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αvβ3 integrin expression increases elasticity in human melanoma cells

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ABSTRACT

Living cells interact with the extracellular matrix (ECM) transducing biochemical signals into mechanical cues and vice versa. Thanks to this mechano-transduction process, cells modify their internal organization and upregulate their physiological functions differently. In this complex mechanism integrins play a fundamental role, connecting the extracellular matrix with the cytoskeleton. Cytoskeletal rearrangements, such as the increase of the overall contractility, impact cell mechanical properties, the entire cell stiffness, and cell deformability. How cell mechanics is influenced via different integrins and their interaction with ECM in health and disease is still unclear. Here, we investigated the influence of αvβ3 integrin expression on the mechanics of human melanoma M21 cells using atomic force microscopy and micro-constriction. Evidence is provided that (i) αvβ3 integrin expression in human melanoma cells increases cell stiffness in both adherent and non-adherent conditions; (ii) replacing αvβ3 with αIIbβ3 integrin in melanoma cells, cell stiffness is increased under adherent, while decreased under non-adherent conditions; (iii) αvβ3 integrin cell stiffening is also maintained when cells adhere to fibronectin, but this phenomenon does not strongly depend on the fibronectin concentration. In all, this study sheds light on the role of αvβ3 in regulating cellular mechanics.

1. Introduction

There is growing evidence that the mechanical properties of the extracellular matrix (ECM) regulate cellular decision-making processes via mechano-transduction [1]. The current understanding of adhesion-mediated environmental sensing is still fragmentary, and several design principles have emerged from experiments. For example, surface biochemistry, namely the presence of diverse matrix proteins, has a strong effect on the selection of specific integrin receptors, and consequently on the initial assembly of integrin complexes. The mechanical properties of the extracellular environment play a more important role in cellular behavior than originally thought. It has been shown that cells (i) upregulate the cytoskeleton and cell-matrix adhesion on stiffer substrates more strongly, and (ii) locomote in favor of stiffer or strained substrates, and that (iii) extracellular mechanical properties and cellular decision-making are connected to the internal force developing at cell-matrix contacts due to cellular acto-myosin contractility [2,3].

Integrins are a superfamily of transmembrane receptors composed of two non-covalently associated distinct subunits α and β [4]. Each subunit is composed of a large extracellular *N*-terminal domain that allows specific ligand binding, a single transmembrane-spanning helix and a short *C*-terminal cytoplasmic sequence that provides docking for signaling, adaptors, and cytoskeletal-associated proteins. In mammalian tissues, subunits α and β combine in a restricted mode forming 24 different integrins, which are widely distributed. They integrate the extra -and intracellular environment through bidirectional signaling, and consequently are highly dynamic modulators of cell function, including cell adhesion, shape, motility, proliferation, differentiation, and survival. Through these processes, integrins are involved in many physiological aspects, including wound healing, immune response, hemostasis as well as in pathological dysfunctions, i.e. fibrosis, inflammatory disease, bleeding, and thrombosis. On top of that, integrins are key elements for the modulation of the mechanics involved in the interaction between cells and the surrounding ECM. Importantly, expression of specific integrin types may have a different impact on the mechanical properties of the cell.

Integrins such as α5β1, αIIbβ3, and αvβ3 are major fibronectin (FN) receptors that bind in an RGD-dependent manner [5]. Redundancy, however, exists with respect to certain integrin–ECM interactions, as some integrins bind the same extracellular ligands, albeit at different affinities and conversely, some ligands are

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recognized by different integrins. For example, recent studies have shown that αvβ3 integrin promotes melanoma tumor survival *in vivo* and that the interaction with the ECM seems to play a critical role in this process [6]. However, its mechanisms are still unknown. The aim of this work was to explore the influence of integrins αvβ3, α5β1, and αIIbβ3 on the mechanical properties of human melanoma cell lines.

