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Increase in Apoptosis by Beta-1 Integrin Blocking Through P21-Dependent and-Independent Mechanisms

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Abstract: Integrins are the primary receptors used by cells to interact with extracellular matrix. Various β_1 and β_4 integrin complexes were shown to be involved in the activation of a number of cell cyclin inhibitors. The β_4 integrin cytoplasmic domain was shown to be involved in the activation of p21 pathways of growth arrest and apoptosis. In this study, the β_1 integrin was blocked and cell were cultured on laminin as ECM, thus only the β_4 integrins binds to the ECM. This way we were able to study the mouse Clara cell cycle progression and death possible through the regulation of p21 in a controlled integrin-ECM binding environment using both wt and p21-/-mice. Upon beta-1 integrin blocking, an increase in apoptosis was observed in Clara cells from both wt and p21 ko mice at 72 and 120 h in culture, the apoptosis rate was higher ($p<0.05$) in cells from wt mice compared to cells from p21 ko mice at 72 h and a decrease ($p<0.05$) in BrdU incorporation was observed in cells from wt mice at 24 and 72 h in culture and in cells from p21 ko mice at 24 h in culture. Cytoplasmic PCNA expression was found to be higher ($p<0.05$) in cells from both wt and p21 ko mice upon beta-1 integrin blocking at 120 h and the expression of nuclear PCNA expression significantly increased ($p<0.05$). In the absence of p21, there was an increase in cytoplasmic PCNA expression but not nuclear PCNA expression. The expression of PCNA increases in cells from both wt and p21 ko mice but p21 is essential for the nuclear PCNA localisation.

Key words: Apoptosis, mechanisms, inhabtors

INTRODUCTION

Integrins are the primary receptors used by cells to interact with extracellular matrix. Both ECM and integrins play an important part in the survival, proliferation and death of cells. When an injury occurs in lung epithelial cells signals through the integrins could significantly effect whether disease or repair occur.

Laminin attaches to a number of integrins including $\alpha_1\beta_1$, $\alpha_2\beta_1$, $\alpha_3\beta_1$, $\alpha_6\beta_1$, α_7 β_1 and $\alpha_6\beta_4$ (Belkin and Stepp[1] Burkin *et al*.,[2]; Damsky and Werb[3]; Fornaro and Languino[4]; Hynes[5]; Klinowsks *et al*.,[6]; Kumar[7]; Pance *et al*.,[8]; Sannes *et al*.[9]; Sheppard[10]; van der Flier and Sonnenberg[11]; Vivinus-Nebot *et al*.,[12]). Various β_1 and β_4 integrin complexes were shown to be involved in the activation of a number of cell cyclin inhibitors (Clarke *et al*.,[13]; Dixiti *et al*.,[14]; Giancotti[15]; Miyata *et al*.,[16]; Montgomery *et al*.,[17]; Pu and Streuli[18]; Ruoslahti and Reed[19]; Schwartz and Assoian[20]; Vivinus-Nebot *et al*.,[12]). $\alpha_6\beta_4$ was shown to activate p53 function in carcinoma cells (Bachelder *et al*.,[21]) and p53 was found to inhibit $\alpha_6\beta_4$ integrin survival signalling (Bachelder *et al*.,[22]). The β_4 integrin cytoplasmic domain was shown to be involved in the activation of p21 pathways of growth arrest and apoptosis (Clarke *et al*.,[13]). T cell proliferation in the human thymus was found to be controlled via $\alpha_6\beta_4$ by laminin 5 (Vivinus-Nebot *et al*.,[12]).

$\alpha_5\beta_1$, a fibronectin binding integrin, was found to negatively regulate cell growth in the absence of attachment to fibronectin (Varner *et al*.,[23]). $\alpha_5\beta_1$ was reported to be involved in the expression of tumour suppressor $p16^{INK4a}$ upon loss of anchorage (anoikis) in a number of human cancer cells (Plath *et al*.,[24]) and also supports survival of cells by up-regulating Bcl-2 expression (Zhang *et al*.,[25]). Trypsin was also found to stimulate integrin $\alpha_5\beta_1$ dependent adhesion to fibronectin Miyata[16]. $\alpha_6\beta_1$ was shown to be an essential step accompanying the neoplastic transformation of hepatocytes Carloni[26].

Previously we have characterised the integrins in pulmony bronchioles and we found that both β_1 and β_4 were present[27]. In this study, the β_1 integrin was blocked and cell were cultured on laminin as ECM, thus only the β_4 integrins binds to the ECM. This way we were able to

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study the mouse Clara cell cycle progression and death possible through the regulation of p21 in a controlled integrin-ECM binding environment using both wt and p21-/-mice.

