RESEARCH ARTICLE

Pathological Adhesion of Primary Human Schwannoma Cells is Dependent on Altered Expression of Integrins

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Mutations in the tumor suppressor gene coding for merlin cause Neurofibromatosis type 2 (NF2), all spontaneous schwannomas, and a majority of meningiomas. Merlin links transmembrane proteins to the cytoskeleton. Accordingly, primary human schwannoma cells lacking merlin show an increased number of lamellipodia and filopodia as well as increased cell spreading. We show enhanced adhesion in primary human schwannoma cells and present evidence that this is dependent on the integrin chains α6β1 and α6β4. We further demonstrate that the integrin chains β1 and β4 are upregulated in schwannomas using different complementary methods, and report higher expression of these integrins per schwannoma cell by fluorescence assisted cell sorting (FACS). Finally we report clustering of the integrin chains α6, β1, and β4 on schwannoma cells. Our findings fit well into recent data on the role of merlin in signaling cascades connected to integrins and help explain pathological ensheathment of extracellular matrix or pseudomesaxon formation which is a hallmark of schwannoma histopathology.

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Introduction

Schwannomas are the hallmark of Neurofibromatosis type II (NF2), a cancer predisposition syndrome inherited on an autosomal dominant basis. NF2 is also characterized by the development of other benign tumors within the nervous system, especially meningiomas and gliomas. NF2 tumors, as well as all sporadic schwannomas and most sporadic meningiomas (13, 21, 25, 33, 53) have been found to develop following mutations in both alleles of the NF2 gene (41, 49), coding for the tumor suppressor merlin/schwannomin. This tumor suppressor is then not detectable at protein level in pathological tissue (3, 29, 42, 51). Merlin is a member of the protein 4.1 superfamily, and presents high degree of homology with the ERM (ezrin, radixin, moesin) cytoskeleton-cell membrane linker proteins. The homology is highest at the N-terminus (50), which is suggested to serve as a connection site to transmembrane proteins. Merlin does however not present the common actin binding site of the ERM proteins, but shows instead a weak actin binding site at the N-terminus as well as binding sites for tubulin and fodrin (8, 22, 45, 52). Similarly to the ERM proteins, merlin has been colocalized with actin to the motile region of the cell (19, 43, 44). As described for the ERM proteins (19), merlin is therefore likely to play a role in cell-cell and cell-substrate adhesion, probably via the regulation of actin filament and plasma membrane interactions (48). This data suggests a regulatory function of merlin in cell movement, shape and adhesion Interestingly it has been shown that the overexpression of merlin in a rat JS RT schwannoma-like cell line indeed leads to decreased adhesion of these cells (23).

Adhesion to the extracellular matrix plays an important role in the regulation of cell growth, differentiation, and survival of many cells including Schwann cells (31). Thus, we hypothesized that altered adhesion might be important to schwannoma development. In line with this assumption, primary human schwannoma cells, show greater cell size and increased cell spreading as well as higher numbers of lamellipodia, filopodia, and membrane ruffles compared to normal Schwann cells (37, 40). One of the most interesting and best characterized family of adhesion proteins in Schwann cells is the integrin family. Interestingly, one integrin chain, integrin β1, has been shown to interact with merlin (36). In vivo integrins form heterodimers consisting of an α- and a β-chain to act as receptors mostly for proteins of the extracellular matrix, and receptors including the β1 and β4 chain have been shown to be expressed at different levels depending on the differentiation of Schwann cells (11, 14-16, 27, 35, 38, 47). Further, integrins are involved in the signal transduction to the cell interior. Here we show increased adhesion in primary human schwannoma cells. Screening for regulated adhesion molecules we found and then confirmed increased expression of the integrins β1 and β4 both at mRNA (cDNA array analysis, quantitative RT-PCR) and protein

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Schwannoma	RNA analysis LightCycler	Protein Western	Protein FACS	expression immunocytochemistry	Adhesion assay
NF 1001_1	■			■	■
NF 355	■			■	■
NF 373	■	■	■	■	■
NF AK		■	■		
NF BI		■		■	
NF GZ3	■			■	■
NF KG	■	■		■	■
NF Reg 2	■	■	■	■	
NF Reg 3	■	■		■	■

Normal nerve	RNA analysis LightCycler	Protein Western	Protein FACS	expression immunocytochemistry	Adhesion assay
MOS 0901_1	■			■	■
MOS DK				■	
MOS GZ2	■			■	■
MOS GZ3	■	■		■	
MOS GZ4	■	■		■	■
MOS GZ5	■		■	■	■
MOS GZ6	■		■		
MOS GZ7	■		■		
MOS GZ8	■	■	■	■	
MOS KA		■		■	
MOS LS	■	■		■	■

Table 1. List of normal nerves and tumors used in this study. The use of the normal nerve and tumor material for each level of analysis is indicated by the gray shadowing, thus establishing that most tumors and nerves were used in three or more complementary experiments.

level (Western blotting, FACS). Furthermore we present evidence that the increased adhesion of schwannoma cells is indeed dependent on the integrins α6, β1, and β4 in vitro. We also show that these integrins are expressed on the schwannoma cell surface in vitro where they form clusters, further indicating that these integrin receptors are functionally important in schwannoma cells. By immunohistochemical staining we were finally able to confirm an identical switch in integrin expression in vivo as that found in vitro, with a high and atypical expression of the β4 chain in the non-myelinating schwannoma cells. This we suggest may also explain pathological ensheathment of extracellular matrix found in schwannomas.

