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Integrin characterization in pulmonary bronchioles

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Abstract

Integrins are a family of cell surface glycoproteins that act as receptors for ECM proteins or for membrane bound counter-receptors on other cells. The integrin receptor family of vertebrates includes at least 16 distinct α subunits and at least 8 β subunits which can associate to form more than 20 distinct integrins. So far, there are no published reports describing integrin characterization in mouse lung tissue and mouse Clara cells. This paper described the characterization of six integrins, mainly α_5, α_v, α_6, β_1, β_3, and β_4, in mouse pulmonary bronchioles and also in Clara cell cultures. α_5, α_v, α_6, β_1, and β_4 integrins were present in Clara cells both in tissue sections and cultures. β_3 integrin was found to be absent in mouse Clara cells.

Keywords: Clara cells; Integrin; Pulmonary bronchioles

Introduction

Integrins are a family of cell surface glycoproteins that act as receptors for ECM proteins or for membrane bound counter-receptors on other cells. Integrin mediated cell–ECM adhesion sites are complex specialized structures termed focal contact or focal adhesions (Aplin et al., 1998). Each integrin is a heterodimer that contains an α and a β subunit with each subunit having a large extracellular domain, a single membrane spanning region, and in most cases (other than β_4), a short cytoplasmic domain (Frisch and Ruoslahti, 1997; Hynes, 1992, 2000). The integrin receptor family of vertebrates includes at least 16 distinct α subunits and at least 8 β subunits which can associate to form more than 20 distinct integrins (Coraux et al., 1998; Frisch and Ruoslahti, 1997; Hynes, 1992; Ingber, 1991; Liapis and Hutton, 1997; Mizejewski, 1999; Schwartz, 1997).

The integrins expressed in diverse cell types display different ligand specificities. In addition, during dynamic and complex processes, such as organ development and tumor progression and metastasis, the cellular distribution and/or intensity of expression may change (Arroyo et al., 2000; Boudreau and Jones, 1999; Brown, 2000; Coraux et al., 1998; Dedhar and Hannigan, 1996; Felding-Habermann et al., 2001; Frisch and Ruoslahti, 1997; Giancotti, 1997; Hemler, 1998; Howe et al., 1998; Hynes, 1992; Ingber, 1991; Legier et al., 2001; Schwartz, 1997; Shyy and Chien, 1997).

There are no published reports describing integrin characterization in mouse lung tissue and mouse Clara cells. Six integrins were investigated: α_5, α_v, α_6, β_1, β_3, and β_4.

Materials and methods

Isolation and culturing of mouse bronchiolar cells

Clara cells were isolated using a modification of methods described previously (Belinksy et al., 1995; Masek and Richards, 1990; Oreffo et al., 1990; Richards et al., 1990; Van Scott et al., 1987).

In brief, 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 0.5 ml pentabarbitone

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(Sagatal™). The ventral surface skin was removed, and a midline incision was made to allow entry into the peritoneal cavity. The gastrointestinal tract was displaced to the right and the major dorsal blood vessels cut. The position of the trachea was located, and a cannula (1 mm Luer cannula, CAN1004, Scientific Lab. Supplies) was inserted from a partial cut at the top of the trachea. The diaphragm was carefully opened by a small incision just below the xiphisternum followed by widening this cut in both directions. The rib cage was removed, and care was taken not to puncture the lungs. A portion of the thymus was removed for easier access to the vessels around the heart.

The lower region of the heart was gripped with a hemostat, and a cannula (1.7 mm Luer cannula, CAN1008, Scientific Lab. Supplies) was inserted into the pulmonary artery through an incision in the pericardium. Sterile saline (0.15 M NaCl) was gravity fed through the cannula, causing an increase in size of the left atrium. An incision was made in the left atrium to allow fluid exit. Lungs were artificially ventilated with a 1.0 ml syringe of air. After 3 ventilations, the lungs were totally free of blood and appeared completely white. The heart was detached and discarded.

A syringe containing preheated trypsin at 37°C was attached to the cannula, and trypsin (T8003, Sigma) was intratracheally instilled. The lungs were detached at the bottom by cutting the esophagus, the posterior vena cava, and any strands of tissue holding them to the diaphragm, and then the whole preparation was turned around so as to work from the top of the trachea. The lung was freed and removed intact from the cavity. The lung was cleaned from any remains of esophagus and any other debris. The lung was transferred into a vial containing preheated phosphate buffer saline (PBS) at 37°C and incubated for 15 min at 37°C.

