Tenascin is overexpressed in vitiligo lesional skin and inhibits melanocyte adhesion

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Summary

The aetiology of vitiligo remains obscure. In this study, the role of integrins in the observed inability of melanocytes to repopulate lesional skin was investigated. Antibodies directed to α_2 , α_3 , α_5 , α_v , α_6 , β_1 and β_3 integrin subunits were used. Immunohistology revealed no marked differences in the overall levels of expression of integrins between control, non-lesional, perilesional or lesional skin. Moreover, no differences were noted in the level of expression of integrins or the adhesive capacity between cultured control cells derived from three separate donors and vitiligo-derived melanocytes from two donors. Rather, it was clearly observed that towards the lesion, vitiligo skin contains increasing amounts of tenascin in the basal membrane and papillary dermis in five patients employing T2H5 antihuman tenascin antibody. The anti-adhesive effect observed *in vitro* for this extracellular matrix molecule using normal melanocytes may contribute to loss of pigment cells in vitiligo or to ineffective repopulation of the lesions.

In vitiligo, melanocytes are gradually lost in depigmenting lesions of the skin.¹ It has been suggested that defective adhesion is involved in loss of melanocytes in this pigmentary disorder.² Moreover, the persistence of depigmented lesions is suggestive of defective melanocyte migration which may be reversed by photochemotherapy,² and therefore baseline expression of adhesion molecules may differ in vitiligo as compared with control skin. Melanocyte adhesion, spreading and migration is mediated by integrins.³ Employing antibodies to integrin subunits, the specific integrins involved in each process have been identified *in vitro*.^{3,4}

Importantly, all integrins thus far identified on melanocytes can also be found on neighbouring keratinocytes. Although immunohistology has been employed to identify integrins expressed on melanocytes *in situ per se*,³ this may not be the method of choice to determine differences in the level of expression by melanocytes. Even by double immunostaining procedures, it is difficult to distinguish between integrin expression by melanocytes vs. neighbouring keratinocytes. Therefore, *in vitro* cultures derived from control as well as from vitiligo non-lesional skin were employed to

investigate further potential differences in adhesive behaviour in vitiligo and control skin. In addition, the use of cultured cells provides the opportunity to perform functional adhesion assays. In this respect it is of great interest to note that cultured melanocytes from non-lesional vitiligo skin appear to retain disease-related characteristics. It has been demonstrated that vitiligo melanocytes have altered growth characteristics under limiting conditions.⁵ Moreover, upon electron microscopic observation, dilated rough endoplasmic reticulum (RER) is apparent in diseased cells from human and mouse vitiligo subjects.^{6,7} Therefore, the use of cultured cells to investigate differences in adhesive characteristics is justified.

The level of expression of relevant integrin subunits by vitiligo melanocytes was compared with baseline expression by control melanocytes. As integrins require activation prior to mediating their respective adhesive functions,⁸ potential differences in intrinsic adhesive behaviour were also investigated. The ability to adhere to different extracellular matrix (ECM) molecules, namely collagen IV, laminin, fibronectin, hyaluronic acid and tenascin was assessed using ⁵¹Cr release as a measure of the fraction of cells that have adhered. Results of these experiments illustrate the importance of the composition of the ECM for the adhesive

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behaviour of cells. In this respect, it has been reported that the concentration of tenascin can increase many-fold in pathological conditions. In skin, elevated tenascin content has been observed during tumorigenesis and wound healing.^{2,9} Moreover, others have reported the abundance of this ECM molecule in vitiligo skin in a preliminary investigation,¹⁰ whereas this ECM molecule may interfere with melanocyte adhesion.^{11–13} Consequently, the tenascin content of the basal membrane in non-lesional, perilesional and lesional vitiligo skin was compared by immunohistology. The combination of functional assays and immunohistological examination provides insight into the role of adhesive mechanisms in the aetiopathology of vitiligo.