Materials and Methods

Preparation of human Schwann cell cultures. Normal human Schwann cells and schwannoma cells were isolated as previously described (24, 40). Peripheral nerves were obtained from surgical patients not carrying any disease predisposing to a peripheral neuropathy. Schwannomas were kindly provided by NF2 patients after informed consent. Diagnosis of NF2 was based on clinical criteria defined by the NIH Consensus Conference on Neurofibromatoses (1). Cells were collected and resuspended in proliferation medium: DMEM, 10% FCS, 0.5 μM forskolin, 10 nM β1-heregulin$_{177\text{-}244}$ (Mark Sliwkowski, Genentech, San Francisco, Calif), 0.5 mM 3-isobutyl-1-methylxanthine (IBMX, Sigma), 2.5 μg/ml insulin (Sigma). Cells were seeded into 6-well plates or

8-well chamberslides (Nunc, Rochester, NY), coated with 1 mg/ml poly-L-lysine (Sigma) and 4 μg/ml natural mouse laminin (Gibco), at a density of 10000 cells/cm^2. Proliferation medium was changed every 3 to 4 days and cells were passaged when confluent or at the latest after 8 days in culture.

Total number of nerves and tumors, purity of Schwann cell cultures, and passage numbers. For this study a total of 9 different schwannomas and 11 different normal nerves were used (Table 1). All RNA and protein preparations were carried out using cells in the second to fourth passage, as then the number of contaminating fibroblasts is negligible (less than 2%) (40). The number of fibroblasts was routinely checked by S100 immunocytochemistry.

Adhesion assay. Two thousand cells were seeded in a 50-μl drop on small areas of pre-coated plastic petri dishes for 3 hours in a humidified atmosphere with 10% CO_2 (34). For blocking experiments anti-integrin β1, anti-integrin β4 (Santa Cruz, Calif) or anti-P0 (all 1 μg/ml) (kind gift from J. Achelos, Graz) was added to the medium used to seed the cells. The adhesion assay was stopped by washing off loosely attached cells. The attached cells were then fixed with 4% PFA/PBS. Adhesion was quantified by counting all attached cells under phase microscopy (500-1200 cells per experiment, n = 5). Results were expressed as a percentage of the number of schwannoma cells left adhering to the PLL/Lam substrate.

RNA preparation. Total RNA isolation was performed as previously described (4) and homogenizing by application on Qiashredder (QIAGEN, Hilden, Germany), followed by treatment with DNase I. RNA integrity was checked on an agarose gel.

Expression analysis by cDNA array. For cDNA array (Human 1.2 Atlas Array, Clontech, Heidelberg, Germany) experiments, probe synthesis was performed using the Clontech Probe Synthesis Kit according to the manufacturer's protocol except that the MMLV reverse transcriptase was replaced by Superscript (Gibco). (^{32}P)dATP labeling of the cDNA synthesized was measured using a beta counter following the purification by ChromaSpin columns (Clontech). Hybridized filters were exposed on a phosphor intensifying screen (Molecular Dynamics, Freiburg, Germany) and scanned at three time points (day 1, 3, and 8). Data were analyzed using ImageQuant software (Amersham, Freiburg, Germany) and arrays were normalized against the house keeping genes dotted on the membrane, for significance a doubling of the signal intensity was requested. The experiment was repeated twice, using RNA of different normal Schwann and schwannoma cells.

Quantitative RT-PCR. One μg total RNA was used for cDNA synthesis using the First Strand cDNA Synthesis Kit for RT-PCR (Roche, Mannheim, Germany). To examine cDNA synthesis efficiency, a PCR of a house keeping gene (histone H2Az) was performed. For LightCycler reactions the LightCycler Fast Start DNA Master SYBR Green I Kit (Roche) was used according to the manufacturer's protocol. Four mM $MgCl_2$ and 0.15 μM primer were used. For normalization a house keeping gene (huHPRT) was run with every reaction.

The integrin primers (TIB molbiol, Berlin, Germany) used were the following: α6-sense, 5′-CAGTT GAGTT GTGTT GCCAA-3′; α6-antisense, 5′-GAAGG TTTAG CAACT CCCG-3′; β1-sense, 5′-GTGGT TGCTG GAATT GTTCT TA-3′; β1-antisense, 5′-AGTGT TGTGG GATTT GCAC-3′; β4-sense, 5′-ATAGA GTCCC AGGAT GGAGG A-3′; and β4-antisense, 5′-GTGGT GGAGA TGCTG CTGTA-3′.

The LightCycler program was the following: 95°C, 10 minutes; 95°C, 10 seconds; 55°C, 8 seconds; and 72°C, 14 seconds for 45 cycles. Melting curves were examined for product control and crossing points were used for expression analysis. Experiments were repeated 4 times independently, using different pairs of RNA from normal Schwann and schwannoma cells.

Western blotting. Protein was prepared from subconfluent cells using a denaturing lysis buffer (1% TritonX-100; 20mM Tris-HCl (pH7.5), 150mM NaCl, 1mM MgCl2, 2mM EDTA, 10% glycerol, 0.1%SDS, 1% sodium deoxycholate, 1:500 small peptide inhibitor mix (Sigma), 1:100 100mM PMSF stock) and concentrations were determined with a detergent compatible protein assay (Biorad, Munich, Germany) according to the manufacturer's protocol. Detection of a house keeping gene such as actin or GAPDH could not be used as a loading control as they are differentially regulated (cDNA-array, data not shown). Thus equivalent loading was further confirmed by coomassie gel stains (Biorad) prior to western blotting, and colloidal gold stains (Biorad) of the membranes thereafter. Proteins (5 μg total cell lysate per lane) were separated by SDS-Page on a 8% polyacrylamid gel. Blocking was done in TBS, 0.1% Tween, 5% milk, 2% BSA, the membranes were incubated with either goat polyclonal anti-α6 integrin

1:100, rabbit polyclonal anti-β1 integrin 1:200, rabbit polyclonal anti-β4 integrin 1:500, rabbit polyclonal anti-NF2 (C18) 1:250 antibodies (Santa Cruz) in blocking solution for 2 hours at room temperature and incubated with appropriate secondary antibodies. ECL (Amersham) was used for detection. Each experiment was repeated at least 3 times, each time using a different pair of normal Schwann and schwannoma cells.

