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Beta-4 Integrin Transfection, Cloning and Functional Assay in Squamous Cell Carcinoma

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Beta-4 Integrin 유전자 주입, 클로닝과 편평상피암에서의 Beta-4 Integrin 기능에 관한 연구

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서 론 : Laminin의 수용기로 알려진 Integrin α6β4의 세포내 표현 정도는 편평상피암을 위시한 여러 악성종양의 전이능력 및 예후와 밀접한 상관관계가 있다고 알려져 있다. 이 Integrin은 Laminin과 같은 세포외 리간드와 결합하면 상피세포의 기저막 지주 구조물인 hemidesmosome의 세포체질 요소(cytoskeletal element)와 연관되어 그 결과 세포의 기저막과 세포내 케라틴을 연결하는 역할을 한다. Integrin α6β4는 구조적으로 다른 많은 integrin들과 달리 β4의 세포질내 영역(cytoplasmic domain)이 특징적으로 크다. 이 세포질내 영역 β4 integrin의 기능은 아직 밝혀지지 않고 있으나 아마 세포 성장의 신호전달 및 악성종양의 특징인 침윤, 전이에 관련할 것으로 보아지고 있다.

재료 및 방법 : 저자들은 우선 β4 integrin의 wild type c-DNA와 β4의 세포질내 영역(cytoplasmic domain) 및 β4의 tyrosine 인산화 반응 부위가 각각 결손된 c-DNA를 PCR을 통하여 합성하여 pRc/CMV 벡터에 삽입한 후 원래 β4 integrin의 발현이 결핍된 인간 방광암 세포에 Calcium phosphate precipitation 방법으로 주입(transfection)시켜 형질변환된 세포를 면역형광법, Flow cytometry 및 Immunoprecipitation 방법으로 클로닝하여 wild type β4-full length(Clone FL), truncated β4-cytoplasmic domain(Clone CD), 및 mutated β4-tyrosine phosphorylation site (Clone M)을 얻었다. 암 세포의 부착 및 침투 능력의 기능적 연구로 모노 클로날 항체와 fibronectin, laminin, Matrigel을 단백질 기질로 사용하였으며 결과 비교를 위하여 pRc/CMV 벡터만 주입시켰던 클로운과 방광암 세포주를 β4 integrin 음성 대조군으로 또한 이 Integrin의 높은 발현을 보이는 두경부 편평상피암 세포주를 양성 대조군으로 이용하였다.

결 과 : 세포 부착능력에 있어서 온전한 β4 cytoplasmic domain이 존재하는 클로운이 laminin에 강한 부착능력을 보였으나 fibronectin의 부착정도는 β4 integrin의 표현정도와 관계없이 모든 클로

운에서 비슷하였다. Matrigel을 투과하는 암세포 침윤 능력에서는 β4 integrin의 표현이 존재하는 클로운들이 투과 능력이 높았으나 세포외 리간드가 없는 control membrane을 사용하였을 때와 비교하여 투과능력의 차이를 보이지 않았다.

결 론 : 유전자 주입(transfection) 방법으로 β4 integrin의 다양한 클로운의 합성이 가능하여 이 Integrin의 암 세포의 부착 및 침투 능력에서의 기능을 규명할 수 있게 한다. β4 integrin은 편평상피 암세포의 부착에 있어서 세포외 리간드 laminin과 특이 결합하여 부착 능력을 높이는 중요한 역할을 하며, 편평상피 암세포의 침투에 있어서는 β4 integrin의 표현이 침투 능력을 높이는 역할을 하나 이 때에는 laminin과 같은 리간드와의 특이 결합에 의존하지는 않는 것으로 사료된다.

중요 단어 : Integrin α6β4 · Laminin · β4 integrin · Transfection · Clone.

Introduction

Tumor biologists have long appreciated that cell adhesion molecules including integrins, CAMs, cadherins and selectins, play an important role in tumor cell invasion and metastasis. Integrins are a large family of homologous cell surface receptors that mediate cell-matrix and cell-cell adhesion and serves as transmembrane linkers between the extracellular matrix and cytoskeleton. More recently, it has become clear that integrins are not simply sticky molecules ; rather, they are true receptors capable of generating intracellualr signals for the regulation of cell growth and differentiation in tumor cells.