We used the following model cells derived from the same cell line: M21, expressing αvβ3 and α5β1; M21L, expressing only α5β1; and M21GPIIb, expressing αIIbβ3 and α5β1, but not αvβ3 [7]. Cells were grown on surfaces without specific coating and/or with fibronectin, and the elasticity was probed using atomic force microscopy to answer the following questions:

(i) Does the expression of αvβ3 integrin in M21 cells influence cellular stiffness?
(ii) Do possible differences in cellular stiffness prevail under adherent and non-adherent conditions?
(iii) Does the binding to fibronectin influence the mechanics of M21 cells? Does it influence the mechanics of M21 cells differently when αvβ3 integrin is expressed? Does it depend on fibronectin concentration?

The results from these measurements were compared with previous stiffness measurements of the same cell lines using a microfluidic device under non-adherent cellular conditions [8].

2. Materials and methods

2.1. Cell culture

Human melanoma cells M21, M21L [9,10], and M21GPIIb [11] were grown in RPMI-1640 medium (Gibco Laboratories, Grand Island, NY) supplemented with 10% fetal calf serum (FCS) from Gibco Laboratories, 100 U/ml of penicillin, and 100 μg/ml of streptomycin in 5% CO_2 within a fully humidified incubator at 37 °C.

2.2. Antibodies and reagents

Mouse monoclonal antibodies against the αvβ3 heterodimer (LM609) and β1 subunit (P5D2) were purchased from Millipore-Sigma. Specific mouse monoclonal antibodies for β3 (BV3) and αIIb (B9) subunits were bought at Origene (Rockville, MD) and Santa Cruz Biotechnology (Dallas, TX), respectively. The APC-labeled goat anti-mouse IgG-specific secondary antibody was obtained from Jackson Immuno-Research (West Grove, PA).

2.3. Flow cytometry

M21, M21L, and M21GPIIb cells were detached from the tissue culture dishes by mild trypsinization and washed in 20 mM Hepes buffered saline (HBS) at pH 7.4 containing 1% bovine serum albumin (BSA). Cells (0.5×10^6) in 100 μl of the same buffer were incubated for 30 min at 4 °C with 20 μg/ml mouse mono-clonal antibodies against the αvβ3 heterodimer (LM609), β3 (BV3), β1 (P5D2), and αIIb (B9) subunits. Cells were then washed with HBS by centrifugation at 500*g* for 5 min. APC-labeled goat anti-mouse IgG-specific antibody at 20 μg/ml was added to the cells for an additional 30 min at 4 °C. The cells were subsequently washed, fixed in 1% paraformaldehyde, and analysed by flow cytometry using a FACSCalibur flow cytometer. The antibody binding level was expressed as mean fluorescence intensity using the FlowJo software.

2.4. Fibronectin coating of petri dishes

We performed two sets of experiments to determine the Young's modulus of M21, M21L, and M21GPIIb cells adherent to (i) cell culture dishes without fibronectin coating and (ii) coated with fibronectin. To assess the effect of fibronectin coating, two different coating conditions were tested. The first condition tested the effect of FN at a high concentration (1 mg/ml), keeping the remaining bare surfaces available for FCS proteins. Thus, the surface of cell culture dishes (Falcon, REF 353004) was coated with a solution of 1 mg/ml fibronectin from bovine plasma (F1141 Sigma-Aldrich) and allowed to air-dry for at least 45 min at room temperature. The second condition tested the effect of FN-only binding, using different fibronectin concentrations, blocking remaining bare surfaces with bovine serum albumin . The surface of cell culture dishes was divided into three separate regions using two

