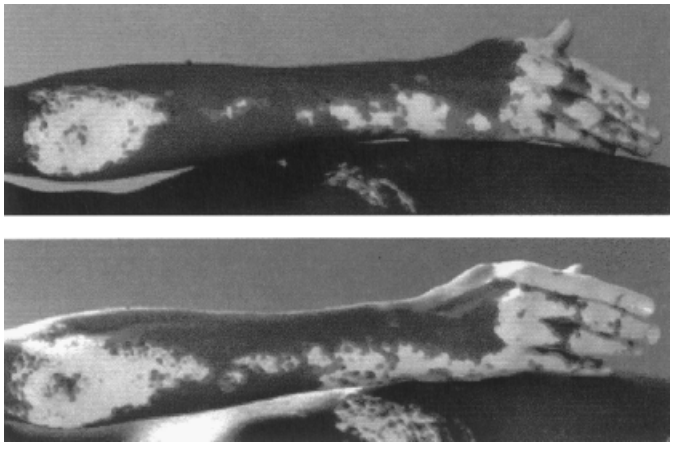

Figure 1. Both arms of a generalized vitiligo (1 in mirror image) emphasizing characteristic symmetrical distribution of depigmented lesions.

UV overexposure, contact with bleaching phenols, or skin injuries such as burns and cuts. Depigmentation ensues only in a subset of individuals prone to develop vitiligo (Das *et al*, 2001); consequently, a hereditary component to this disease has been the subject of intense investigation.

GENETIC PREDISPOSITION

The prevalence of vitiligo is markedly increased in some communities where consanguinial marriages are common. For example, within a community in Bangalore, India, up to one in every five individuals develops depigmented skin lesions (Ramaiah *et al*, 1988). Vitiligo is also more prevalent among autoimmune patients, particularly those with autoimmune thyroiditis (Hegedus *et al*, 1994). Moreover, an association with specific HLA haplotypes has been demonstrated within several populations (Venneker *et al*, 1993; Buc *et al*, 1996). These findings have prompted research into specific genes underlying the predisposition to depigmentation, but investigations have been complicated by the multifactorial nature of the disorder. The involvement of multiple genes was predicted by analyzing familial inheritance patterns (Nordlund and Majumder, 1997).

One approach to finding vitiligo-associated genes has come from investigations showing consistent morphologic aberrancies in melanocytes from vitiligo patients. These aberrancies include dilated RER profiles in the cell and compartmentalization of melanosomes (Boissy *et al*, 1991). Because these aberrancies are maintained in patient-derived melanocyte cultures for multiple passages, they are likely intrinisic to the pigment cell and can be studied by comparing gene expression profiles for melanocytes from vitiligo patients against those from controls. From such studies, the VIT1 gene has been identified (Le Poole *et al*, 2001). Reduced expression of the VIT1 transcript by vitiligo melanocytes is associated with enhanced expression of the hMSH6 mismatch repair gene. It has been proposed that VIT1 can regulate hMSH6 expression through the formation of RNA–RNA hybrids (Le Poole *et al*, 2001). Enhanced expression is suggestive of a need for G/T mismatch repair. Inadequate mismatch repair leaves cells vulnerable when exposed to mutagens such as UV.

Another gene associated with vitiligo encodes catalase (Casp *et al*, 2002). Mutations in or near the gene can render the cells more susceptible to damage—a finding in accordance with improved maintenance of melanocyte cultures in the presence of added catalase (Medrano and Nordlund, 1990). Interestingly, family studies have also linked vitiligo to a novel autoimmune susceptibility gene, AIS1 (Alkhateeb *et al*, 2002). Moreover, genes associated with vitiligo, as revealed by linkage analysis, include those involved in antigen processing and presentation, such as LMP7, TAP1, and CTLA-4, further supporting the involvement of an immune mechanism in depigmentation (W.T. McCormack, personal communication).