From the point onwards, the isolation was carried out in a sterile laminar flow tissue culture hood (Class II) using aseptic techniques and sterile solutions. Digested lungs from 8 mice were place in a sterile plastic petri dish and chopped with sterile scissors to small pieces of about 1 mm. Fetal bovine serum (2 ml/lungs) was added to the minced lungs to inhibit further trypsin activity. The minced lungs were transferred to a 50 ml centrifuge tube, and DNase I solution (3 ml/lungs) was added. Treatment of DNase I reduce viscosity. The mixture was hand shaken for 4 min. The suspension was filtered through 150 μ and 30 μ nylon filters and topped-up to 50 ml with DNase 2 solution. The tube was centrifuged at low speed of 32*g* at 10°C for 6 min in order to obtain functionally active Clara cells. The supernatant was removed, and 50 ml of DNase 2 solution was added to the pellet at the bottom of the tube. The tube was hand shaken for 1 min and centrifuged at 32*g* at 10°C for 6 min.

The supernatant was removed, and 10 ml medium (1:1 M199/HamF12, 1% glutamine, 1% streptomycin) was added and hand shaken for 1 min. At this point, the isolation consists predominantly of bronchiolar cells, macrophages, and fibroblasts. The latter two cell types have an increased capacity for adherence than epithelial bronchiolar cells. The suspension was transferred to a sterile non-tissue culture petri dish and incubated at 37°C for 2 h at 5% CO_2 in air to allow differential attachment.

The suspension was transferred to a 50 ml centrifuge tube and centrifuged at 100×*g* for 5 min at 10°C. The supernatant was removed, and an appropriate culture medium (1:1 mixture of Hams F12 (Gibco) and M-199 medium (Gibco) supplemented with 2 mM L-glutamine, 10 μg/ml insulin, 5 μg/ml transferrin, 100 ng/ml hydrocortisone, 10 ng/ml EGF, 0.1 ng/ml retinyl acetate, and Pen/Strep. (Belinksy et al., 1995; Masek and Richards, 1990; Van Scott et al., 1987; Van Winkle et al., 1996) was added to an approximately 1×10^4 clumps/ml. 50 μl aliquots were taken for an approximate cell count.

Cells were counted using a hemocytometer. However, this apparatus is designed primarily for quantification of single cell suspension. Isolated Clara cells are usually in clumps and with typical 'bunch of grapes' morphology. Thus, for cell estimation, the number of clumps and individual cells were recorded and an estimate made of total cell number.

Once isolated, cells were plated onto 16-well glass chamber slides (Gibco) which had been pre-coated with 50 μg/ml fibronectin and incubated at 37°C, 5% CO_2 in 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 fixed at time 24, 72, and 120 h by methanol or methocarn (100 ml methanol; 50 ml chloroform; 25 ml glacial acetic acid) at −20°C.

Lung tissue sections

Lung tissues were fixed in buffered formalin for 16 h and then processed to paraffin wax in a vacuum impregnating processor. Paraffin embedded sections were cut on a microtone to a thickness of 3 μm.

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.

Antigen retrieval. Two methods of antigen retrieval were used: trypsin antigen retrieval and microwave antigen retrieval.

a. Trypsin antigen retrieval. A glass slide container was prewarmed in 37°C incubator. Trypsin solution (100 mg trypsin, 100 mg calcium chloride, 100 ml H_2O, adjusted to pH 7.6 using 0.2 M Tris) was prewarmed for 5–10 min in a 37°C in a water bath. Slides that were de-waxed and rehydrated (as described above) were placed in glass slide container containing the pre-warmed trypsin for 30 min at

Table 1
Integrin characterization

	α_6	α_5	α_v	β_1	β_3	β_4
Tissue sections	++	++++	+++	+++	0	++++
24 h	++	++++	+++	+++	0	++++
72 h	+	+++	+++	+++	0	++++
120 h	+	++++	+++	+++	0	++++

Key: ++++, very strong positive. +++, strong positive. ++, moderately positive. +, weak positive. ±, very weak positive. 0, no stain.

37°C. Then, slides were rinsed with H_2O and equilibrated in Tris-buffered saline (TBS).

b. Microwave antigen retrieval. DAKO antigen retrieval solution was prepared by dilution 1:100 with distilled water. A microwave dish was filled up with antigen retrieval solution and preheated 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 preheated 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/cytochemistry studies

Most of the antibodies, mainly α_5, α_v, α_6, β_1, and β_3, were obtained as a gift from Prof. Hideo Yagita, Department of Immunology, Juntendo University School of Medicine, Tokyo, Japan, while β_4 antibody was commercially available from Chemicon. The β_1 antibody obtained from Prof. Yagita is now commercially available from PharMingen (Catalogue No. 553837), and this is a functional β_1 blocking antibody (Burns et al., 2001; Noto et al., 1995). β_4 antibody (AB1922, Chemicon) was used at a concentration of 1/100, while α_5, α_v, α_6, β_1, and β_3 antibodies were used at a concentration of 1/10.