METHODS

Clara cell isolation and culturing: Mice (C3H/He strain or p21 ko mice either male or female, between 4 to 8 weeks old) were sacrificed by lethal intraperitoneal injection of 0.5ml pentabarbitone (Sagatal™). P21 ko mice wee kindly provided by Dr. Philip Leder, Harvard Medical Shool, Boston. Clara cells were isolated and cultured as previously described[27].

Once isolated, cell were plated onto 16-well glass chamber slides (Gibco) which had been pre-coated with appropriate laminin (50 μg mL^{-1}) and incubated at 37°, 5% CO_2/air. Cells were allowed to attach overnight after which the medium was replaced to remove dead and unattached cells. Medium was subsequently replaced every 2 days. Cells were usually fixed at days 1, 3 and 5 by methanol at-20°.

Integrin blocking experiments: Clara cells from both wt and p21 ko mice were cultured on Laminin at a concentration of 50 μg mL^{-1} and incubated with either a β_1 blocking antibody (HMβ_1) or with a cytokeratin 8 antibody as a control. Both antibodies were used at a concentration of 10 μg mL^{-1}. The β_1 integrin antibody was obtained as a gift from Prof. Hideo Yagita, Department of Immunology, Juntendo University School of Medicine, Tokyo, Japan. This antibody is now commercially available from PharMingen (Catalogue No. 553837). The HMβ_1 antibody has been shown previously to react with mouse β_1 and has an inhibitory effect on cell adhesion to ECM proteins this is a functional β_1 blocking antibody[28]. The cytokeratin 8 antibody (10526 ICN) does not have an inhibitory function.

Feulgen staining: After the culturing cells were fixed in Bouvin fixative overnight at 4°. The slides were incubated in denaturing solution (5M HCl) for 45 min at room temperature. The slides were washed for about 15 min using tap water. Slides were incubated in Schiffs reagent for 1 h at room temperature and then washed in tap water until pink colour developed. Slides were counterstained in 0.1% Light Green and mounted in Cedarwood oil and stored at 4° in the dark.

Tissue sections/antigen retrieval: Paraffin tissue sections were place in xylene for 15 min. The sections were then transferred in a series of alcohol gradients from 100% to 74% to 64% and then tap water for 10 min each. DAKO antigen retrieval solution was prepared by dilution 1:100 with distilled water. A microwave dish was filled up with antigen retrival solution and pre-heated in a microwave to boiling point. Slides that were de-waxed and rehydrated (as described above) were placed in a plastic slide rack and placed in a microwave dish containing the pre-heated antigen retrieval solution and were microwaved three times for 5 min each. The slides were allowed to cool for 30 min and then were rinsed with H_2O and equilibrated in TBS.

Immunohisto-chemistry: Slides were equilibrated in TBS for 5 min. The slides were block with an appropriate serum in which the secondary antibody was raised. PCNA antibody (P8825, Sigma) (concentration of 1/5000) was applied for 2 h, followed by three 5 min washes with TBST. Secondary antibody was put on for 30 min then washed three times for 5 min each with TBST. An Alexa conjugated secondary antibody (diluted 1:200 in serum) was put on the slide and incubated for 3 min. The slides were then washed three times for 5 min each and mounted using DAKO fluorescent mounting medium. The slides were then visualised either under the fluorescent microscope or confocal microscope.

Proliferation counts: Cultured Clara cells were exposed to medium containing 10μM BrdU for 6 h, after which they were fixed using 80% ethanol at 4° overnight. After fixation cells were rinsed in PBS for 10 min and incubated in 5M HCl for 45 min at room temperature. Cells were then washed four times in PBS for 5 min each and incubated in 1% H_2O_2 for 10 min. Cells were washed twice in PBS for 5 min and incubated in blocking solution (PBS + 20% rabbit serum + 0.05% Tween) for 10 min. The slides was drained and incubated in rat anti-BrdU antibody (Beohringer Manneheim) diluted 1:10 in blocking solution for 60 min. The slide was then washed three times in PBS for 5 min each and DAB solution (100 μl DAB + 4.8ml 0.05M Tris + 100 μl 1% H_2O_2) was added for 5 min or until the colour developed. The slides was then rinsed with water and counterstained with haematoxylin, time according to strength of solution. The slide was mounted using DAKO aqueous mounting medium.