RESISTANCE TO APOPTOSIS

Progressive vitiligo is not associated with excessive inflammation. Melanocytes are thus likely eliminated by apoptosis, avoiding recruitment of an inflammatory infiltrate (Norris *et al*, 1994). Melanocytes from vitiligo patients clearly exhibit resistance to apoptosis, however, both *in vitro* and *in vivo*. High levels of Bcl-2 are found in melanocytes from both patients and controls (van den Wijngaard *et al*, 2002b). Bax levels are likewise comparable among vitiligo and control melanocytes (van den Wijngaard *et al*, 2002b). In general, melanocytes from patients and controls respond to apoptotic stimuli such as UV-B with equal levels of cell death (van den Wijngaard *et al*, 2002b). This is also consistent with an equal extent of resistance to CD95L- mediated apoptosis noted for control and vitiligo melanocytes. Melanocytes can be induced to express cFLIP, yet resistance to CD95L-mediated apoptosis is independent of cFLIP expression.[1] These findings cumulatively indicate that vitiligo melanocytes are no more prone to apoptosis than are control melanocytes.

Interestingly, exposure to bleaching phenols induces increased expression of the A2B receptor for adenosine specifically by epidermal melanocytes, and adenosine receptor expression has been associated with increased susceptibility to apoptosis (Harning *et al*, 1991). At present, it is not known whether depigmentation following exposure involves or circumvents an immune response to melanocytes. Where T cells are present to mediate depigmentation, cytoxicity is likely mediated by a granzyme/perforin mechanism for induction of melanocyte apoptosis in vitiligo (van den Wijngaard *et al*, 2002b). In this respect, perforin expression as well as granzyme expression (**Fig 2**) was observed in marginal skin from progressive vitiligo patients (van den Wijngaard *et al*, 2000).

DEPIGMENTATION AND SERUM AUTOANTIBODIES

The first studies linking vitiligo to autoimmune responses involved the detection of serum autoantibodies to melanocytes in vitiligo patients, with autoantibody levels elevated in patients with progressive disease (Harning *et al*, 1991). Patients exhibited serum autoantibodies reactive with melanocyte antigens (Naughton *et al*, 1983). *In vitro* studies were used to demonstrate that melanocytes are vulnerable to complement-mediated killing and ADCC (Norris *et al*, 1988). Complement-activated killing may occur specifically in vitiligo patients where aberrant expression of complement regulatory proteins, membrane cofactor protein (MCP), and decay-accelerating factor (DAF) leaves melanocytes sensitive to complement-mediated lysis (Venneker *et al*, 1998; van den Wijngaard *et al*, 2002a). *In vivo*, vitiligo serum is capable of reducing melanocyte numbers in xenotransplanted human skin (Gilhar *et al*, 1995). The revelation of multiple intracellular proteins as targets for autoantibodies rendered a causative contribution of antibodies to depigmentation unlikely until the discovery of humoral responses to membrane antigens, such as the melanin-concentrating hormone receptor (MCH-R1) (Kemp *et al*, 2002). Recognition of MCH by neighboring keratinocytes appears to be mediated by a different receptor, leaving epidermal MCH-R1 expression restricted to melanocytes (Burgaud *et al*, 1997). In this respect, the binding of antibodies to MCH-R1 theoretically contributes to depigmentation by the introduction of pigment cell damage as well as by receptor inactivation and subsequent inhibition of melanogenesis. Moreover, the melanosomal antigen TRP-1 can be targeted because it is expressed on the cell membrane during trafficking to the melanosome (van den Wijngaard *et al*,

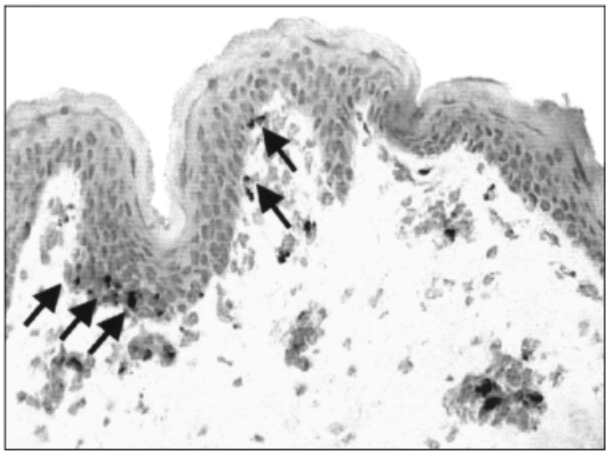

Figure 2. Granzyme expression detected by immunohistochemical analysis of perilesional skin from a patient with progressive, generalized vitiligo. Granzyme-expressing T cells in close proximity to the epidermis, where remaining melanocytes are located, are marked with arrows.