Materials and methods

Skin sections

Expression of integrin receptors was evaluated in non-lesional, perilesional and lesional limb skin from three female Caucasian patients with progressive ($n=2$) or stationary vitiligo prior to medication (mean age 34 years) and compared with expression in control foreskin (16 years). Absence of melanocytes in lesional skin was confirmed as described previously¹ using antibody NK1-beteb.¹³ Four millimetre biopsies were obtained and snap-frozen in liquid nitrogen. Six micrometer frozen sections were fixed for 10 min in cold acetone and stored at -20°C until use. Similarly, the presence of tenascin was evaluated in non-lesional, perilesional and lesional skin from five generalized vitiligo patients with progressive depigmentation. Biopsies were taken either from the limbs ($n=4$) or from the trunk. The patient group of four males and one female had a mean age of 45 years and an average duration of 13 years of vitiligo.

Melanocyte culture

Melanocytes were isolated from blister roof tops¹⁴ or from surgical specimens¹⁵ essentially as previously described. The culture medium consisted of Ham's F10 (Gibco Life Technologies, Breda, the Netherlands) with 1% v/v Ultrosor G (Gibco), 10 ng/mL tetradecanoyl phorbol 13-acetate (Sigma-Aldrich, Zwijndrecht, the Netherlands), 0.1 mmol/L 3-isobutyl-methylxanthine (Sigma), 100 IU/mL penicillin/100 mg/mL streptomycin (Gibco Life Technologies) and 2 mmol/L glutamine (Gibco Life Technologies). Contaminating cell types were selectively eliminated by addition of 100 $\mu\text{g}/\text{mL}$ geneticin (Gibco Life Technologies) to the culture

medium for 3 consecutive days.¹⁶ Elimination of contaminating cell types was confirmed by phase contrast light microscopy as well as by fluorescence-activated cell scanning (FACS) analysis (see below) using antibody NK1-beteb^{13,16} as the primary antibody. Control melanocyte cultures used in this study were derived from foreskin ($n=2$) or abdominal skin ($n=1$) from donors of varying pigmentation with a mean age of 16 years. Vitiligo melanocytes were derived from Caucasian non-lesional limb skin. Patients had a mean age of 38 years at the time of cell isolation.

Immunohistology

To detect expression of integrin subunits, frozen skin sections were incubated with a primary antibody in predetermined optimal dilution for an hour. Subsequently, peroxidase-labelled rabbit antimouse antiserum (Dakopatts, Glostrup, Denmark) was applied 1 : 100 for 30 min. To enhance the signal, peroxidase-labelled swine antirabbit antiserum (Dakopatts) was applied 1 : 50 for 30 min. Finally, aminoethylcarbazole (AEC) (Sigma) dissolved in acetate buffer pH 4.9 was used to reveal the presence of the antigen under study. Non-specific staining was prevented both by pre-incubation with 10% normal swine serum and by washing with Tris(hydroxymethyl)aminomethane-buffered saline (TBS) throughout the staining procedure. For the α_2 and α_3 integrin subunits, double stainings with antibody NK1-beteb specific for the gp100/Pmel17 protein of melanocytes were performed to demonstrate loss of melanocytes in conjunction with levels of integrin expression.

In the skin, the double-staining procedure was performed as follows. A mixture of primary antibodies in optimal dilutions for single stainings was applied to skin sections for 1 h at room temperature. After thorough washing, a mixture of secondary antibodies was applied for 1 h at room temperature. The mixture consisted of peroxidase-labelled goat antimouse IgG2b and alkaline phosphatase-labelled goat antimouse IgG1 (1 : 20 each, Southern Biotechnology, Birmingham, AL, U.S.A.). Alkaline phosphatase was localized with Fast Blue BB (Sigma), followed by revealing peroxidase activity using AEC as mentioned under single stainings.

Alternatively, for tenascin single immunostainings skin sections were pretreated with 10% normal goat serum (Dakopatts) for 15 min to block aspecific binding of primary antibodies. Primary antibody T2H5¹⁷ was optimally diluted in 10% normal human serum in TBS. Skin sections were incubated for 1 h at room temperature with the primary antibody at relevant dilutions