Feulgen staining: After the culturing cells were fixed in Bouvin fixative overnight at 4°. The slides were incubated in denaturing solution (5M HCl) for 45 min at room temperature. The slides were washed for about 15 min using tap water. Slides were incubated in Schiffs reagent for 1 h at room temperature and then washed in tap water until pink colour developed. Slides were counterstained

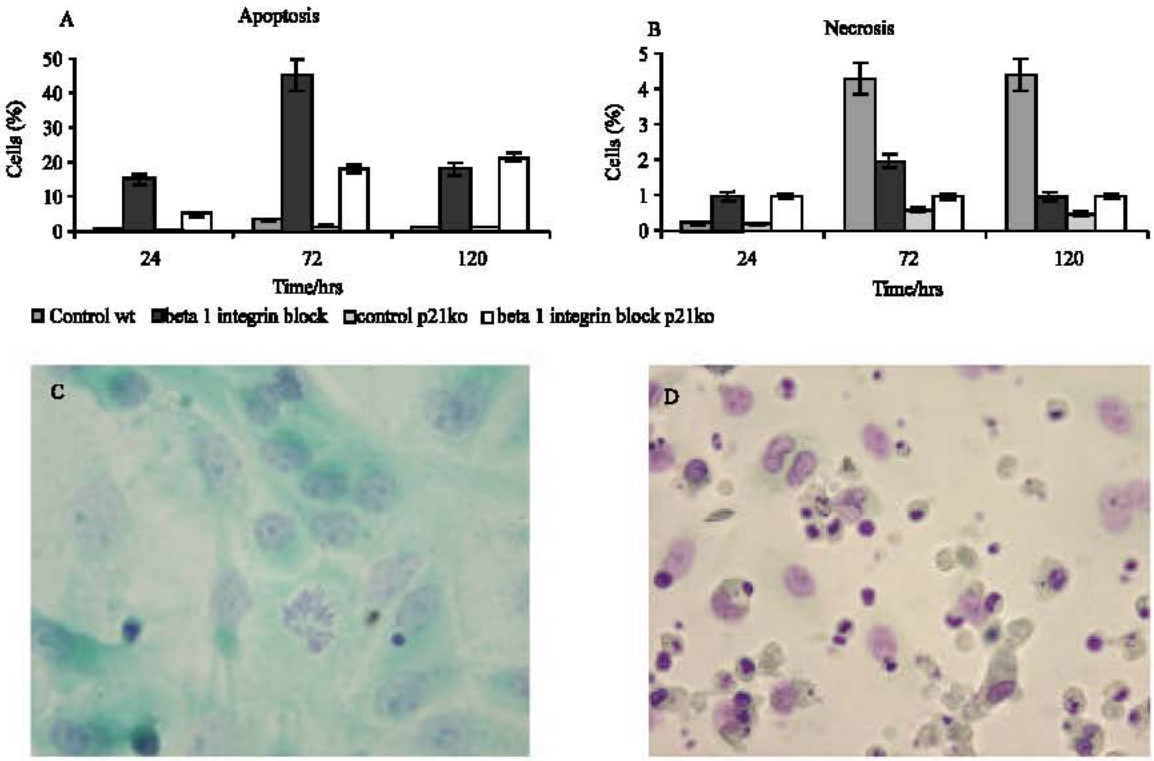

Fig. 1: Changes in Clara cells apoptosis and necrosis upon beta-1 integrin blocking in wt and p21 ko mice. A significant increase ($p<0.05$) in apoptosis was observed upon beta-1 integrin blocking in Clara cells from wt and p21 ko mice at 72 and 120 h in culture (A). No significant changes in necrosis were observed upon beta-1 block in cells from both wt and p21 ko mice (B). (D) represents the increase in apoptosis upon beta-1 blocked in cells from wt mice as compared to control (not beta-1 blocked) (C). Magnification x200

in 0.1% Light Green and mounted in Cedarwood oil and stored at 4° in the dark.

Cell counting: A wide range of variation in the degree of immunohistochemical staining was observed. Thus, strongly stained cells were considered as positive staining, while negatively or weakly stained cells were considered as negative. Experiments and counts were repeated at least three times and standard deviation was calculated using Microsoft Excel. Counts of 500 cells were sufficient to achieve a stable running mean.

Statistical analysis: Statistical analysis was carried out using Microsoft Minitab software. The general linear model test (ANOVA) with Bonferoni corrections for multiple tests, was used to find out significant changes in cell behaviour upon cell-matrix disruption and to find out differences in Clara cells from wt and p21 ko mice. Experiments and counts were repeated at least three times. For all tests a p value less than 0.05 was considered significant.

