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EXPERIMENTAL STUDIES

IMPLICATION OF INTEGRIN α5β1 IN SENESCENCE OF SK-Mel-147 HUMAN MELANOMA CELLS

N.I. Kozlova, G.E. Morozevich, N.M. Gevorkian, L.K. Kurbatov, A.E. Berman*

Institute of Biomedical Chemistry,
10 Pogodinskaya str., Moscow, 119121 Russia; *e-mail: 1938berman@gmail.com

Downregulation of α5β1 integrin in the SK-Mel-147 human melanoma culture model sharply inhibits the phenotypic manifestations of tumor progression: cell proliferation and clonal activity. This was accompanied by a 2-3-fold increase in the content of SA-β-Gal positive cells thus indicating an increase in the cellular senescence phenotype. These changes were accompanied by a significant increase in the activity of p53 and p21 tumor suppressors and components of the PI3K/Akt/mTOR/p70 signaling pathway. Pharmacological inhibition of mTORC1 reduced the content of SA-β-Gal positive cells in the population of α5β1-deficient SK-Mel-147 cells. A similar effect was observed with pharmacological and genetic inhibition of the activity of Akt1, one of the three Akt protein kinase isoenzymes; suppression of other Akt isozymes did not affect melanoma cell senescence. The results presented in this work and previously obtained indicate that α5β1 shares with other integrins of the β1 family the function of cell protection from senescence. This function is realized via regulation of the PI3K/Akt1/mTOR signaling pathway, in which Akt1 exhibits a non-canonical activity.

Key words: tumor progression; cellular senescence; integrins; signaling; non-canonic function of Akt protein kinase

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INTRODUCTION

This work is a continuation of previous studies aimed at elucidating the role of integrins, the main signaling mediators between the extracellular matrix and intracellular processes, in the development and progression of tumors. In various tumor cell types, integrins of the β1 and β3 families were shown to mediate signals that stimulate tumor progression, in particular, increase invasive activity, resistance to anchorage-dependent apoptosis (anoikis), and neutralize the mechanisms of cellular senescence [1–7]. These data are consistent with the results of numerous studies on the role of integrins in oncogenesis [8]. However, we found that in some cell types, these receptors can have a blocking effect on certain parameters of progression. This effect may be based on a specific for certain integrins ability to initiate unusual (non-canonical) signaling pathways. For example, in the model of the MCF-7 cell line of human breast adenocarcinoma, stimulation of the signaling activity of β1 integrins increased the sensitivity of cells to anoikis [9]. However, a study on the SK-Mel-147 human melanoma cells showed that these receptors increased cell resistance to this type of apoptosis [5, 6]. In the same model, differences were found in signaling pathways mediated by α2β1, α3β1, and α5β1 integrins involved in the mechanisms of anoikis and invasive activity of tumor cells [5, 6, 10]. It turned out that protein kinase Akt is involved in both of these pathways and plays an unusual (non-canonical) function of suppressing, rather than stimulating, various phenotypic manifestations of tumor progression. It should be noted that the above pathways differ in which of the Akt enzymes exhibits non-canonical activity in each of them, so that Akt1 non-canonical activity was determined in the invasion-controlling pathway, while the Akt2 non-canonical function was observed in the anoikis-controlling pathway.

The differences found between the integrin-dependent mechanisms that control two phenotypic markers of malignant progression suggested the importance of studying other characteristics of the tumor phenotype, in particular, the mechanisms that could control cellular senescence. In recent studies on melanoma cells, we have shown that α2β1 and α3β1 integrins initiate senescence-protecting mechanisms based on a non-canonical activity of Akt1 [7, 11]. However, a significant difference between these integrins is that senescence induced by deficience of α2β1, unlike that, resulted from α3β1 depletion, was accompanied by a marked decrease in cell proliferation.