2001; Trcka *et al*, 2002). Thus, a causative role for such autoantibodies to melanocytes in vitiligo etiology is entirely possible.

INFILTRATING T CELLS

T cell infiltrates in vitiligo have long been overlooked because they are minute and can be found spanning the margin only in patients with progressive disease. In exceptional cases of inflammatory vitiligo, in which more immunocytes infiltrate marginal skin surrounding expanding lesions than in generalized vitiligo, these infiltrates are more easily characterized. They have been found to contain CD4 and CD8 T cells as well as CD68+ macrophages, but no B cells (Le Poole *et al*, 1996), and invariably they are seen at the site of depigmentation. Findings for inflammatory vitiligo have been extended as well to patients with generalized vitiligo, who represent the vast majority of vitiligo patients (van den Wijngaard *et al*, 2000). Infiltrates have not been described for segmental or occupational vitiligo, leaving room for further investigation. In inflammatory and generalized vitiligo, cytotoxic T cells are juxtaposed to remaining melanocytes, suggesting a causative role for them in depigmentation.

Exciting new data specifically relating to vitiligo patients further support a causative role for T cells. T cells were propagated from marginal skin of HLA-A2+ progressive vitiligo patients. They were maintained without antigenic stimulation, and resulting cultures were characterized for cytokine profiles, peptide specificity, and cytotoxic activity toward melanocytes. These investigations revealed that both helper and cytotoxic T cells from progressing margins generate predominantly type 1 cytokines, namely IFN-γ and TNF-α, whereas CD8+ T cells frequently recognize the modified MART-1 peptide ELAGIGLTV. This is in accordance with prior data showing elevated MART-1 reactivity in peripheral blood from patients with progressive disease (Ogg *et al*, 1998). Furthermore, the cytotoxicity of CD8+ T cells toward HLA-matched melanocytes was demonstrated (Wankowicz-Kalinska *et al*, 2003).

Candidate antigens to be targeted by T cells in vitiligo were derived from melanoma research, as most T cells infiltrating melanoma tumors were found to respond to melanosomal differentiation antigens that are also expressed by normal melanocytes (Sakai *et al*, 1997). These include tyrosinase, gp100, MART-1, TRP-2, P protein, and, to a lesser extent, TRP-1 (Sakai *et al*, 1997). Because of their discovery as tumor-associated antigens, these differentiation markers have been targeted in numerous immunotherapy trials to combat melanoma (Engelhard *et al*, 2002). As these antigens are shared among normal melanocytes and melanoma tumor cells, it follows that immunotherapy directed against melanoma cells can lead to destruction of normal melanocytes and result in progressive depigmentation, as demonstrated in mice (Overwijk *et al*, 1999; Van Elzas *et al*, 2001).

Specific properties of the melanosome (Marks *et al*, 2003) the organelle harboring the majority of melanoma tumor–associated antigens, may explain the immunogenic nature of these differentiation antigens. Interestingly, the melanosome found in melanocytic cells is very similar to lysosomes of other cell types. As with lysosomes, targeting antigens to the melanosomal compartment increases their immunogenicity (Wang *et al*, 1999). Although presentation of antigens is a feature of professional antigen-presenting cells, melanocytes are capable of presenting antigens in the context of MHC class II molecules (Le Poole *et al*, 1993). Under exceptional conditions, as in melanoma or vitiligo, melanocytic cells become HLA-DR+ and thus present the contents of the melanosome in the context of MHC class II (Le Poole *et al*, 1996; van den Wijngaard *et al*, 2000). These epitopes are likely excluded from peripheral tolerance, so class II–expressing melanocytes can become targets for cytotoxic CD4 T cells or they can activate *in situ* T cell help for activation of adjacent cytotoxic CD8 T cells, thereby breaking tolerance to CD8 epitopes (Overwijk and Restifo, 2000).