Slides were equilibrated in TBS for 5 min. The slides were blocked with an appropriate serum in which the secondary antibody had been raised. Primary antibody at a concentration specified in the Table 1 was applied for 2 h followed by three 5 min washes with Tris-buffered saline Tween-20 (TBST). Secondary antibody was put on for 30 min then washed three times for 5 min each with TBST and then visualized using the ABC kit. For immunofluorescence, a secondary antibody conjugated to an Alexa™ dye was used as described below. A negative control was carried out by using TBS instead of the primary antibody.

After the primary antibodies, the slides were washed three times of 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 visualized using either a ultraviolet (UV) microscope or confocal microscope.

Cell counting. In a wide range of variation, the degree of immunocyto/histochemical staining was observed. Thus, strongly stained cells were considered as positive staining,

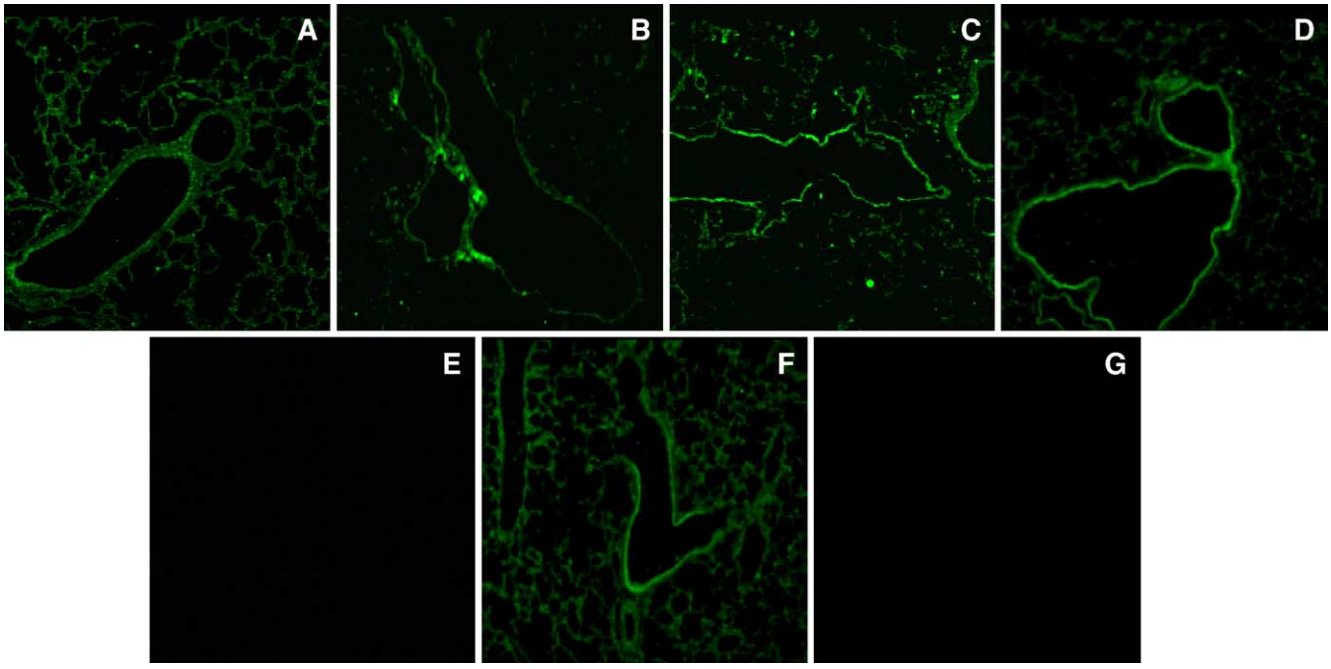

Fig. 1. Integrin characterization in mouse bronchiolar region of lung tissue sections using Alexa™ 488 conjugated secondary antibody. Integrins α_5, α_v, α_6, β_1, and β_4 were present in mouse small airways (A, B, C, D, and F respectively), while integrin β_3 was absent (E). Panel (G) shows a typical negative control, whereby the primary antibody was omitted. Magnification ×200.