RESULTS

Necrotic and apoptotic cells were counted in primary Clara cell cultures at times 24, 72 and 120 h respectively using Feulgen staining technique (Fig. 1). A significant increase ($p<0.05$) in apoptosis was observed upon beta-1 integrin blocking in Clara cells from wt and p21 ko mice at 72 and 120 h in culture. Apoptosis was higher ($p<0.05$) in cells from wt mice compared to cells from p21 ko mice at 72 h upon beta-1 integrin blocking.

No significant differences in necrosis were observed upon beta-1 integrin blocking in both cells from wt and p21 ko mice.

Effects of beta 1 integrin blocking on clara cell proliferation in wt and p21 ko mice: Clara cell proliferation was evaluated by three different methods: BrdU incorporation, mitosis count and PCNA immunocytochemistry and counts. The expression of cytoplasmic PCNA was significantly higher ($p<0.05$) in cells from wt and p21 ko mice upon

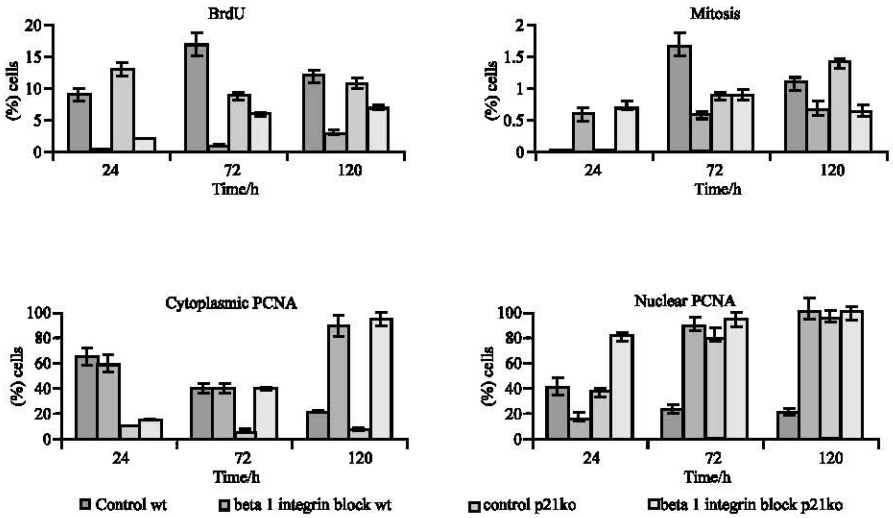

Fig. 2: Changes in Clara cell proliferation upon beta-1 integrin blocking in wt and p21 ko mice. A decrease ($p<0.05$) in BrdU incorporation upon beta-1 blocking was observed in cells from wt mice at 24 and 72 h in culture and in cells from p21 ko mice at 24 h in culture (A). No significant changes in mitosis were observed (B). Cytoplasmic PCNA expression levels increased ($p<0.05$) in cells from wt and p21 ko mice upon beta-1 blocking at 120 h in culture (C). Nuclear PCNA expression increased ($p<0.05$) in cells from wt mice upon beta-1 blocking at 72 and 120 h in culture (D)

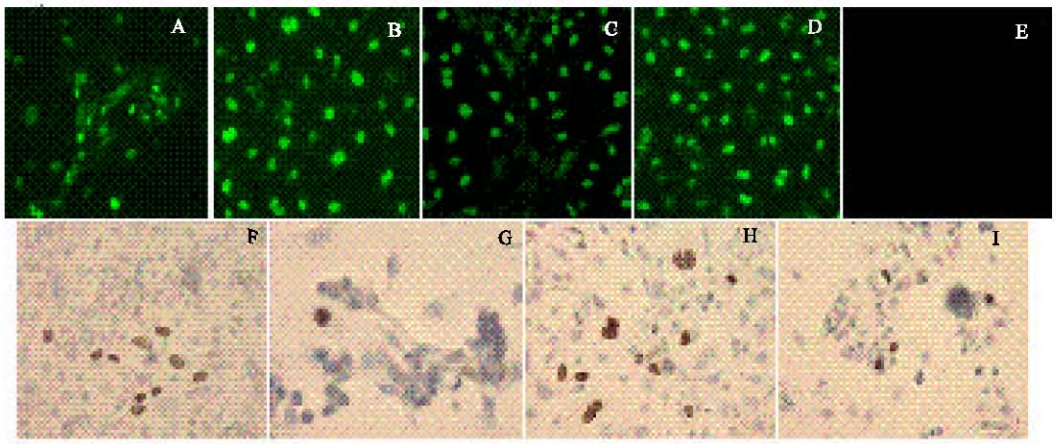

Fig. 3: Changes in Clara cell proliferation upon beta-1 integrin blocking in wt and p21 ko mice. Upon beta-1 integrin blocking, the cytoplasmic PCNA expression increased ($p<0.05$) in Clara cells from wt (B) and p21 ko mice (D) as compared to control cells (not beta-1 blocked) [wt (A) and p21 ko (C)] at 120 h in culture. Nuclear PCNA expression increased ($p<0.05$) in cell from wt mice (A) upon beta-1 blocking at 120 h in culture (B). A decrease ($p<0.05$) in BrdU incorporation upon beta-1 blocking was observed in cells from wt (G) and p21 ko mice (I) at 24 and 120 h in culture as compared to control cells [wt (F), p21ko (H)]. E is the negative control for immunofluorescence whereby primary antibody was omitted. Magnification x 200