Integrins bind to a variety of extracellular matrix proteins, including fibronectin, laminin and collagens. Among them, the α6β4 integrin is a laminin/kalinin receptor[1)2)] and the only known integrin found in hemidesmosome, the anchoring structure in the basal membrane of epithelial cells[3)]. As for binding to an extracellular ligand(laminin), α6β4 associates with cytoskeletal elements of hemidesmosomes(intermediate filament), thereby linking the basement membrane to the keratin filament system. The β4 cytoplasmic domain is unusually large and contains toward its C-terminus two pairs of type Ⅲ fibronectin-like repeats and is necessary for localization to hemidesmosome[4-6)], but not for association with α6[5)].

Laminins play an active role in tumor progression and not simply as a passive barrier that impedes stromal invasion and carcinoma interactions with laminins are mediated largely by integrin[7-10)]. At least nine different integrins can function as laminin receptor[9)] but our interest has focused on the α6 integrins : α6β1 and α6β4. The α6β1 integrin, which is expressed in most cell types, links extracellular laminins with the actin cytoskeleton and it can be associated with focal adhesions[11)12)]. The α6β4 is a receptor not only for laminin-1, but also for several other isoforms as well[2)5)9)]. In the hemidesmosomes of keratinocytes, α6β4 interacts with laminin-5 and links them indirectly to the cytokeratin network[13)]. Laminin binding to α6β4 causes activation of the associated kinase and consequently tyrosine phosphorylation of the β4 subunit cytoplasmic domain to influence cell proliferation and differentiation[14)].

In this study, we sought to gain insight into the function of α6β4 by studying the behavior of the clones made by the transfection of UC-2 cells, which are β4-deficient urinary bladder carcinoma cells, with wild type β4-full length cDNA(Clone FL), truncated β4-cytoplasmic deletion cDNA(Clone CD), β4-tyrosin phosphorylation site mutation cDNA(Clone M), and pRc/CMV vector only(Clone CMV).

We then compared the above clones with UM-SCC-22B cells, which comprise the squamous carcinoma cell line derived from human hypophayrngeal cancer, that express high surface levels of this integrin. The data obtained revealed that α6β4 expression increases the adhesive strength of these cells on laminin matrices, which appeared to require an intact β4 cytoplasmic domain. However, regardless of the α6β4 expression, all the cells showed similar adhesive

ability on fibronectin, suggesting the ligand-specific role for α6β4 in increasing cell adhesion. And the expression of α6β4 facilitated the cells' ability to invade the Matrigel matrices, and it also increased invasiveness on the control membrane, which has no ECM protein, suggesting a ligand-independant role for α6β4 in promoting cell invasion.

Materials and Methods

1. Cells and cell culture

Human Squamous cell carcinoma cell line UM-SCC-22B was established in our laboratory[15] and was used as an α6β4 positive control. The human bladder carcinoma cell line UM-UC-2[16,18] was used for β4 transfection. Cells were maintained in Dulbecco's modification of Eagle's minimal essential medium supplemented with 2mL L-glutamin, 1% nonessential amino acids(Sigma Chemical company, St. Lo-uis, MO), penicillin(100μg/ml), streptomycin(100μg/ml), and 10 % fetal calf serum(Hyclone Laboratories Inc., Logan, UT)(M10 medium). Confluent cultures were passaged using porcine trypsine(0.1% wt/vol)(Sigma) and 0. 0002% EDTA in Puck's saline A.

2. Monoclonal Antibodies and Proteins

Hybridoma supernatants containing anti-β4 monoclonal antibody UM-A9[19], anti-α6 antibody GoH3[16], and anti-β1[20] were used . Human fibronectin(Gibco-BRL, Gaithersburg, MD), mouse laminin-1, and Matrigel(Becton Dickinson, Bedford, MA) were used as substrates for adhesion and invasion assay.