Fluorescence assisted cell sorting analysis (FACS). Cells were resuspended in ice-cold FACS-PBS (PBS/1% BSA/0.1% sodium azide) to a concentration of 7×10^5 cells/ml, and kept on ice. Ten μl of anti-integrin α6 (c = 0.025 μg/μl), β1 (c = 0.2 μg/μl), β4 (c = 0.025 μg/μl) antibodies (Becton-Dickinson, Heidelberg, Germany) diluted in 20 μl of FACS-PBS were added to each sample, and incubated in the dark at 4°C for 30 minutes. Control probes for auto-fluorescence, and propidium iodide staining were analyzed. Data was acquired on a FACS scan (FacsCalibur-Becton-Dickinson, Heidelberg, Germany), further analyzed with Cellquest (Becton Dickinson). Dead cells were excluded from the results. For statistical analysis, the fluorescence values on a logarithmic scale, were converted into a linear scale with 256 channels: $64 \times \log_{10}$ (peak fluorescence value) = linear peak channel number.

This experiment was repeated 4 times, using different pairs of normal Schwann and schwannoma cells.

Immunocytochemistry. Cells on 8-well chamberslides were washed with PBS and blocked in 2 mg/ml BSA/PBS for 10 minutes at room temperature. Incubation with the first antibody (goat polyclonal anti-α6, rabbit polyclonal anti-β1, and rabbit polyclonal anti-β4, all 1:100 in BSA/PBS, Santa Cruz) was carried out for 30 minutes at 4°C, followed by 3 washing steps with BSA/PBS and fixation in 4% PFA. Secondary antibodies (RAG-Cy3 1:600, GAR-Cy3 1:800, GAR-Cy2 1:600, Dianova, Hamburg, Germany) were added for 40 minutes at room temperature, followed by counterstaining with DAPI (Sigma). Cells were coverslipped using VectaShield mounting medium (Vector Laboratories, Burlingame, Calif) and stored at 4°C. As control the first antibody was omitted once in every experiment. Analysis of the cells was carried out on a Zeiss Axiovert Fluorescence microscope (Zeiss, Göttingen, Germany), and all pictures were taken through a ×63 Zeiss Plan Neofluar objective.

Immunohistochemistry. Normal nerve sections were kindly provided by AAWM Gabreels-Festen (Nijmegen), schwannoma frozen material by Laura Papi (Florence). The latter were cut to 12-μm thick sections on a Leica cryotome, dried on poly-L-lysine slides and stored at -80°C. Defrosted tissue sections were fixed in acetone and immunostained for integrin α6 (1:200), integrin β1 (1:500), and integrin β4 (1:2000) (all SantaCruz) with routine immunohistochemical methods, using avidin-biotin complex reagents (Vector Laboratories, Burlingame, Calif), 3, 3′-diaminobenzidine chromagen and hematoxylin counterstain. Secondary antibodies (goat anti rabbit biotin, rabbit anti goat biotin [Vector]) were diluted 1:200. Two different normal nerves and 2 schwannomas from different patients were used; experiments were repeated 2 to 3 times.

Statistical methods. Paired (related) 2-tailed t-tests were used. In each case we compared Schwann and schwannoma cells, the differing variable between populations being per definition presence or absence of merlin. In the case of the adhesion assay, we compared either normal Schwann cells with schwannoma cells under the same conditions, or schwannoma cells with each other under differing antibody treatments. Results were considered significant when $p \leq 0.05$.

Results

Increased adhesion of schwannoma cells. The in vitro (37, 40) and in vivo (10, 12, 18) data describing the altered morphology of human schwannoma cells prompted us to hypothesize that the adhesive characteristics of schwannoma cells must be different from those of normal Schwann cells. To test this hypothesis, we performed a comparative adhesion assay with normal Schwann and schwannoma cells. We found the number of Schwann cells (normal and tumoral) adhering to poly-L-lysine (PLL) coated dishes to be much lower (only 30%, data not shown) than that attaching to PLL/laminin (Lam). This was to be expected as laminin is part of the extracellular matrix in vivo, whereas PLL is not. Comparing the normal Schwann cells and schwannoma cells seeded on PLL/Lam coated dishes, we found a difference in their adhesive characteristics. Schwannoma cells had already started to spread out when incubation on the dish was stopped after 3 hours, while normal Schwann cells were still rounded. Further, more than twice as many schwannoma cells adhered to the substrate ($p = 0.01$, $n = 5$; Figure 5).We therefore concluded that adhesion of schwannoma cells is increased on laminin in vitro. Furthermore the extensive lamellipodia and filopodia formation of schwannoma