Published data on the role of integrins in cell senescence are scarce and contradictory, besides, their effects on cell behavior are highly dependent on the type of cells. For example, αvβ3 integrin is critical to the ability of glioblastoma cells to counteract senescence owing to the αvβ3-induced p21 suppression. However, these cells did not show a similar requirement for other integrins, including αvβ5, which is close to αvβ3 in ligand properties. Furthermore, αvβ3 was not found to be critical in protecting epithelial cells from senescence [12]. Moreover, in intestinal cells and in cultured fibroblasts, a clear correlation was found between

the αvβ3 expression and the development of the cellular senescence phenotype [13, 14]. The β1 family integrins also differ in their effects on senescence. In cultured fibroblasts, suppression of α5β1 greatly increased the signs of senescence, indicating a senescence-protective capacity of this receptor [15] in contrast to integrin α6β1, which showed a senescence-promoting effect in the same model [16, 17]. On the other hand, an enhanced senescence phenotype was observed in cultured osteosarcoma cells in response to general activation of β1 integrins [18].

The above results of our studies and others confirm the well-known feature of integrins: not only members of the same or different families, but an individual receptor can initiate different cellular reactions in normal and tumor cells. In order to elucidate the mechanisms underlying this poorly investigated phenomenon, it is important to perform a comparative analysis of various receptors and signaling pathways initiated by them.

The aim of this work was to investigate the involvement of α5β1 integrin in tumor cell senescence and to characterize the signaling pathways mediated by this receptor. We used the same as in previous studies model of cultured melanoma cells to correctly compare the α5β1-initiated pathways with the pathways initiated by other β1 integrins. We have shown that downregulation of α5β1 stimulated melanoma cell senescence through a signaling mechanism based on non-canonical activity of the Akt1 protein kinase.

MATERIALS AND METHODS

Cells and Reagents

The human melanoma cell line SK-Mel-147 was obtained from the Sloan Kettering Memorial Cancer Center (USA). Cell cultivation was performed as described previously [10]. Sigma reagents (USA), except otherwise stated were used. Polyclonal antibodies to the α5 subunit were obtained from Chemicon (USA). Polyclonal antibodies to protein kinases and their phosphorylated forms (Akt, pAkt Ser473, pErk Thr202/Tyr204, pmTOR Ser2448), p53 and p21 proteins were obtained from Cell Signaling Tech (USA). The Akt1-specific inhibitor XXIII, Akt2-specific inhibitor XII and mTOR inhibitor rapamycin were obtained from Calbiochem (USA).

Cell Senescence

Cells were passaged in 12-well plates for 24 h, washed with phosphate buffer. Subsequent processing was carried out according to the protocol and with reagents from Bio Vision (USA). Cells with signs of senescence (containing green-colored products of β-galactosidase reaction) were visualized under a microscope and their percentage (%) in the total cell population was determined [7].

Transduction of Cells with shRNA

Bacterial glycerol clones containing the pLKO.1-puro lentiviral plasmid vector with shRNA for the α5-integrin subunit or "empty vector" were obtained from Sigma. Lentiviral clones with vectors containing shRNA specific for Akt protein kinase isozymes (Akt1, Akt2, and Akt3) were obtained from GeneCopoeia (USA). Lentiviral particles were obtained and infection of cells was carried out as described previously [4].

Cell proliferation was analyzed according to the described method [19]. Briefly, $(1–2)\times10^4$ cells were passaged in 96-well plates in DMEM with serum for various time intervals. The cells were then stained with the crystal violet dye, extracted with methanol, and the optical density was determined at 570 nm in a Tecan Genios Plus plate photometer (Switzerland).

Clonal Activity (Colony Formation)

Cells were passaged in 1% methylcellulose gel in DMEM medium with serum, as described previously [10]. Colonies were stained with crystal violet. The colony-containing plates were scanned and the number of colonies in the image was counted.

Polyacrylamide Gel (PAAG) Electrophoresis and Immunoblotting

Preparation of cell lysates, electrophoresis, and electrotransfer of proteins onto the PVDF membrane were performed as described previously [20]. After reaction with specific primary antibodies, the membrane was incubated with immunoglobulins conjugated with horseradish peroxidase; the blots were developed using ECL reagents (Amersham, UK) and analyzed in the ChemiDoc imaging system (Bio-Rad, USA). The system is a part of the basic equipment of the "Human Proteome" project at Institute of Biomedical Chemistry. Relative quantitative analysis of immunoblot proteins was performed using the Image Lab program (Bio-Rad).

Statistical Analysis

Differences between groups were assessed using the Student's *t*-test. Differences were considered as statistically significant at $p<0.05$.