Not surprisingly, melanoma-associated skin depigmentation is considered a positive prognostic sign for melanoma patients (Nordlund *et al*, 1983). Skin depigmentation represents an active and effective immune response against melanosomal differentiation antigens that may eliminate distant melanoma metastases (Nordlund *et al*, 1983; Kawakami *et al*, 1998). As explained earlier, melanoma patients treated with experimental immunotherapy that targets melanocyte differentiation antigens can develop vitiligo (Yee *et al*, 2000). T cells infiltrating depigmenting skin were found to be identical to T cells infiltrating adjacent tumors, indicating that the same cells responsible for tumor reduction are also responsible for depigmentation (Becker *et al*, 2002). This leaves the question of whether T cells are similarly responsible for depigmentation when there is no malignancy and thus no obvious reason for breaking tolerance to self antigens.

STRESS AND NEW LESIONS

Prior to the onset of progressive episodes, patients frequently experience trauma to the skin in the form of excessive UV exposure; cuts, bruises or burns; or contact with bleaching chemicals, including parasubstituted phenols encountered in large quantities in occupational settings such as rubber manufacture (O'Malley *et al*, 1988). Stress induces the expression of protective heat shock proteins (HSP) and glucose-regulated proteins (GRP) that bind cellular transcripts and proteins in order to put the cell on hold until the stress passes. We recently reported aberrant expression of such stress proteins, specifically HSP70 and HSP27 in vitiligo skin.[2] Under excessive stress, the cytoprotective effect of stress proteins can be inadequate to prevent cell death and the proteins spill over into the environment. When taken up by passing dendritic cells, stress proteins invoke an immune response specifically to chaperoned peptides and proteins (Srivastava and Amato, 2001). If these proteins are melanosomal antigens, the resulting immune response will be directed to melanocytes. Moreover, several stress proteins, including HSP70 and grp94, can induce dendritic cell activation and enhance uptake of antigens and subsequent T cell activation (Basu *et al*, 2002). Our findings also indicate that stress proteins upregulate expression of ICAM-1 by melanocytes. The significance of this finding is supported by the

[2]Le Poole IC, Curry J, Qin J, Stennett L, Nickoloff BJ. Role for heat shock proteins in the immunopathogenesis of vitiligo. 63rd Annual Meeting SID, Los Angeles CA, abstr. #776, 2002

observation that ICAM-1 expression is found in the basal layer of the epidermis of marginal vitiligo skin (Le Poole *et al*, 1996; van den Wijngaard *et al*, 2000).

A POTENTIAL CONTRIBUTION FOR DENDRITIC CELLS

Dendritic cells were recently reported to be cytotoxic to tumor cells, engaging TNF family members expressed on the dendritic cell membrane to induce apoptosis in their targets (Janjic *et al*, 2002; Lu *et al*, 2002). Specificity for tumor cells is maintained by expression of receptors for TNF, TRAIL, and lymphotoxin-α by malignantly transformed target cells (Janjic *et al*, 2002). Interestingly, upregulation of these receptors was reported for normal keratinocytes after UV-B treatment and such keratinocytes are effectively killed by purified recombinant TRAIL (Maeda *et al*, 2001). It is likely, that melanocytes are similarly vulnerable to dendritic cell–mediated killing under stress. Reduced turnover rates for melanocytes compared to keratinocytes likely dictate harsher consequences for the pigment cell population. Our preliminary observations using melanocyte cell line PIG1 targeted by stress protein-activated dendritic cells support this concept.[3]

VITILIGO AND MELANOMA: FLIP SIDES OF THE SAME COIN?