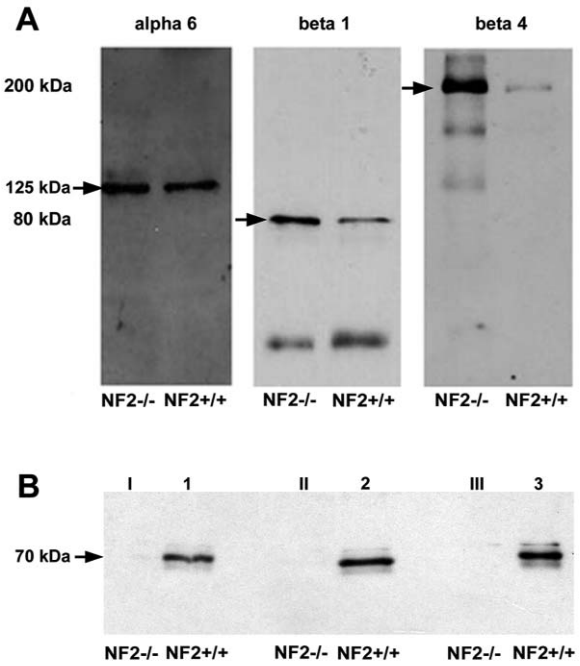

Figure 1. Increased expression of integrin chains b1 and b4 in schwannoma cells lacking merlin expression. **A.** Western blot analysis, staining for integrin chains α6, β1, and β4 on whole cell lysates of Schwann (*NF2*$^{+/+}$) or schwannoma (*NF2*$^{-/-}$) cells. Blots show increased expression of integrins β1 and β4, but not α6 (n = 6 for all 3 chains). Arrows indicate the main protein band, and size in kDa. **B.** Western blot analysis staining for C-terminus merlin, showing that merlin is only expressed in normal Schwann cells (*NF2*$^{+/+}$). None of the schwannoma cell lysates (*NF2*$^{-/-}$) presents a merlin band. I-III are total protein of 3 different schwannoma cell cultures (*NF2*$^{-/-}$),1-3 total protein of three different normal Schwann cell cultures (*NF2*$^{+/+}$), all used in this study. Merlin is detected at 70 kDa, indicated by the arrow.

cells on PLL/Lam coated dishes could not be found when schwannoma cells were seeded on PLL only (data not shown), suggesting that this specific morphology depends on the substrates found in the extracellular matrix (ECM).

Expression of integrin chains β1 and β4 is upregulated in schwannomas at RNA and protein level. The integrin family plays an important role in cell-ECM (eg, laminin) adhesion. Furthermore integrin clusters are found in cell extensions such as lamellipodia and filopodia which are significantly more present in schwannoma cells. Integrin β1 has previously been described to interact with merlin (36), and it is known to form heterodimers with the integrin chains $\alpha_{1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, \text{and } V}$ in vivo. Thus considering the increased adhesion found in schwannoma cells, and the relevance of integrin

	Array data (n=2)	Quantitative RT PCR (LightCycler, n=5)
Integrin α6	4.5	3 ± 0.30
Integrin β1	2.0	2 ± 0.15
Integrin β4	2.5	45 ± 5.20

Table 2. Expression data of integrins α6, β1, and β4 determined by array analysis and quantitative RT-PCR.

Expression of the integrins α6, β1, and β4 in schwannoma cells is referred to the expression of these integrins in normal Schwann cells, which was set to a relative number of 1. For the quantitative RT-PCR the SEM is indicated.

mediated adhesion in Schwann cells, we were curious to find out whether this disrupted function might be correlated with the differential regulation of integrin expression at the cell surface. We therefore performed a cDNA array (Human 1.2 Atlas Array, Clontech), to investigate whether the expression of integrin chains and/or of other adhesion molecules may be altered in schwannoma cells compared to normal Schwann cells. Using this method we were able to detect an upregulation of the integrin chains α6 and β1 by factor 4.5 and factor 2, respectively in schwannoma cells, as well as that of the integrin chain β4 (factor 2.5). This is interesting as both integrins β1 and β4 form heterodimers with α6 in vivo, and are reported differentially expressed during Schwann cell development (11, 14-16, 35, 38, 47). None of the other integrin chains dotted on the cDNA array ($\alpha_{1, 2b, 3, 4, 5, 6, 7, X, V, M, \text{and } L}$ and $\beta_{1, 2, 3, 4, 5, 6, \text{and } 8}$) were detected as regulated by this method. To verify these results we controlled the mRNA expression levels of these integrin chains by means of a quantitative RT-PCR on a LightCycler (Roche) and were able to confirm the upregulation of the integrin chains α6 (factor 3), β1 (factor 2), and massively β4 (factor 45) (Table 2).

In order to assess whether this transcriptional regulation was related to an increase in translated protein, further experiments were carried out. In Western blot analysis (Figure 1A) the α6 chain band migrates to 125 kDa, the only one of the 3 α6-isoforms to be detected in Schwann cells. No significant difference in the expression of this integrin chain could be detected between Schwann and schwannoma cells. Conversely both β1 and β4 integrin chains did prove to be more highly expressed in the tumoral cells (Figure 1A). The β1 band is split, running at about 85 kDa for the main band and 45 kDa for the second. This was originally surprising as the β1 integrin chain is expected to migrate between 100 and 130 kDa depending on the splice variant (β1 A-D), and the degree of glycosylation. In preliminary tests