RESULTS AND DISCUSSION

The effect of α5β1 Downregulation on Proliferation and Senescence of SK-Mel-147 Cells

Increased intracellular secretion of lysosomal enzymes is a characteristic phenotypic sign of cellular senescence, which can be quantitatively evaluated

by assaying β-galactosidase (SA-β-Gal) activity. The role of α5β1 in the mechanism of senescence was assessed by changes in the activity of SA-β-Gal in a population of cells with blocked integrin expression. Downregulation of α5β1 was carried out by transducing cells with a plasmid vector carrying shRNA specific for the α5 subunit.

Figure 1A shows that transduction of SK-Mel-147 cells with α5-specific shRNA led to a two-fold decrease in α5β1 expression and induced a 4-fold increase in the number of the cell population with increased SA-β-Gal activity (Fig. 1C). These changes were accompanied by a sharp inhibition of the proliferative activity of melanoma cells (Fig. 1B) and their ability to form colonies (clonal activity) (Fig. 1D), a characteristic feature of tumor progression [21].

It should be noted that, in contrast to α5β1, the enhancement of the senescence phenotype in SK-Mel-147 cells in response to suppression of the α3β1 receptor did not affect their proliferative

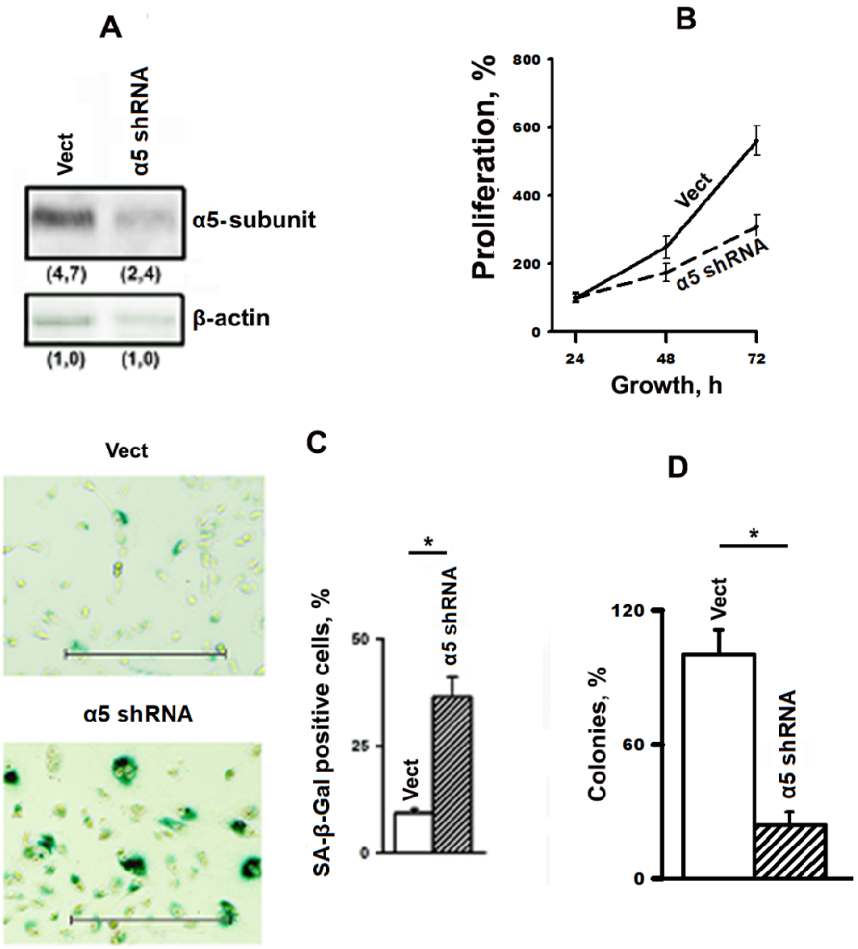

Figure 1. Downregulation of α5β1 integrin stimulates senescence in SK-Mel-147 cells. (**A**) Immunoblot analysis of the efficiency of α5β1 integrin downregulation. Cells were transduced with a vector containing non-specific (Vect) or α5-specific shRNA (α5 shRNA) as described in the Materials and Methods section. Proteins of the cell lysate (30 μg) were separated by PAAG electrophoresis followed by electrotransfer, treatment with antibodies to the corresponding proteins (1:1000 dilution), staining, and densitometry as described in the Materials and Methods section. Numbers in brackets represent signal protein densitometry data normalized to β-actin. Data from a typical experiment are presented. (**B**) The effect of α5β1 downregulation on proliferation of SK-Mel-147 cells. Cells transduced with a control vector or a vector containing α5 shRNA were passaged in 96-well plates in DMEM with serum for the indicated time, and cell number was assessed by crystal violet color. Proliferation was expressed as % of the number of cells after 24 h of growth in culture. Data represent results of three experiments (M±SEM). (**C**) The effect of α5β1 downregulation on senescence of SK-Mel-147 cells. Cells transduced with the corresponding vector were passaged in 12-well plates for 24 h, fixed, and incubated overnight at 37°C in a dye solution containing X-Gal substrate. The cells were visualized under a microscope and the number of cells containing green inclusions was counted; scale bar 300 μm. Senescence was quantified by the percentage (%) of SA-β-Gal-positive cells in the total cell population. Data represent results of three experiments (M±SEM). Here and below, an asterisk indicates statistically significant differences between the compared groups: * – $p<0.02$. (**D**) The effect of α5β1 downregulation on the clonal activity of SK-Mel-147 cells. 2000 cells transduced with the corresponding vectors were passaged in 1% semi-liquid methylcellulose in Petri dishes for 14 days as described in the Materials and Methods section. Colonies on Petri dishes were stained with crystal violet, scanned and the number of colonies was counted. The number of colonies formed by cells transduced with Vect was defined as 100%. Data represent results of three experiments (M±SEM).