in TBS. In the second step, mouse monoclonal antibodies were recognized by biotin-labelled F(ab)2 rabbit-antimouse antiserum (Dakopatts) used 1:200. To improve antigen detection, peroxidase labelled biotin and streptavidin were mixed according to the manufacturer's protocol of a Dakopatts streptABC detection kit. Peroxidase activity was detected as above. Antibodies used for *in situ* detection of integrin subunits α_2 ,¹⁸ α_3 ,¹⁹ α_5 ,²⁰ α_6 ,²¹ and β_1 ,²² are further described in Table 1. Pre-evaluated optimal antibody concentrations used for immunohistology were on average 2.5 times those used for FACS analysis. Abundance and intensity of staining was evaluated by two investigators independently. The abundance of subepithelial tenascin was determined as the average thickness of the subepithelial layer immunostained by T2H5. Quantification was performed by image analysis using a Zeiss Interactive Digital Analysis System (ZIDAS) (Zeiss, Thornwood, NY, U.S.A.). Abundance was measured as the subepithelial immunostained surface area (mm²) divided by length of the dermoepidermal junction (mm²).

FACS analysis

Cultured adherent cells were scraped for three-step indirect immunofluorescent stainings. Cells were incubated with optimally diluted primary antibody in phosphate-buffered saline (PBS) containing 10% normal human serum and 0.01% azide for 1 h at 4 °C. Cells were washed in 2% fetal calf serum (FCS) containing PBS with 0.01% azide. In the second step biotin labelled rabbit antimouse antiserum (Dakopatts) 1:50 was added for 30 min, followed by phycoerythrin labelled streptavidin (Dakopatts) 1:25 for 30 min. Fluorescence was measured in 5000 cells per sample by FACS analysis employing a Becton Dickinson FACS-can (Lincoln Park, NJ, U.S.A.) and evaluated by the Becton Dickinson LYSIS II computer program. Anti-

bodies directed to integrin subunits α_2 ,¹⁸ α_3 ,¹⁹ α_5 ,²⁰ α_v ,²³ α_6 ,²¹ β_1 ,²² β_3 ,²⁴ and β_4 ,²⁵ are described further in Table 1.

Adhesion assays

Cultured melanocytes from control and vitiligo donors as described under 'melanocyte culture' were used to perform adhesion assays. Adhesion assays were performed as described previously by Danen *et al.*²⁶ and briefly recapitulated below. Ninety-six well flat-bottom microtitre plates were coated with 20 µg/mL of one of the following: human plasma-derived laminin (Sigma), human foreskin fibroblast-derived fibronectin (Sigma), human placental collagen IV (Sigma), rooster comb-derived hyaluronic acid (Sigma) or human glioblastoma cell line-derived tenascin (Sigma). Wells coated at 4 °C overnight were washed in PBS containing 0.25% bovine serum albumin (BSA). For assays where the inhibitory effect of tenascin for melanocyte adhesion was to be determined, some wells precoated with fibronectin were subsequently coated with tenascin as mentioned above.

Fibronectin was chosen as a model ECM component to investigate potential inhibition of adhesion by tenascin because melanocytes readily adhered to fibronectin. Therefore, a potential inhibitory effect is clearly detected. Cultured cells were detached using 0.02% ethylene diamine tetraacetic acid (EDTA) in PBS and labelled with 500 µCi Na₂CrO₄ in a volume of 100 µL Dulbecco's modified Eagle's medium (DMEM) containing 0.25% BSA for 90 min at room temperature. Washed cells were allowed to adhere for 75 min at 2 × 10⁴ cells per well at 37 °C and 5% CO₂. Non-adherent cells were removed by washing with PBS containing 0.25% BSA. Attached cells were lysed by addition of 1% Triton-X-100 in PBS. Radioactivity of the supernatant was measured in a gamma counter. Percentage

Table 1. Antibodies to integrins used in this investigation

Integrin subunit	Antibody	Reference	Source	Dilution FACS
α_2	10G11	18	Dr C.G.Figdor, Nijmegen University, the Netherlands	1:25
α_3	J143	19	Dr C.G.Figdor	1:50
α_5	SAM1	20	Dr C.G.Figdor	1:50
α_v	NKI-M7	23	Dr C.G.Figdor	1:50
α_6	GoH3	21	Dr A.Sonnenberg, NKI, Amsterdam, the Netherlands	1:2.5
β_1	4B4	22	Telios, San Diego, CA, U.S.A.	1:100
β_3	C17	24	Dr A.Sonnenberg	1:25
β_4	3E1	25	Telios	1:25

Figure 1. Double immunostaining to detect the expression of the α_3 integrin subunit by antibody J143 (shown in blue) as well as the presence of melanocytes using the highly specific antibody NKI-beteb (shown in red) in non-lesional (N), perilesional (P) and lesional (L) skin. Loss of melanocytes in lesional skin is apparent. Moreover, the difficulty to differentiate between integrin expression by melanocytes vs. expression by neighbouring keratinocytes is demonstrated. (Original magnification 100 $\times$.)

adhesion was determined as 100 $\times$ the mean value obtained from five wells/total cell count. PBS/BSA-coated wells were used to determine baseline adhesion values.

