

# Identification of Melanocytes Antigens in Vitiligo

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Following is a progress report on vitiligo-related research conducted in my laboratory during the past year. These studies were supported in part by the grant we received from the NVFI. The major accomplishments were as follows:

## 1. Elucidating one of the mechanisms by which PUVA may cause repigmentation in vitiligo

PUVA is probably the most effective treatment for vitiligo. Despite its use for this purpose for many decades, the manner in which it works is still unknown. It could work by suppressing immune mechanisms that cause vitiligo; or it could work by directly stimulating melanocyte proliferation and/or indirectly by stimulating these cells by inducing epidermal or dermal cells to release factors that result in melanocyte proliferation. To investigate the latter possibility, sera from stable non-segmental vitiligo (n=8) collected before and following 2 and 4 months of PUVA therapy were incubated at different concentrations (10%, 5%, and 2.5%) with normal neonatal human melanocytes (mel). Sera of active untreated non-segmental vitiligo pts (n=10) and healthy individuals (n=10) were similarly incubated with mel. Proliferation of Me1 was measured by <sup>3</sup>H-thymidine incorporation day 6 of incubation. There was a significant stimulation of Me1 by sera obtained following 4 months of PUVA therapy compared to sera obtained from the same pts prior to therapy, i.e. mean of stimulation index was  $2.97 \pm 1$  vs.  $0.89 \pm 0.9$  at 10% serum concentration respectively ( $p < 0.01$ ). By contrast, there was no difference in proliferation between sera of vitiligo pts collected prior to PUVA, health individuals, or untreated active vitiligo pts., i.e. mean of stimulation index was  $0.89 \pm 0.9$  vs.  $0.32 \pm 0.4$  vs.  $1.1 \pm 0.6$  at 10% serum concentration respectively ( $p > 0.05$ ). Similar but less striking effects were measured at 5% and 2.5% serum concentrations. Proliferation did not result from the presence of 8-MOP or DMSO solvent in the sera of PUVA treated pts., as neither agent significantly affected melanocyte proliferation.

These findings indicate that sera of PUVA treated pts. contain a factor that stimulate melanocyte proliferation and suggest that one of the mechanisms of PWA induced repigmentation in vitiligo is release of circulating factor(s) that induce melanocyte proliferation.

These observations are being submitted for presentation at the annual meeting of the SID.

## 2. Further characterization of "vitiligo" antigens

In prior studies, we have shown that most patients with active vitiligo have antibodies to melanocytes. We suspect these antibodies are involved in the cause of vitiligo because there is a relation between the presence and level of the antibodies and the extent and activity of the vitiligo. The antigens on melanocytes which are targeted by these antibodies may also be important in the pathogenesis of the disease. In earlier studies we have shown that the antibodies are usually targeted to one or more antigens with MWs of approximately 40-45, 74 and 90 kDa which are expressed on the surface of melanocytes. In our most recent study, we have examined whether these "vitiligo" antigens are possibly related to previously known pigment cells antigens. To further characterize these "vitiligo" antigens, we examined their relation to antigens defined by a panel 25 monoclonal antibodies (moab) to pigment cell antigens. We found by immunoprecipitation and SDS-PAGE analysis of <sup>125</sup>I labeled, detergent soluble, human melanocyte macromolecules, that 24 (82h) of 29 pts. with vitiligo had antibodies to one or more, vitiligo antigens vs. 2 (7%) of 28 control individuals. Seventeen of the 25 moabs did not react with any labeled antigen in the same lysate. Of the remaining 8 moabs, only 4 precipitated an antigen that co-migrated with one of the vitiligo antigens. Moab TA99, HMSA-5, and TMH-1 (all directed to a 75 kD tyrosinase-related protein) co-migrated with the 75 kDa vitiligo antigen. Moab W6/32 (directed to class I HLA antigen) co-migrated with the 40-45 kDa vitiligo antigen. Immunodepletion studies with vitiligo antibodies selectively depleted the antigen defined by W6132 but not the antigen defined by TA99 and HMSA-5. The vitiligo antigens were easily labeled by the lactoperoxidase technique but poorly labeled with <sup>35</sup>S-methioine, suggesting they are expressed on the cell surface. These studies indicate that 90 kDa and 75 kDa vitiligo antigens differ from antigens defined by currently available moabs to pigment cell antigens. The 40-45 kDa vitiligo antigen appears to share a cross-reactive epitope, or be tightly bound to, class I HLA antigen.