activity [11], while stimulation of senescence in these cells caused by downregulation of α2β1 was accompanied by a decrease in the mitogenic activity of cells [7]. These differences between receptors of the same family are probably associated with the different effects induced by downregulation of each receptor on the downstream signaling pathway Ras/Raf/MEK/Erk that controls mitogenic activity. In our studies, downregulation of α2β1 and α5β1 led to a decrease in the expression of active forms of Erk, while downregulation of α3β1 did not affect the activity of this enzyme in SK-Mel-147 cells [6, 7].

An increase in SA-β-Gal activity is one of the manifestations of the senescence-associated secretory phenotype (SASP), i.e., excessive secretion of various metabolites, particularly, lysosomal enzymes [22]. The development of SASP, as well as inhibition and arrest of growth, depends on the strength of the stress signal, which is the disruption of matrix-cell connections due to downregulation of integrins [23]. The revealed differences in the effect of β1 integrins on the senescence phenotype of melanoma cells suggest the existence of signaling pathways specific for individual receptors that control tumor progression and are of interest in the context of targeted antitumor therapy.

The Role of the p53/p21 and Akt/mTOR Signaling Pathways in α5β1-Controlled Senescence of SK-Mel-147 cells

Cell senescence is an alternative to apoptosis, the end of their life, and both processes are mediated by the p53/p21 signaling pathway [24]. The choice between these alternatives is determined by the balance between the apoptogenic protein p53 and its effector, the inhibitor of the mitotic cycle p21. Elevated p21 stimulates senescence, while high p53 activity decreases p21 activity [24].

In recent studies, we have found that increased senescence caused by deficiency of α2β1 or α3β1 lead to a significant (about 2-fold) increase in the level of phosphorylated (active) forms of p21 and p53 proteins in SK-Mel-147 cells, but this did not increase of these cells [7, 11]. Figure 2 demonstrates that downregulation of α5β1 was accompanied by a similar stimulation of p21 and p53 in melanoma cells while their mitotic cycle analysis showed no increase in apoptosis (data not shown).

Figure 2. Immunoblot analysis of the effect of α5β1 downregulation on the expression of signaling proteins in SK-Mel-147 cells. Lysate proteins from cells transduced with Vect or α5 shRNA were separated by PAAG electrophoresis followed by immunoblotting as described in the Materials and Methods section. Numbers in brackets represent signal protein densitometry data normalized to β-actin. Data represent results of a typical experiment.