Results

Expression of integrin subunits in situ

Integrin subunits α_2 , α_3 and α_6 were expressed in the basal layer of the epidermis in frozen sections obtained from control skin as well as in sections obtained from non-lesional, perilesional and lesional skin obtained from three vitiligo patients. No *in situ* expression of α_5 was observed.

In Fig. 1, a representative α_3 (blue)/melanocyte (red) double staining is shown. No difference in the level of integrin expression is apparent between non-lesional, perilesional and lesional vitiligo skin. The loss of melanocytes towards lesional skin is represented by loss of NKI-beteb immunostaining in lesional skin. In the perilesional skin from this progressive vitiligo lesion, remnants of melanocytes were observed. Similar results were obtained in α_2 /melanocyte double stainings. Similarly, with antibodies to α_6 and β_1 no differences in the levels of integrin expression were observed among non-lesional, perilesional and lesional vitiligo skin.

Integrin expression by cultured melanocytes

Aberrations in the level of expression of integrins specifically in melanocytes were investigated by FACS analysis. In Fig. 2, baseline expression of integrin subunits is compared between control and vitiligo melanocytes. It must be noted that among antigens, differences in mean

Figure 2. Baseline expression of integrin subunits by control (three cultures) and vitiligo (two cultures) melanocytes, represented as mean fluorescence intensities $\pm$ standard error of the mean (SEM).

Figure 3. Adhesion to extracellular matrix components by control (three cultures) and vitiligo (two cultures) melanocytes, represented as the percentage of cells that have adhered $\pm$ SEM.

fluorescence intensities may reflect differences in affinity of the antibody for the corresponding antigen. No significant difference in the level of expression of any of the integrin subunits under study was observed upon comparison of control and vitiligo cells.

Adhesion of control and vitiligo melanocytes to extracellular matrix components

As shown in Fig. 3, no intrinsic difference in adhesive capacity to fibronectin, laminin, collagen IV or hyaluronic acid was observed between vitiligo and control melanocyte cultures. In culture, melanocytes adhere

Figure 4. The effect of tenascin on neonatal melanocyte adhesion to fibronectin. Results from two representative experiments, represented as the average percentage of adherent cells in five wells $\pm$ standard deviation (SD). FN: fibronectin-coated wells, FN/TN: wells coated with fibronectin and tenascin. TN: tenascin-coated wells. BSA: mock-coated wells, pretreated with bovine serum albumin. In one experiment, a fibronectin to tenascin ratio of 1:1 w/w was used, whereas in the other experiment shown the tenascin concentration used for coating was increased fivefold.

most readily to ECM component fibronectin. However, laminin and collagen IV also stimulate adhesion as compared with uncoated plastic. Adhesion to hyaluronic acid-coated plastic was comparable with that observed to uncoated plastic. In Fig. 4, the adhesive capacity of melanocytes to fibronectin in the presence and absence of tenascin is compared at two different tenascin concentrations. Evidently, tenascin inhibits adhesion of melanocytes to fibronectin to an extent increasing with rising tenascin concentration. Importantly, percentages of adherent cells can vary per experiment as a function of several experimental variables beyond the scope of this investigation. Thus, results are best compared within experiments.

Tenascin in vitiligo skin

In Fig. 5, the average thickness of the tenascin-containing layer beneath the epidermis is representative of the abundance of tenascin in control vs. vitiligo skin. Interestingly, in all progressive vitiligo patients, the abundance of tenascin increases towards the lesion. Moreover, the increase in tenascin abundance tends to be inversely correlated to the duration of the disease. Figure 6 shows a representative example of T2H5 immunostaining in non-lesional, perilesional and lesional skin from a vitiligo patient, demonstrating an increase in tenascin content of the papillary dermis towards the lesion.