beta-1 integrin blocking at time 120 h in culture as compared to the control (using a non-blocking antibody) The expression of nuclear PCNA increasedsignificantly ($p<0.05$) in cells from wt mice upon beta-1 integrin blocking at time 72 and 120 h in culture (Fig. 2 and 3).

A significant decrease ($p<0.05$) in BrdU incorporation was observed in cells from wt mice upon beta-1 integrin blocking at times 24 and 72 h in culture. A decrease ($p<0.05$) in BrdU incorporation was noticed in cells from p21 ko mice upon beta-1 blocking at 24 h in culture (Fig. 2 and 3) No significant changes in the mitosis were

observed both in cells from wt and p21 ko mice upon beta-1 integrin blocking.

DISCUSSION

Apoptosis rate increases upon beta-1 integrin blocking: Upon beta-1 integrin blocking, an increase in apoptosis was observed in Clara cells from both wt and p21 ko mice at 72 and 120 h in culture. This result was expected since a number of studies have shown that when cells are detached from substrate or anchorage is presented they usually undergo apoptosis[19,29,30,2,31,32-37,17,13]. Thus disruption of Clara cell- beta-1 integrin interactions results in apoptosis.

Upon beta-1 integrin blocking, the apoptosis rate was higher ($p<0.05$) in cells from wt mice compared to cells from p21 ko mice at 72 h. In the absence of p21, there was a reduction of the apoptosis rate, thus p21 seems to induce apoptosis in Clara cell culture upon beta-1 integrin blocking. p21 has been previously shown to be able to both induce[38,39] and inhibit apoptosis[40-43].

Beta-1 integrin is involved in Clara cell proliferation: Upon beta-1 integrin blocking, a decrease ($p<0.05$) in BrdU incorporation was observed in cells from wt mice at 24 and 72 h in culture and in cells from p21 ko mice at 24 h in culture. Thus the cell-beta-1 integrin interaction could be promoting proliferation in Clara cells and disruption of this interaction could result in a decrease in proliferation. β_1 integrin complexes has been previously shown to be involved in cell proliferation[16,23,25,26]. $\alpha_5\beta_1$, a fibronectin binding integrin, was found to decrease cell proliferation in the absence of attachment to fibronectin and that ligation of this receptor with fibronectin reverses this process[23]. Trypsin was shown to stimulate the integrin $\alpha_5\beta_1$ dependent adhesion to human gastric carcinoma cells to fibronectin and increases cell proliferation[16]. Since trypsin was used to isolate Clara cells, the $\alpha_5\beta_1$ integrin complexes could have been stimulated but since beta-1 integrin was blocked and thus was not bound to a suitable receptor, there was a decrease in cell proliferation.

PCNA expression is increased upon beta-1 integrin blocking but PCNA requires p21 for its nuclear localisation: Interesting results were obtained with regards to the PCNA expression. Cytoplasmic PCNA expression was found to be higher ($p<0.05$) in cells from both wt and p21 ko mice upon beta-1 integrin blocking at 120 h. The expression of nuclear PCNA expression significantly increased ($p<0.05$) in cells from Clara cells from wt mice only upon beta-1 blocking at 72 and 120 h in culture. In the absence of p21, there was an increase in cytoplasmic PCNA expression but not nuclear PCNA expression. Thus upon beta-1 blocking the expression of PCNA seems to be increase in cells from both wt and p21 ko mice but p21 is essential in the nuclear PCNA localisation. The role of p21 in the nuclear localisation of PCNA and the importance of this complex in cell cycle progression have been previously described.

CONCLUSION

Cell-beta 1 interactions were described as a potential factor in cell proliferation. The lack of binding of beta-1 integrin to an appropriate receptor could lead to a decrease in cell proliferation.

Beta-1 integrin disruption could also lead to an increase in PCNA expression but for an increase in nuclear PCNA p21 was shown to be an essential factor. Thus as originally hypothesised, integrins that bind to laminin are important for cell cycle progression through p21-dependent or -independent mechanisms.

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