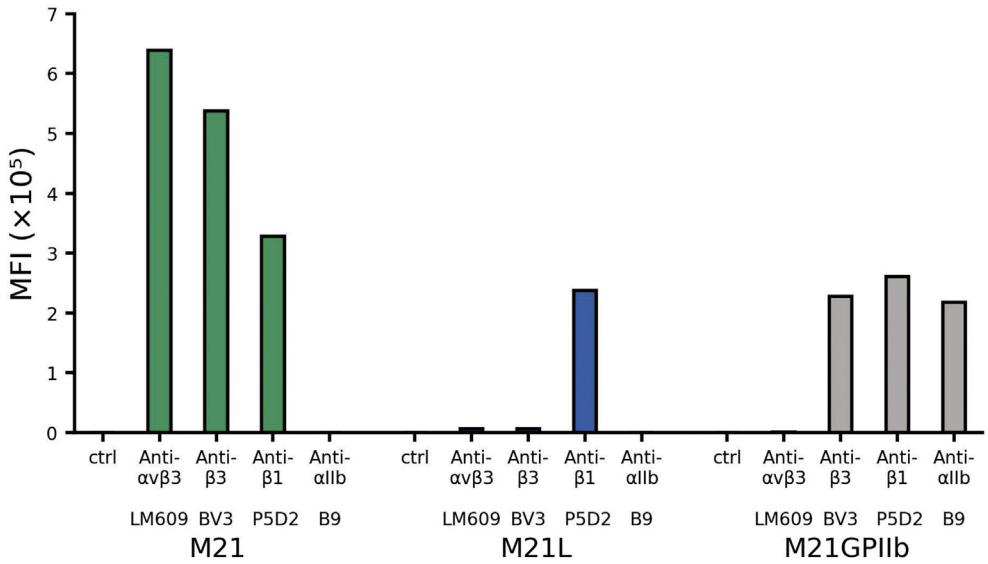

Fig. 1. Flow cytometric analysis shows the surface expression of integrins expressed on the melanoma cell panel used in this study. After the cells were detached, they were stained with specific antibodies against the avβ3 heterodimer, β3, β1, and aiib subunits. The histogram shows the integrin surface expression level (mean fluorescence intensity, MFI) analysed by flow cytometry.

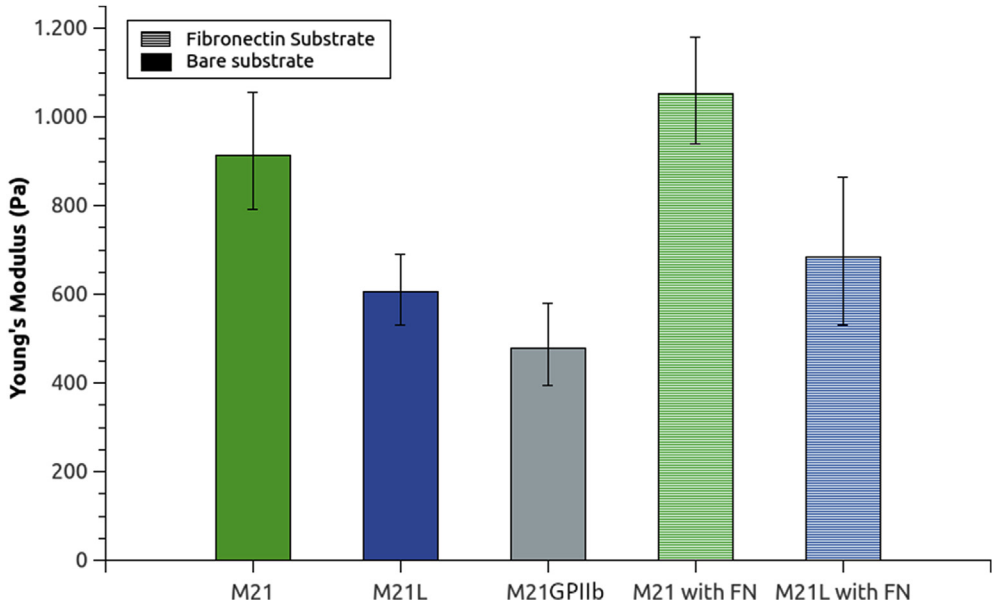

Fig. 2. Young's modulus of M21, M21L, and M21GPIIb cells grown on non-coated (left) and M21 and M21L grown on fibronectin-coated dishes (right). The values represent mean and standard error of the mean of the log-transformed data.