Figure 5. Quantification of tenascin in non-lesional (n), perilesional (p) and lesional (l) vitiligo skin relative to expression in non-lesional skin. The duration of vitiligo is noted for the patients under study.

Figure 6. Tenascin expression in non-lesional (N), perilesional (P) and lesional (L) vitiligo skin, revealed by T2H5 immunostaining (original magnification 50×).

Discussion

From this investigation it can be concluded that there is no overall difference in the level of expression of integrins at the cell surface of non-lesional vitiligo and control melanocytes. In addition, functional studies did not reveal any significant difference in adhesive capacity between non-lesional vitiligo melanocytes vs. melanocytes derived from healthy control skin. These findings are in accordance with the fact that no difference was observed in migratory capacity of melanocytes

in the epibolic outgrowth of explants cultured from non-lesional vs. control human skin.²⁷ As no intrinsic aberrations were observed in the function of integrins on the vitiligo melanocyte cell surface, it follows that conclusions drawn from adhesion and migration studies performed employing control melanocytes will also apply to melanocytes derived from vitiligo patients.

This is of major importance as for repigmentation, melanocytes surrounding vitiligo lesions and melanocytes from hair shafts²⁸ need be stimulated to re-enter vitiligo lesional skin. Observations that LTC-4 and nerve growth factor (NGF) stimulate melanocyte migration²⁹ are, thus, of great interest for the development of new therapeutic modalities. However, results presented here concerning the abundance of tenascin in vitiligo skin also indicate that vitiligo lesions present a hostile environment for melanocytes. Tenascin has been described as an anti-adhesive molecule which is explained at least in part by its ability to bind and/or shield molecules otherwise mediating adhesion of several cell types.^{30–32} The presence of tenascin has been suggested to augment dispersion of the malignant derivative of melanocytes, thereby contributing to metastasis of malignant melanomas.⁹ In this study it was observed that vitiligo patients exhibited an increase in tenascin content of the ECM in contact with epidermal melanocytes. In addition, it was shown that tenascin markedly inhibits adhesion of melanocytes to fibronectin. Interestingly, as it has been reported that the composition of the ECM can influence melanogenesis,³³ it appears that other aspects of melanocytes in addition to adhesion may be affected by the observed increase in tenascin content.

It is yet to be determined whether the observed tenascin increase in vitiliginous skin precedes loss of melanocytes or occurs as a consequence of local immune reactivity. Cytokines produced as a consequence of local immune reactivity may contribute to an accumulation of tenascin. Evidence is accumulating to support the role that cell-mediated immunity is involved in the aetiopathogenesis of vitiligo.^{35,36} This is most overtly observed in inflammatory vitiligo.³⁷ In this respect it is of interest to note that, in inflammatory vitiligo patients, the tenascin increase is reportedly most marked within the inflammatory rim, whereas in generalized vitiligo patients, the increase can be greatest in the lesional area. This suggests that tenascin induction is a relatively late event and may interfere with repigmentation rather than induce depigmentation. The cell type responsible for the observed overproduction remains obscure. In this respect it is of interest that

elevated concentrations of transforming growth factor- β (TGF- β) can induce tenascin synthesis in fibroblasts.³⁷ However, tenascin produced by fibroblasts would be likely to result in overproduction in the reticular dermis rather than in the basement membrane. It appears to be highly unlikely that melanocytes are responsible for the observed increase, as the highest tenascin content is observed in lesional skin, where melanocytes are absent.¹ Moreover, using melanocytes cultured from several donors, no tenascin production was observed by Western blotting, ELISA assays or immunohistology (results not shown). This is in accordance with observations reported by Herlyn *et al.* concerning a lack of tenascin production by melanocytes.⁹ Considering the location of the increased tenascin content observed in vitiligo, keratinocytes constitute the most likely source of tenascin production in vitiligo perilesional and lesional skin. This is supported by the observation that keratinocytes are capable of tenascin synthesis.^{38–40} It can, therefore, be speculated that the data presented here are in accordance with the hypothesis that keratinocytes are involved in the aetiopathogenesis of vitiligo.

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