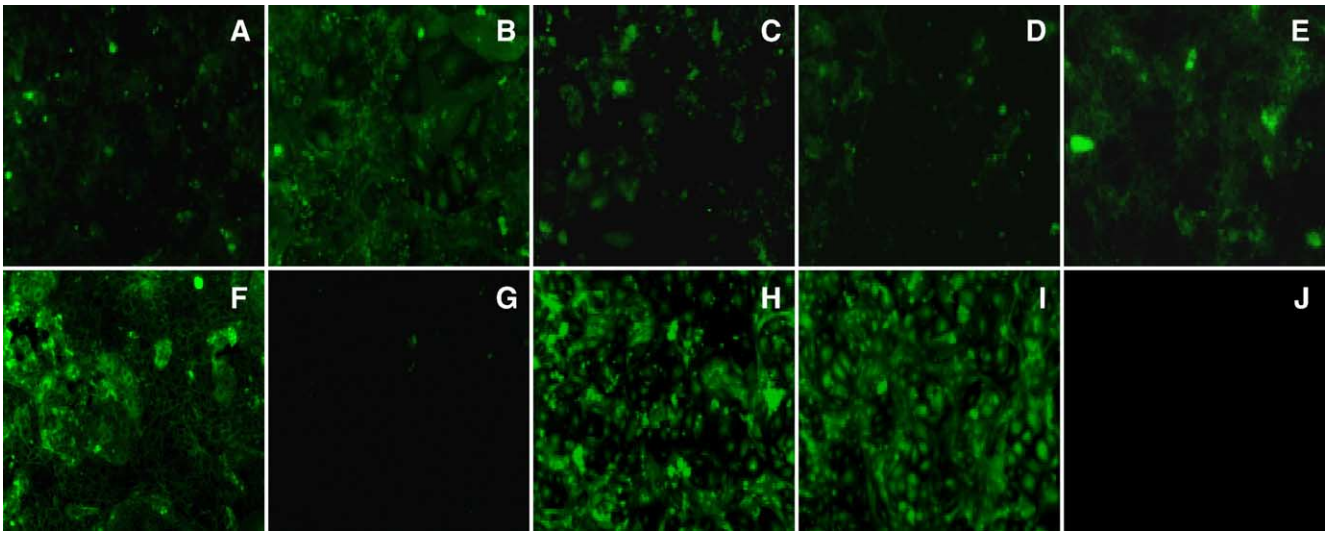

Fig. 2. Integrin characterization in Clara cell cultures using Alexa™ 488 conjugated secondary antibody. An increase in the expression of α_5 integrin was observed in Clara cells from 48 h (A) to 72 h (B) in culture. A decrease in the expression of α_6 was observed from 24 h (C) to 120 h (D). No changes in the expression of integrins α_v (E), β_1 (F), and β_4 (H and I). β_3 integrin was not expressed in primary Clara cells at all time points (G). Panels (E, F, I, and G) are Clara cells at 120 h in culture; (H) Clara cells at 72 h. Panel (J) is the negative control, whereby the primary antibody was omitted. Magnification ×200.

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.

Results

α_v, α_6, β_1, and β_4 were all present in both mouse lung tissue sections and in primary Clara cell culture. The integrin β_3 was absent in both mouse lung tissue (Fig. 1) and primary Clara cell culture (Fig. 2). Although various modifications were carried out (as explained above), the integrin β_3 was still absent. The absent β_3 integrin in mouse lung and Clara cells could be either due to the non-reactive nature of the antibody or because integrin β_3 was not present. The latter hypothesis is more plausible since β_3 integrin is absent from the human bronchiolar region (Pilewski et al., 1997).

Discussion

Clara cells have been successfully isolated and cultured in serum free medium for up to 120 h. Most of the cells were healthy looking Clara cells, the characteristics of which were described previously in McBride et al. (2000).

Various conditions of antigen retrieval techniques were evaluated in order for the detection to be successful. The best results were obtained when the microwave antigen retrieval method was used on methocarn fixed tissue sections. The use of microwave heating on formalin fixed tissue for integrin detection has not been successful (Cattoretti et al., 1993), with the exception of a relatively recent report (Gladson et al., 1996; Liapis and Hutton, 1997). Liapis and Hutton (1997) were still unable to detect specific immunoreactivity with several antibodies. A number of alternative buffers (Alsbeh and Battifora, 1995), varying the buffer pH (Grossfeld et al., 1996), or use of newly developed tissue fixatives (Muller et al., 1996) have been described. Even though the exact mechanism by which microwave heating works is still unclear (Shi et al., 1991, 1995), signal detection for many commonly used antibodies is superior compared to other methods such as autoclave or water bath (Tani and Phillips, 1995).

Six integrins were investigated α_5, α_v, α_6, β_1, β_3, and β_4. α_5, α_v, α_6, β_1, and β_4 integrins were present in Clara cells both in tissue sections and cultures. β_3 integrin was found to be absent in mouse Clara cells. β_3 integrins have been previously described to be absent in human bronchiolar epithelium (Damjanovich et al., 1992; Pilewski et al., 1997). During the culture, there seems to be a decrease in α_6 from 24 h to 120 h in culture. There was a decrease in α_5 integrin expression from 24 h to 72 h in culture.

In conclusion, functional Clara cells have been isolated and cultured in serum free medium for up to 120 h. Clara cells express α_5, α_v, α_6, β_1, and β_4 integrins, but β_3 subunit was absent in both tissue sections and cultured cells.

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