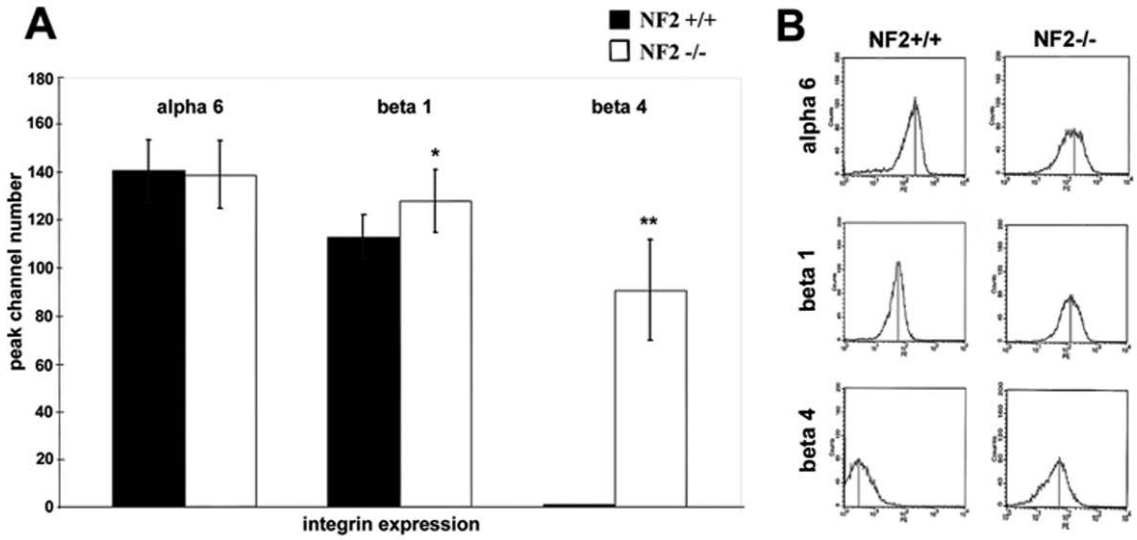

Figure 2. Enhanced expression of integrin chains β1 and β4 at single cell level of Schwann cells and schwannoma cells. Fluorescent cell sorting analysis staining for membrane bound integrin chains α6, β1, and β4 on live Schwann (*NF2*$^{+/+}$) and schwannoma (*NF2*$^{-/-}$) cells, showing increased expression of β1 and β4 at single cell level. Graph (**A**) represents mean data from 4 independent experiments, using different schwannoma (white) and normal Schwann cell cultures (black). Bars indicating the standard error of the mean (SEM), stars indicating the statistical significance measured by paired t-test (α6 $p = 0.67$; β1 $p = 0.018$; β4 $p = 0.006$). Raw FACS data histograms (**B**) show the staining of normal Schwann cells and schwannoma cells for integrin α6, β1, and β4. The X-axis indicates the fluorescence intensity per cell, the Y-axis the number of cells counted (relative units, logarithmic scale). Maximum fluorescence intensity is marked by the black bar.

with the antibody, we were able to detect the 125 kDa β1 band in Schwann cells using boiling SDS buffer (data not shown). However when this same tissue was lysed in the standard denaturating lysis buffer we used, this 125 kDa band almost disappeared, and the 85 kDa and 45 kDa bands were comparatively reinforced. We therefore suggest that the aforementioned bands are cleaved forms of the β1 chain. Nevertheless we chose not to prepare protein from the primary Schwann cells in boiling SDS buffer, as preliminary results proved a much lower protein yield, and cell material was not sufficient. The 85 kDa band is most likely specific for several additional reasons. Firstly 88 kDa corresponds to the size of the non-glycosylated protein (Swissprot. Ref.: PO5556) and secondly a specific band of similar size has been described previously (30). This being the case, qualitative comparison of β1 band intensity was performed on the 85 kDa band, which again is more prominent in tumoral cells. The 45 kDa band might be the proteolysed second segment, as 85 + 45 kDa = 130 kDa.

The β4 chain(205 kDa), shows the highest increase in band intensity, and therefore expression in favor of schwannoma cells (Figure 1A).

In order to ensure that merlin expression was as stated present in Schwann cells and absent in schwannoma cells in primary culture, we performed western blot analysis of merlin expression using the total cell lysates also used for analyzing for integrin expression (Figure 1B). Three randomly selected pairs representing material from 3 different normal nerves and 3 different schwannoma are depicted. Merlin migrates as expected to 70 kDa, and is only to be detected in lysates originating from normal Schwann cells.

Thus, it is to be suspected that the loss of merlin expression in schwannoma cells is in part responsible for the differential expression of these integrin chains.

Increased expression of integrins β1 and β4 on single schwannoma cells. To analyze expression of integrins at the surface of single cells fluorescent assisted cell sorting analysis (FACS) was used (Figure 2). Expression of the α6 integrin chain was not found to be significantly different in schwannoma compared to control cells; however, both the β1 and the β4 integrin chains showed a significant increase in expression as shown by the shift of the peak channel number. This suggests an enhanced integrin coverage of tumoral cells, as well as a surprisingly high expression of integrin chain β4 which does not seem to be prominently expressed by normal Schwann cells in culture. Thus schwannoma

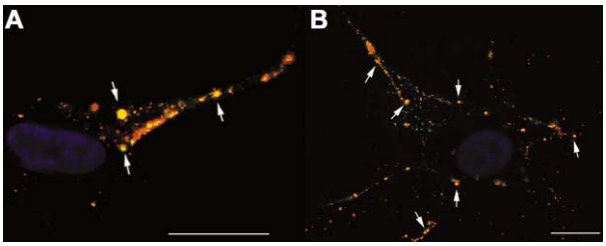

Figure 3. α6/β1 and α6/β4 colocalize in schwannoma cells, indicating their expression as functional integrin receptors. Immunocytochemical stains of live cells showing colocalization of α6/β1 (**A**) and α6/β4 (**B**) in schwannoma cells (α6 shown in red [Cy3], β1 and β4 shown in green [Cy2]), arrows indicate some points of colocalization (yellow). Bars indicate 20 μM.

cells express not only higher levels of integrin chains, but also a different pattern compared to their non-tumoral counterparts.

Integrin α6, β1, and β4 form clusters in schwannoma cells. As integrins are only functional as heterodimers, we were interested in finding out whether the upregulated integrin chains we found in vitro also corresponded to working receptors. Thus we investigated the colocalization of integrin α6 and β1 as well as α6 and β4 in schwannoma cells by immunocytochemistry. For both integrin combinations (α6β1 and α6β4) colocalization was found in fixed cells (data not shown) as well as after staining of unfixed (living) cells (Figure 3). Schwannoma cells show bright surface staining and clustering of the integrin chains α6, β1, and β4 (Figure 4). By contrast in normal Schwann cells the staining is very weak and hardly seen on the surface. This was to be expected as integrins are receptors to the ECM, and clustering is therefore only awaited "under" the cell. Furthermore, as can be observed in Figure 4, it was necessary to excite the fluorophore for much longer in normal Schwann cells to obtain a micrograph. Thus even the strong nuclear staining is somewhat faded by the time this channel is taken. In all cases the weakest stain was excited first in order to minimize fading. Therefore, not only are α6β1 and α6β4 present as heterodimerized receptors on living cells, but they are also to be found on the cell's "open" surface, away from the ECM, suggesting a loss of polarity of the tumoral cells.