## 3. Relation between vitiligo and melanoma

Several observations suggest there is a link between vitiligo and melanoma. In particular vitiligo is more common in individuals with melanoma, and the presence of vitiligo improves the survival of patients with melanoma. In addition, there is a particular breed of pigs (Sinclair swine) which is born with melanoma and where the melanoma spontaneously disappears in conjunction with the development of vitiligo. These observations suggest that common links between the two diseases might be due to the presence in both diseases of immune responses to pigment cell antigens.

In an attempt to attack this question, we examined whether anti-pigment cell antibody responses are present in Sinclair swine with regressing melanoma, and if so the relation of these antibodies to the appearance of Vitiligo and progressive melanoma. Thirty-eight sera specimens collected at different times from 13 swine born with melanomas were tested for melanoma antibodies by immunoprecipitation and SDS-PAGE analysis of <sup>125</sup>I labeled swine melanoma macromolecules. Antibodies to melanoma were present in 13 (100%) of the swine vs. in 1 of 3 control swine. The antibodies were directed to antigens with approximate MWs of 45,68-75, or 100 kDa. These antigens were also expressed on human melanomas and normal melanocytes but weakly on only 1 of 5 unrelated tumors. The incidence and level of these antibodies increased with time. Antibodies to the 45, 68-75, and 100 kDa antigens were present in 36%, 55% and 9% respectively of sera collected prior to 7 weeks of age, but in 80%, 100%, and 37% of sera collected between 7-20 weeks ( $p < 0.05$ ). The rise in melanoma antibodies preceded or appeared together with tumor regression and loss of pigmentation. These findings indicate that Sinclair swine with melanomas have antibodies to antigens preferentially expressed on pigment cells, and support the hypothesis that the regression phenomenon and the vitiligo-like skin depigmentation result from immune responses to common antigens shared by normal and malignant swine pigment cells.

#### **4. Relation of vitiligo antigens to melanoma antigens**

To further investigate the link between vitiligo and melanoma, we studied whether patients with vitiligo or with melanoma develop immune responses to similar antigens. We tested 30 pts. with melanoma, 29 pts. with vitiligo, and 28 pts. with unrelated conditions for antibodies to human melanocyte antigens using an immunoprecipitation SDS/PAGE analysis assay. Antibodies to melanocytes were present in 24 (80%) of pts. with melanoma, 24 (83%) of those with vitiligo, and in 2 (7%) of controls ( $P < 0.01$ ). The antibodies in pts. with melanoma or vitiligo were directed to similar antigens with MWs of approximately 40-45, 75, and 90 kDa. The frequency of antibody responses to each of these antigens was similar in both diseases. By sequential immunodepletion the antigens defined by antibodies in both diseases were similar. These antigens were also expressed on melanoma cells.

Thus, most pts. with melanoma or with vitiligo develop antibodies to similar antigen which are present on both melanocytes and on melanoma cells. These findings support the hypothesis that clinical link between the two diseases results from immune responses antigens and shared by normal melanocytes and malignant melanoma cells.

#### **5. Isolation and culture of melanocytes derived from hair follicles**

An unusual aspect of vitiligo is that the melanocytes present in the hair follicles of human skin appear to be relatively spared in this disease. This is readily evidenced by the fact that when re-pigmentation occurs in vitiligo, it is usually seen to begin around the hair follicles and to spread from there. Discovering why hair follicle melanocytes are spared in vitiligo could lead a method of increasing the resistance of all melanocytes to the damaging effects of the disease. As a first step in this direction, we have for the first time been able to isolate hair follicle melanocytes and grow them in purified form in culture.

Normal human scalp was trisected 1 mm below the epidermis and hair follicles in the remaining dermis were isolated by collagenase treatment. Contaminating dermal tissue was removed by exhaustive washing, and single cell suspensions were prepared from hair follicles by trypsin/ethylenediamine tetraacetic acid treatment. After contaminating fibroblasts and keratinocytes were removed, cells with 2 distinct morphologies remained in the cultures and accounted for >99% of remaining cells. These included: large dendritic cells, which were deeply pigmented, did not proliferate, and which disappeared from cultures by the third passage, and small bipolar cells which were initially unpigmented, proliferated very rapidly and became weekly pigmented after approximately 4 months in culture. Both cell types were melanocytes as evidenced by staining with antibodies to S-100 and G<sub>D3</sub>.

The availability of cultured hair follicle melanocytes will facilitate investigations of the role of these cells in abnormal diseases of pigmentation.

#### **6. Induction of vitiligo in animals by passive transfer of anti-melanocyte antibodies**

This was the specific goal of the grant we received from the NVFI. I regret to say that despite considerable effort, we were unable to directly test this hypothesis because of technical difficulties in getting grafts of human skin to take in nude mice. We are continuing our efforts to get such grafts to take, as we are anxious to complete these experiments.