Table 1
Average Young's moduli obtained for the cell lines M21, M21L, and M21GPIIb cultured on the fibronectin-coated and non-coated petri-dishes.

Cell line	**Bare Substrate**			
	ln(YM[Pa])	**YM[Pa]**	+	−
M21	6.82 ± 0.14	913	142	123
M21L	6.41 ± 0.13	606	85	74
M21GIIB	6.17 ± 0.19	478	102	84
Cell line	**Fibronectin (FN)**			
	ln(YM[Pa])	**YM[Pa]**	+	−
M21	6.96 ± 0.11	1054	128	114
M21L	6.53 ± 0.25	685	180	155

polydimethylsiloxane (PDMS) pools to coat the surface with three different fibronectin concentrations (1, 10, and 100 μg/ml). The surface was incubated for at least 45 min, then the excess solution was removed by aspiration, and finally the surface was blocked by BSA (100 μg/ml) for 1 h at room temperature to saturate non-specific cellular interactions.

2.5. Atomic force microcopy (AFM)

All AFM measurements were performed on a NanoWizard IV Atomic Force Microscope (JPK, Bruker, Santa Barbara, CA, USA) coupled with an inverted optical microscope Ti-Eclipse (Nikon, Japan). During the first experiments, a Novascan V-shaped

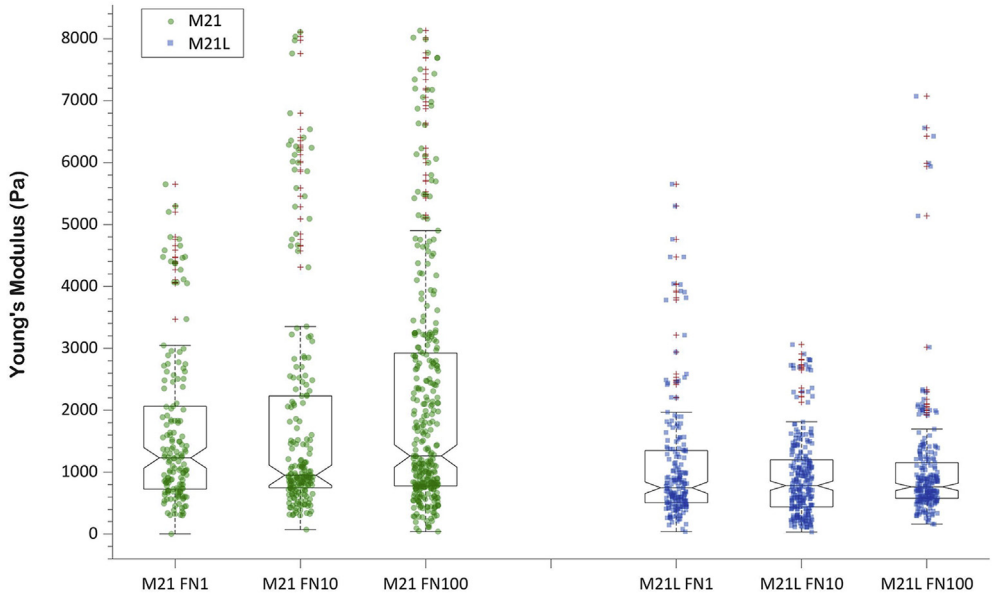

Fig. 3. Young's modulus of M21 and M21L cells growing on FN-only coated substrates at different concentrations (FN1, FN10, and FN100 stand for 1 μg/ml, 10 μg/ml, and 100 μg/ml, respectively). The values represent mean and standard error of the mean of the log-transformed data.