Increased adhesion of schwannoma cells is integrin-dependent. To test whether the altered adhesive behavior of schwannoma cells was truly related to the upregulation and clustering of these integrin chains, we conducted an adhesion assay in which cells were seeded in the presence of anti-integrin β1, anti-integrin β4, or anti-P0 as a control. Using antibodies against either integrin β1 or β4 the number of adherent cells was found to be significantly decreased in schwannoma cells (β1 $p=0.02$; β4 $p=0.005$; $n=5$), while addition of the antibody against adhesion protein P0 did not show any effect (Figure 5). Schwann cell adhesion was not influenced by the addition of antibodies, although they present high levels of integrin β1, thus suggesting that expression alone is not sufficient to influence adhesive qualities of the cell. Thus the increased adhesion of schwannoma cells depends on integrin mediated adhesion, and the clustering of integrin chains is necessary for this effect to be relevant.

In vivo all schwannoma cells express integrins α6, β1, and β4. We finally investigated the expression of the integrin chains α6, β1, and β4 in frozen sections of NF2 schwannoma and normal nerve sections as control (Figure 6). In normal nerve, the antibodies showed classical staining, with β1 staining preferentially non-myelinating Schwann cells, β4 myelinating Schwann cells, and α6 both cell types. Interestingly, almost all cells in schwannomas showed staining for all 3 (α6, β1, and β4 integrin) chains, although these cells are by definition non-myelinating.

Thus, our data support the hypothesis that schwannoma cells indeed do show a different and enhanced adhesion when compared to normal Schwann cells, and this difference in adhesion is fuelled by differential expression and clustering of integrin chains, most importantly β1 and β4.

Discussion

Schwannomas are benign tumors which show only slightly increased proliferation indices (40). Though the tumors grow slowly it is unlikely that this characteristic is sufficient to explain the development of the Schwann cell tumors. Prompted by the fact that human schwannoma cells show enhanced cell spreading as well as an increased number of lamellipodia, filopodia, and membrane ruffles (37, 40), we hypothesized that the adhesive characteristics of schwannoma cells must be different from those of normal Schwann cells. We here show that schwannoma cell adhesion is much stronger than that of normal human Schwann cells. Complementarily Bashour and colleagues reported a direct relation between merlin expression and the adhesive characteristics of human schwannoma cells as abnormal ruffling and cell spreading could be reversed by TAT mediated merlin transduction (2).