The immunoblot analysis data (Fig. 2) show that in α5β1-depleted SK-Mel-147 cells, the expression of active forms of Akt protein kinase and its downstream signaling mediators, mTOR and p70S6 protein kinases, significantly increased. It is known that the PI3K/Akt/mTOR signaling axis plays a key role in various cell reactions and, particularly, in control of the activity of p53/p21, the cell senescence effectors [25, 26]. The fundamental (canonical) function of Akt-mediated signaling consists in participation in the processes aimed at cell survival: stimulation of proliferation and growth, blockade of the mechanisms of cell death and senescence. This function is realized by Akt-mediated signaling pathways that block p53 and apoptogenic proteins of the BH3-only family [27].

However, in some stress situations, Akt exhibits "reversed" (non-canonical) activity and mediates pathways that activate p53/p21 and stimulate the development of cell senescence [25, 26, 28]. In normal cells, senescence-initiating stress signals include telomere erosion, decreased telomerase activity, DNA damage, etc.; they lead to so-called replicative senescence. Tumor cells are characterized by oncogene-induced senescence (OIS) [25, 26]. In both cell types, the senescence mechanism is based on the Akt-mediated activation of p53 and p21 [25, 28].

According to our results, senescence of melanoma cells as a response to stress caused by the downregulation of α2β1 and α3β1 receptors is also realized through the non-canonical Akt activity, the activation of these oncosuppressors [7, 11].

The activation of Akt/mTOR in SK-Mel-147 cells with reduced expression of α5β1 and an enhanced senescence phenotype, found in this study, suggests that this receptor also mediates a mechanism based on the non-canonical function of these protein kinases.

In order to test this assumption, we have investigated the effect of protein kinase inhibitors on senescence of the studied cells. Figure 3 shows that treatment with rapamycin, a specific inhibitor of mTOR protein kinase, significantly blocked senescence of SK-Mek-147 cells induced by α5β1 deficiency.

These data provide direct evidence for the non-canonical function of the Akt/mTOR signaling in the α5β1-dependent mechanism of melanoma cell senescence. They are consistent with previously obtained results on a similar role of this signaling pathway not only in senescence, but also in other manifestations of tumor progression, which are realized with the participation of β1-integrins [5–7, 10, 11]. An important feature of the Akt signaling in melanoma cells found in these studies is that different Akt isoenzymes (among three identified) can exhibit non-canonical activity depending on a particular integrin, engaged in signal transmission, and, apparently, a specific phenotypic parameter of progression, controlled by this integrin. For example, Akt1 isozyme exhibited non-canonical properties in the α3β1- and α5β1-initiated signaling pathways that control the invasive phenotype of melanoma cells, while, in the same cells, these receptors regulate anchorage-dependent apoptosis (anoikis) through the pathways in which the "reversed" (non-canonical) activity was performed by Akt2 isoform [6]. To compare, in the pathways that mediate α2β1-initiated control of anioiks in melanoma cells, a non-canonical activity was performed by Akt1 isoform [5].

In the present work, an increased level of the active (phosphorylated) form of Akt1 and, to a much lesser extent, Akt2 (Fig. 2), may be considered as indirect evidence of the non-canonical function of Akt isoforms in stimulation of SK-Mel-147 cell senescence caused by α5β1 deficiency. In order to obtain direct evidence that blockade of α5β1 stimulates the non-canonical activity of these Akt isoforms, we have investigated the effect of isozyme-specific inhibitors on the senescence of SK-Mel-147 cell. Figure 4 shows that pharmacological inhibition of the Akt1 isoform caused a 30% decrease in the proportion of cells with senescent phenotype in the total population of α5β1-deficient cells, but did not affect the phenotype of intact cells (highly expressed α5β1). The blockade of Akt2 did not affect cell senescence, regardless of the expression level of this receptor.