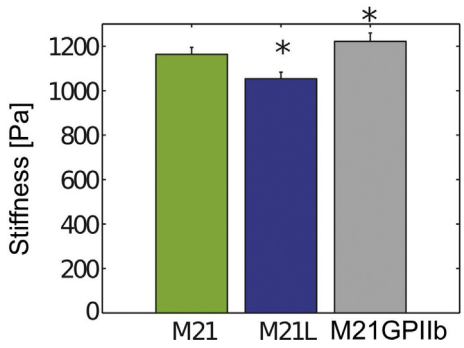

Fig. 4. Influence of αIIbβ3 on cell mechanics by micro-constriction analysis. The stiffness of M21L and M21GPIIb cells compared to the. control population M21 were calculated from the power-law fitting routine [15].

cantilever was used with a spherical silica bead of 1 μm radius as a tip with a spring constant of 0.03 N/m. During the second experiment, a V-shaped cantilever (MLCT-D from Bruker, Santa Barbara, CA, USA) was used, and a silica bead of 10 μm diameter with a spring constant of 0.042 N/m. The cantilever spring constant was calibrated according to the Sader method in air, and the optical lever sensitivity was determined from the thermal spectrum in liquid [12–14], using a custom algorithm based on Matlab (Mathworks, Natick, MA). Force curves were generated in force mapping mode using a square array of 8 × 8 or 5 × 5 points across the perinuclear region of the cells. The size of the grid was chosen according to the dimension of each cell (between 10 × 10 and 20 × 20 μm^2), and 5 curves per point were recorded. To minimize the acquisition time, the approach velocity was set to 15 μm/s. A set point of 0.4 nN was defined to obtain an indentation of at least 200 nm.

2.6. Micro-constriction analysis

Cell mechanical properties of M21 and M21L cells were also assessed by the micro-constriction method [15] with a constriction width of 7.7 μm and a device height of 18.9 μm. M21 cells were cultured as shown in Ref. [8] and harvested at a confluency of approx. 80%.

2.7. Data processing and statistics

The Hertz model was used, avoiding the substrate bottom effect. A reference force curve was acquired on the substrate near every cell to estimate the cell thickness at all points of the force curve map. The thickness of the cell at each point of the force curve map (h) was determined from the cantilever vertical position at the point of contact with the cell, and at the point of contact with the substrate, which was used as reference (h = 0). To avoid the effect of the underlying substrate, only data points fulfilling an indentation of <0.1h were considered to determine the Young's modulus [16]. The geometric mean of all values, from each tested cell line, was used.

3. Results

3.1. Integrin cell surface level

The histogram in Fig. 1 shows the expression level of integrins on the surface of human melanoma cells, M21 using specific antibodies [7].

3.2. AFM measurements

We compared the average Young's modulus (cell stiffness) of (n = 15) M21, M21L, and M21GPIIb cells cultured on treated, non-coated FN petri dishes and the same number of M21 and M21L cells on FN-coated petri dishes. As shown in Fig. 2 and Table 1, the Young's moduli of M21 cells on FN-coated and non-coated dishes were ~50% higher compared to M21L cells. The mechanical properties (elasticity) of M21GPIIb cells, expressing α5β1 and αIIbβ3 instead of αvβ3 integrins, were compared to M21 (αvβ3 and α5β1) and M21L (only α5β1) cells (Fig. 2). It was hypothesized that the replacement of αvβ3 by αIIbβ3 integrin might restore the mechanical properties of M21 control cells, as both integrins have a similar molecular structure; however, the expectation could not be verified by these measurements; M21GPII showed a lower Young's modulus than M21 and M21L cells.

FN-coated surfaces at high concentration (1 mg/ml) led to an increase of ~15% of the Young's modulus on both M21 and M21L cells (Fig. 2). The Young's modulus of cells growing on fibronectin-only surfaces at different concentrations is shown in Fig. 3. As reflected, fibronectin-only binding did not lead to major changes on cell stiffness when varying the concentration. The results suggest that integrin αvβ3 influences the mechanical properties of M21 cells, while the interaction between this integrin and fibronectin has a moderate effect on the cell's Young's modulus.