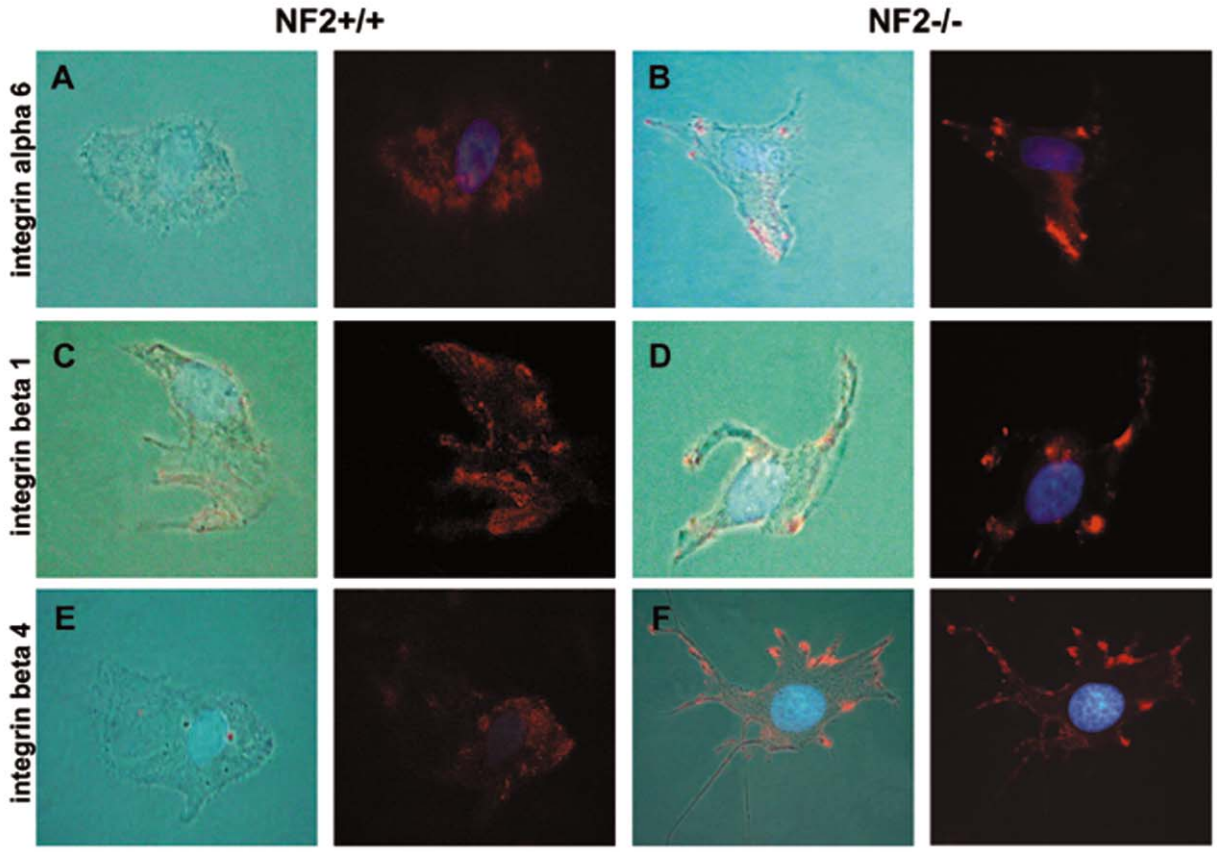

Figure 4. Clustering of integrin chains α6, β1, and β4 in schwannoma cells, indicates increased levels of integrin activity. Immunocytochemical stains for integrins α6 (**A, B**), β1 (**C, D**) and β4 (**E, F**) in live (non fixed) Schwann (*NF2*$^{+/+}$; **A, C, E**) and schwannoma (*NF2*$^{-/-}$; **B, D, F**) cells. All integrins are stained by a Cy3-labeled secondary antibody (red). The nucleus is stained with DAPI (blue). Each pair of pictures shows a phase contrast picture overlaid with fluorescence (left panel) and the corresponding fluorescent picture (right panel). Experiments were repeated 4 times independently, using different schwannoma and normal Schwann cell cultures.

Using a cDNA array to screen for regulated adhesion molecules underlying the altered adhesion we found 3 integrin chains (α6, β1, and β4) to be upregulated in schwannoma cells. We then confirmed that the integrin chains α6, β1, and β4 are significantly upregulated at RNA level by quantitative RT-PCR. Enhanced expression at protein level of the integrin chains β1 and β4 , but not α6 was also detected both on whole cell lysate (Western) and at single cell level (FACS). Our findings are remarkable in that these integrin chains can form the in vivo heterodimers α6β1 and α6β4 and are therefore functional integrin receptors. Secondly, β1 is known to interact with merlin (36). Thirdly, the integrin receptors α6β1 and α6β4 are both receptors for laminin, a prominent component of the extracellular matrix in vivo. Moreover, conversely to most common and widely distributed integrins, the integrin receptors α6β1 and α6β4 have been described to have a prominent importance during Schwann cell differentiation (11) and are known to be differentially regulated in Schwann cells (15, 20). The integrin receptor α6β1 is classically expressed at a higher level by non-myelinating than by myelinating Schwann cells (15), whereas integrin α6β4 expression is restricted to myelinating Schwann cells (11, 15). As the schwannoma cells are definitely not myelinating the clear staining for integrin β4 suggests that the tumoral cells express this integrin chain independently from the presence of the axon, and therefore develop pathological adhesive characteristics which might in part explain their loss of polarity (integrin clusters at the

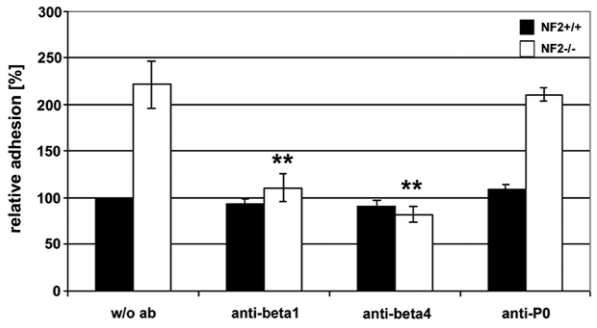

Figure 5. Schwannoma cell adhesion is enhanced compared to that of normal Schwann cells, and dependent on integrins β1 and β4. Bar chart representing the relative number of cells which have adhered to a culture dish (coated with PLL/laminin) after 3 hours of incubation. For ease of comparison the number of normal Schwann cells (*NF2*$^{+/+}$) adhering in the absence of antibodies was set to 100%. Schwannoma cells (*NF2*$^{-/-}$) adhere 2.5 times better than normal Schwann cells under basal conditions. However incubation of these cells with antibodies against either integrin β1 or β4 results in a reduction of the adhesion to 80 to 100% of the *NF2*$^{+/+}$ basal level. The adhesion of normal Schwann cells is not affected. Bars indicate the standard error of the mean (SEM), stars indicate the statistical significance determined by paired t-test (w/o ab $p = 0.01$; beta1 $p = 0.02$; beta 4 $p = 0.005$). The Y-axis indicates the relative adhesion of the cells referring to the adhesion of normal Schwann cells to laminin as 100%. w/o ab, without antibody; anti-beta1 (or beta4), antibody to integrin β1 (or β4); anti-P0, antibody to myelin protein. n = 5.

"top" of the cells), and the ensheathment of the extracellular matrix, or pseudomesaxon formation (10, 12, 18).

Integrin β1 and β4 are upregulated in schwannoma, whereas the expression of α6 at protein level remains unchanged. However, one has to take into account that integrin β4 is only expressed at a very low level in normal Schwann cells and integrin α6, which is already strongly expressed in normal Schwann cells is the only known heterodimer partner for integrin β4 (15). Moreover, integrin α6 forms more stable heterodimers with integrin β4 than with integrin β1 (15, 17). Thus, it is possible that when integrin β4 is upregulated the integrin α6 chain preferentially tends to form heterodimers with this chain, thus leaving at least some of the upregulated integrin β1 chain to bind with other α chains present. This would unbalance the adhesive qualities of the cell. The integrin receptors expressed by human Schwann cells are α2β1, α5β1, α6β1, and α6β4 (26, 27, 39, 48). Therefore the integrin chains α2 and α5 would be additional candidates for the heterodimer formation with β1. However as found in the cDNA array α5 integrin is not expressed in cultured Schwann and schwannoma cells,