Since inhibitors of protein kinases close in substrate specificity can have a cross effect at certain concentrations, we have studied the effect of genetic inhibition of Akt isoforms on the senescence

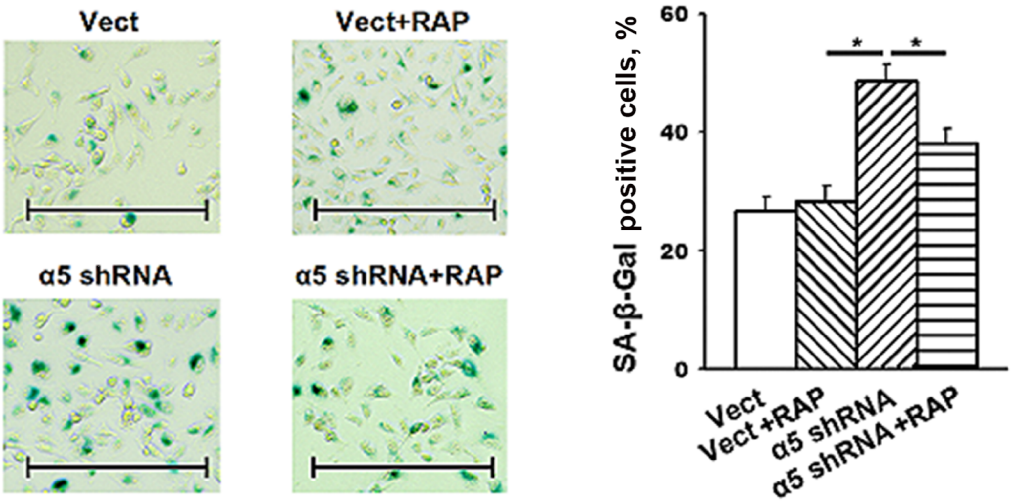

Figure 3. The effect of an mTOR protein kinase inhibitor on senescence in α5β1 downregulated SK-Mel-147 cells. Cells transduced with Vect or α5 shRNA were incubated overnight in serum-reduced medium in the presence of 200 nM rapamycin and were stained for SA-β-Gal positive cells as indicated in the legend to Figure 1. Data represent results of three independent experiments (M ±SEM); scale bar 300 μm. RAP, rapamycin.

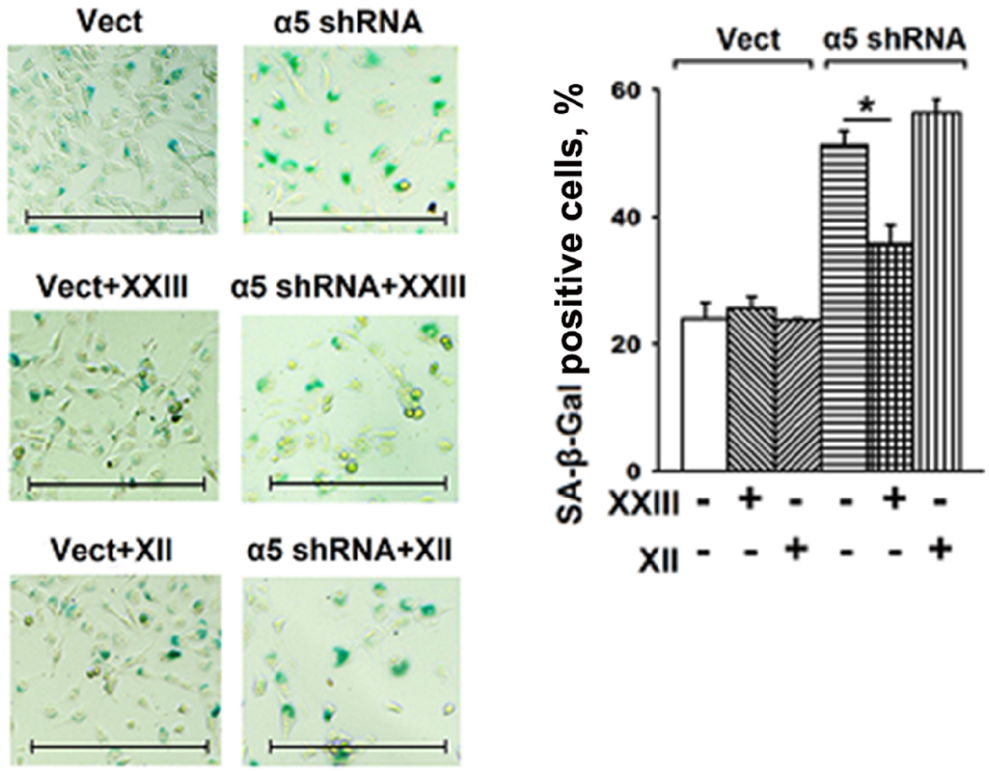

Figure 4. The effect of pharmacological inhibition of Akt isozymes on senescence of SK-Mel-147 cells. Cells transduced with Vect or α5 shRNA were treated with Akt1-specific inhibitor XXIII (3 μM) or Akt2-specific inhibitor XXII (5 μM) for 24 h at 37°C, stained to detect SA-β-Gal positive cells as described in the legend to Figure 1. Data represent results of three independent experiments (M±SEM); scale bar 300 μm.