3.3. Micro-constriction measurements

Two independent measurements were conducted, and the results were pooled. Using the power-law fitting routine of the scatter (t_{entry} vs. εmax/Δp), a cell stiffness of 1,215 ± 17Pa for M21 cells expressing αvβ3 and α5β1 integrins was calculated. M21L cells, which only express α5β1 integrin, showed a significant reduction in stiffness to 1,065 ± 17Pa. The power-law exponents were inversely correlated and markedly different (0.07 ± 0.01 for M21 and 0.09 ± 0.01 for M21L cells). The expression of αvβ3 integrin was correlated with increased stiffness and a decreased power-law exponent in both cell lines.

Moreover, the mechanical properties of M21GPIIb cells, expressing α5β1 and αIIbβ3 instead of αvβ3 integrins, were calculated in comparison to M21 (αvβ3 and α5β1) and M21L (only α5β1) cells (Fig. 4). In contrast to AFM measurements, expression of αIIbβ3 led to an increase in the stiffness of M21GPIIb cells in suspension.

4. Discussion

In this study, we show that individual expression of different integrins, even those with specificity for the same ligand, are influential to the mechanical status of the cell probed by two different technologies, AFM and micro-constrictions.

A systematic approach, comparing the cell mechanical properties under suspended or adherent conditions, provides insight into the effect adhesion may have on cell mechanics. We have chosen a panel of three M21 melanoma cells expressing the fibronectin receptor α5β1 alone (M21L), in conjunction with αvβ3 (M21), or with αIIbβ3 (M21GPIIb). In summary, a systematic effect of αvβ3 integrin expression on the cellular mechanics was found. In M21 cells, an increase in cell stiffness is correlated with the expression of αvβ3 integrin. This could be due to its association with the actin cytoskeleton, namely via a link between integrin, focal adhesion-associated proteins, and actin cytoskeleton that leads to higher connectivity inside the cell and/or to increased actin polymerization [17–19]. Support for this notion comes from a decreased actin content measured by flow cytometry in M21L compared to

M21 cells and from the finding that M21 cells expressing αvβ3 integrin show a higher cell volume (results not shown). In the case of M21GPIIb, which expressed αIIbβ3 instead of αvβ3, the stiffness of cells could not be restored, having a different effect on the stiffness on attached (Fig. 2) and suspended (Fig. 4) cells [8]. These results might be due to a less deformable nucleus in M21GPIIb cells or to a softer perinuclear area of the cell with a weaker adhesion to the substrate; this points in the direction that micro-constriction experiments are more sensitive to the nuclear deformability, while the AFM measurements are probing the perinuclear area.

Our AFM data on FN-only coated substrates, blocked with BSA (Fig. 2), showed that M21 cells are only slightly affected by FN. In contrast, data shown in Fig. 3, on FN substrates not blocked with BSA, to which fetal calf serum (FCS) proteins may adhere, such as vitronectin and fibrinogen, M21 cells appear stiffer. It suggests that M21 cells require ligands other than FN to reinforce their mechanical properties via integrin αvβ3 binding. This is in agreement with previous work indicating that α5β1 mainly determines adhesion strength, while αvβ3 would control adhesion reinforcement and mechano-transduction [20]. Thus, αvβ3 binding to FN-only seems to be insufficient to regulate cellular mechanics.

Future work entailing gene expression knock-down and a more detailed analysis using cell populations expressing different levels of only αvβ3, αIIbβ3, or α5β1 will be required to finally assess the differential contribution of these integrins to integrin-mediated cellular stiffness. Moreover, experiments using other ligands such as vitronectin and/or fibrinogen, specifically targeting only some of the integrins, will be required to obtain a more complete picture of integrin regulation on cell mechanics.

Declaration of competing interest

This is to confirm that the publication of this manuscript does not conflict with any financial or any other interests.

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