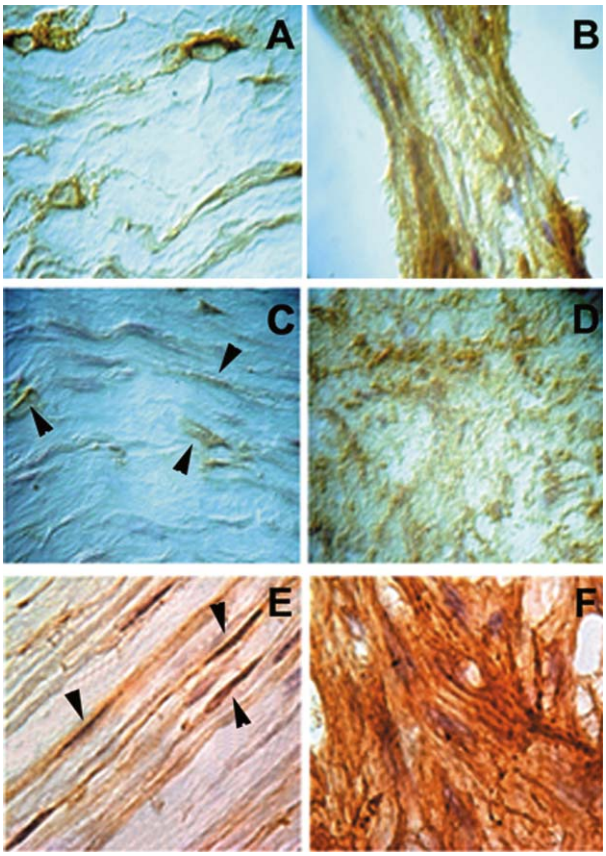

Figure 6. Human schwannoma expresses α6, β1, and β4 integrins in all cells in vivo. Immunohistochemistry of frozen sections of human schwannoma and normal nerve. **A, C,** and **E** show normal nerve controls staining for integrin chains α6, β1, and β4, respectively. β1 stains preferentially non-myelinating Schwann cells (C-arrow heads), whereas β4 is expressed almost exclusively on myelinating Schwann cells (E-arrow heads). β6 is expressed in both cell populations. **B, D,** and **F** show the staining of tumor tissue with anti-integrin α6, anti-β1, and anti-β4, respectively. Notably, although no axon is present in the schwannoma tissue, all cells express high levels of integrin β4. All pictures were taken through a ×63 PlanNeofluar objective (Zeiss). For (**A-D**) phase contrast was used.

and α2 integrin was not detectable neither by PCR nor by Western blotting (data not shown).

To further determine the functional relevance of the upregulated integrin receptors in schwannoma cells we examined their adhesion in the presence of antibodies to integrin β1 and β4. Anti-integrin α6 was not used for this experiment as the binding site for laminin is found on the β chain of the integrin receptor. The blocking of the integrin receptors led to a reduction of adhesion of schwannoma cells, thus suggesting that the upregulation of the integrin chains α6β1 and α6β4 is indeed functionally relevant to the altered adhesion of schwannoma

cells on the ECM component laminin. This assumption was further supported by the finding that a clustering of the integrin chains α6, β1, and β4 could be detected in schwannoma, but not Schwann cells. Clustering of integrins is known to occur at focal contacts of the cell and plays a crucial role in maintaining cell shape (28). Correspondingly, Schwann cells which expressed integrin β1, without presenting abnormal clustering of this integrin chain, were not impeded in their adhesion to PLL-laminin by the addition of antibody against this integrin chain.

It has recently been shown that expression of an activated mutant of the small Rho GTPase Rac triggers dramatic spreading of T-cells and increases their integrin dependent adhesion (7). The Rac-induced spreading was accompanied by specific cytoskeletal rearrangements as well as clustering of integrins at sites of cell adhesion and at the peripheral edges of cells. These observations fit well into the phenotype of human schwannoma cells which present increased cell spreading, higher numbers of lamellipodia and filopodia as well as increased adhesion. Interestingly indirect evidence for increased Rac activity in $NF2^{-/-}$ fibroblasts showing increased activity of the downstream c-jun N-terminal kinase (JNK) pathway compared to those transfected with wild-type merlin has recently been published (46).

In addition to the Rac inside/out signaling to integrins, integrins are known to play an important role in the signal transduction from the extracellular milieu to the cell interior (outside/in). The MAP kinase pathways of Erk (extracellular regulated kinase) and JNK have been described to be activated by the engagement of integrin α6β4 (5, 32, 47) in a Ras dependent manner. Ras itself is able to activate Rac, which can in turn activate Rho (9). Activated Rac and Rho could explain stress fiber formation and lamellipodia, which have also been reported in schwannoma cells (37). It has been shown that the activation of JNK in response to α6β4 ligation is suppressed by dominant negative Ras and Rac1 (32).

Accordingly our group has recently shown that Rac1 and down-stream JNK1&2 are expressed at higher levels and are more active in human primary schwannoma cells compared to control Schwann cells (Kaempchen et al, submitted). These results fit well into the understanding of the data presented here. Moreover, Rac in turn can induce the transcription of integrin chains (6), thus inducing a positive feed-back loop.

We suggest that our observations of increased adhesion and altered morphology of schwannoma cells is due to the enhanced expression and clustering of integrin α6, β1, and β4 and is possibly both a consequence and cause of activation of Rac/MAP kinase pathways. This leads one to suggest that schwannoma formation might be in part caused by an abnormal switch in the integrin coverage of schwannoma cells, thus leading to different adhesive qualities of the tumoral cells. This is supported in vivo by the fact that histological studies of schwannoma describe ensheathment of the extracellular matrix by the tumoral Schwann cells, suggesting disrupted adhesion.

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