of SK-Mel-147 cells. For this purpose, expression of isozymes was blocked in cells depleted in α5β1 by transfection with isozyme-specific shRNA. The data of immunoblot analysis (Fig. 5A) demonstrated almost complete blockade of the expression of each of the Akt isoenzymes in cells transduced with the corresponding shRNA, and a high specificity of the effect, i.e., the lack of cross-blocking during transduction of each of them.

Figure 5B shows that genetic suppression of the activity of Akt isozymes had the same effect on the senescence of intact and α5β1-depleted cells that was observed during pharmacological inhibition: blockade of Akt1 prevented the enhancement of senescence of SK-Mel-147 cells associated with a deficiency of this receptor. Downregulation of other isozymes did not affect the senescence of these cell populations.

Thus, α5β1 integrin shares with other β1-family receptors the function of protecting melanoma cells from cell senescence and, like them, implements this function by controlling the PI3K/Akt1/mTOR signaling pathway, in which Akt1 exhibits non-canonical activity. The similarity of the β1-family integrins in the signaling pathway that controls tumor progression suggests that blockade of the β1-subunit common to these receptors represents a potential approach in antitumor therapy.

CONCLUSIONS

In the model of the cultured SK-Mel-147 human melanoma cells integrin α5β1 plays a positive role in the mechanism of tumor cell protection against senescence. This mechanism involving α5β1 and other β1-family receptor mediated signaling pathways employs a non-canonical activity of protein kinase Akt1. The results of this work and previously obtained data are of interest in the context of targeted anticancer therapy.

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COMPLIANCE WITH ETHICAL STANDARDS

This work is not related to research using humans and animals as research subjects.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