In vitiligo, an unwanted immune response to melanosomal self antigens is triggered by stress, stimulating immunocyte influx and expression of type 1 cytokines. *De novo* presentation of melanosomal antigens in the context of MHC class II and upregulation of costimulatory molecules on the surface of melanocytes contribute to an immune response directed against melanocytes. An overview of the autoimmune response contributing to skin depigmentation is shown in **Fig 3**. In melanoma, vitiligo is considered a desirable side effect of effective immunity to melanocytic cells. An increase in the abundance of melanocyte differentiation antigens supported by a proinflammatory cytokine environment can overcome tolerance to self antigens. Similar to vitiligo, *de novo* expression of MHC class II may factor into this equation and enhance immunity to melanosomal antigens. Cases have been reported, however, where depigmentation of skin and hair were not accompanied by effective tumor shrinkage (Yee *et al*, 2000). Melanoma tumor cells can develop immune escape mechanisms by downmodulation of MHC class I molecules at the cell surface or reduced processing and presentation of target antigens, leaving tumor cells invisible to infiltrating T cells (Marincola *et al*, 2000). In fact, such immune escape can be the very consequence of IFN-γ secretion by activated T cells (Le Poole *et al*, 2002). Cytokine-induced downmodulation of melanosomal antigens interferes with T cell recognition of melanoma cells. Exposure to IFN-γ does not affect recognition of normal melanocytes, likely because these targets retain above-threshold levels of antigen expression. Thus, IFN-γ secretion may contribute to tumor escape as well as to skin depigmentation through T cell activation and reduced expression of melanogenic enzymes.

In both melanoma and vitiligo, depigmentation involves cytotoxic T cell infiltrates specific to similar antigens (Wankowicz-Kalinska *et al*, 2003b). Future investigations will reveal whether an effector function for dendritic cells contributes significantly to the loss of melanocytic cells in either disease. In general, it is of interest to further characterize the similarities as well as the specific differences among target cells and immunocytes interacting in either disease in order to design effective therapy for both.

[3]Le Poole IC, Curry J, Qin J, Stennett L, Nickoloff BJ. Role for heat shock proteins in the immunopathogenesis of vitiligo. *J Invest Dermatol* 119:337, 2002 (abstr.).

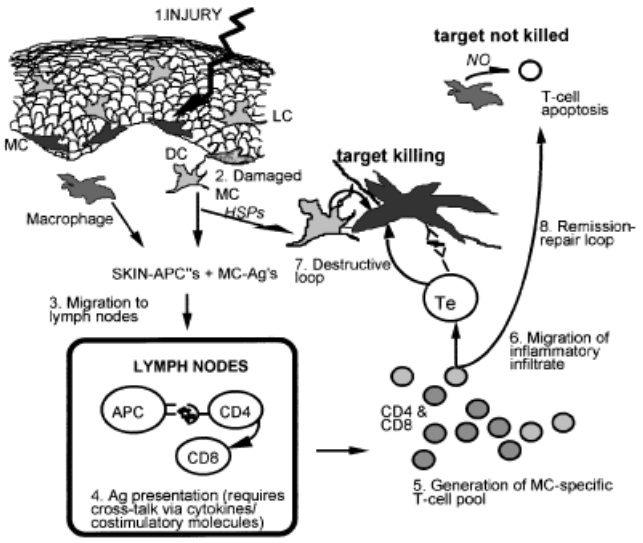

Figure 3. Pathways potentially involved in autoimmune destruction of melanocytes. (1) Injury induced by UV irradiation, mechanical damage, infection, or exposure to bleaching chemicals results in melanocyte damage and upregulation of stress proteins. (2) Damaged melanocytes release heat shock proteins (HSP), inducing expression of TNF family members on dendritic cells and potentiating their cytotoxic ability. HSP can activate dendritic cells to take up and process melanocyte-derived antigens and migrate to draining lymph nodes (3). Presentation of melanocyte-derived antigens to specific effector T cell pools depends on the cytokine environment and costimulation (4). A pool of CLA + T cells and other immunocytes is generated (5). Immunocytes enter the circulation and subsequently migrate toward skin (6). Immunocytes either engage in melanocyte destruction (7) or become inactivated when regulated through a remission-repair loop, inducing T cell apoptosis (8).

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