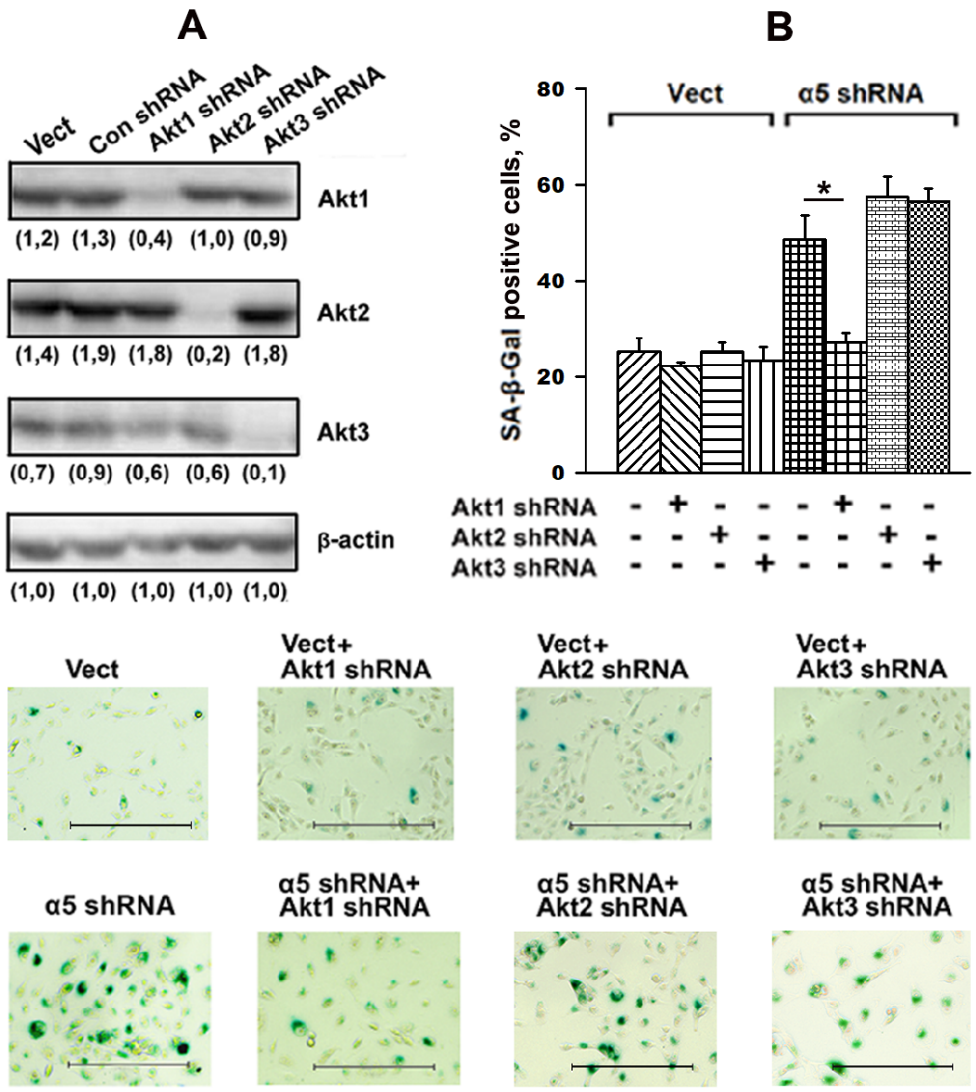

Figure 5. The effect of genetic inhibition of Akt isozymes on senescence of SK-Mel-147 cells. (**A**) Immunoblot analysis of the efficiency of cell transduction with the Akt isozyme-specific shRNA. Vect-transduced cells were transduced with vectors containing Akt1-, Akt2-, or Akt3-specific shRNAs as described in the GeneCopoeia manual. PAAG electrophoresis and subsequent processing were performed as described in the Materials and Methods section and in the legend to Figure 1. (**B**) Effect of suppression of Akt isozymes on senescence of SK-Mel-147 cells. Cells transduced with Vect or α5 shRNA were transduced with isozyme-specific shRNA followed by identification of SA-β-Gal positive cells as described in legend to Figure 1. Data represent results of three independent assays (M±SEM); scale bar 300 μm.

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РОЛЬ ИНТЕГРИНА α5β1 В МЕХАНИЗМАХ СТАРЕНИЯ КЛЕТОК SK-Mel-147 МЕЛАНОМЫ ЧЕЛОВЕКА

Н.И. Козлова, Г.Е. Морозевич, Н.М. Геворкян, Л.К. Курбатов, А.Е. Берман*

Научно-исследовательский институт биомедицинской химии им. В.Н. Ореховича,
119121, Москва, ул. Погодинская, 10; *эл. почта: 1938berman@gmail.com

Подавление экспрессии интегрина α5β1 в культуральной модели линии SK-Mel-147 меланомы человека резко тормозит фенотипические проявления опухолевой прогрессии — пролиферацию и клональную активность клеток. Наблюдаемое при этом 2-3-кратное увеличение содержания SA-β-Gal положительных клеток свидетельствовало об усилении фенотипа клеточного старения. Эти изменения сопровождались существенным ростом активности опухолевых супрессоров p53 и p21 и компонентов сигнального пути PI3K/Akt/mTOR/p70. Фармакологическое ингибирование mTORC1 снижало содержание SA-β-Gal положительных клеток в популяции SK-Mel-147 клеток, дефицитных по α5β1. Аналогичный эффект наблюдали при фармакологическом и генетическом ингибировании активности Akt1 — одного из трёх изоферментов протеинкиназы Akt; супрессия других изозимов Akt не влияла на старение меланомных клеток. Представленные в настоящей работе и ранее полученные результаты свидетельствуют, что α5β1 разделяет с другими интегринами β1-семейства функцию защиты клеток от старения и, как и они, реализует эту функцию путём контролирования сигнального пути PI3K/Akt1/mTOR, в котором Akt1 проявляет неканоническую активность.

Полный текст статьи на русском языке доступен на сайте журнала (http://pbmc.ibmc.msk.ru).

Ключевые слова: прогрессия опухолей; клеточное старение; интегрины; сигналинг; неканоническая активность протеинкиназы Akt

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