3. Expression constructs

The pRc/CMV eukaryotic expression vector containing the full length β4 subunit cDNA termed pCMV/β4 FL was obtained from Dr. Fillipo Giancotti[4,5]. This vector(Invitrogen, San Diego, CA) contains the cytomegalovirus(CMV) promotor which derives high expression of the mammalian gene, and the neomycin resistant gene. In addition, a β4 cDNA with a mutation at the tyrosine phosphorylation site was generated using a site-directed mutagenesis strategy. To first get a PCR product containing a mutation on the tyrosine phosphorylation site corresponding to nucleotides 4936-4938(amino acid 1604 : TAC) of the β4 cDNA located between Not I and Xba I site, four oligonucleotide primers were prepared : primer A containing Not I site(5'-TTGTGCGA GCGGCCGCTGC), primer B(5"-GTCACCAGGAA GCCGAC), primer C(5'-GTCGGCTTCCTGGTGAC) incorporating one base change to induce amino acid changes from tyrosine(TAC) to phenylalanine(TTC) and primer D containing Xba I site(5'-GCCCTCTA-GAACTAGTGG). Two sets of PCR : PCR 1 using primer A and B and PCR 2 using primer C and D were performed separately. The products were separated from excess primers, mixed, denatured and allowed to anneal. DNA chain extension on the recombinants with recessed 3' ends lead to a molecule that can be amplified with the original outside PCR primers to extract the desired, combined DNA fragments. These PCR products were then subcloned into a pGEM-T vector which could efficiently ligate PCR products, adding 3' terminal thymidine to both ends, and cleaved with Not I and Xba I to give a 1066 base-paired β4 insert with desired mutation. To ensure that amplified products of the expected size represented the target sequence, the products were digested with the restriction enzymes known to cut within the target sequence. After verifying the PCR products of this restriction analysis by electrophoresis examination on 6% polyacrylamide gels stained with ethium bromide and viewed under UV ilumination, the β4 tyrosine phosphorylation-defective mutant(β4 M) was constructed following digesting with Not I and Xba I from pGEM-T vector and ligating into the pRc/CMV vector via unique Not I and Xba I site in this vector to produce pCMV/β4 M. The β4 cDNA with a truncated cytoplasmic domain(amino acid 854-1752) termed pC MV/β4 CD was also obtained from Dr. Fillipo Giancotti(Fig. 1).

4. Transfection and cloning of the β4 integrin subunits

UM-UC-2 grown in M10 was transfected with

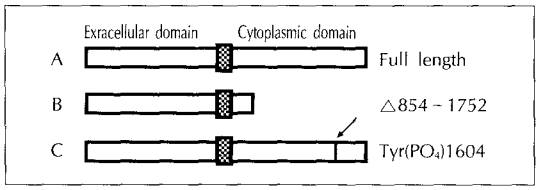

Fig. 1. Schematic diagram of the cDNA-encoded β4 integrin.
A : Full length(wild type)
B : Cytoplasmic deletion(truncated)
C : Tyrosine phosphorylation site-mutated

20μg of cDNA of pCMV/β4 FL, pCMV/β4 M, pCMV/β4 CD, and pRc/CMV vector alone by the calcium phosphate precipitation method[4-6]. Clones expressing the neomycin resistant gene were selected in 1000μg /ml geneticine(G418, GIBCO, Grand Island, NY), a concentration previously determined to kill the parental UM-UC-2 cells within 7 days. Resistant colonies were transferred to 24-well plates, then to coverslips in 6-well plates and tested for β4 expression using immunoflurorescence. Flow cytometry was used to assess the intensity of surface β4 expression. After selection of the clones exhibiting positive β4 expression from immunoflurorescence and flow cytometry, these positive clones were subcultured and tested by immunoprecipitation. Clones were frozen at early passage and while in culture the cells were fed periodically with G418 medium to prevent the outgrowth of revertant clones.

5. Immunofluorescence(IF) staining and Flow Cytometry

The relative level of expression of β4 was compared by IF staining and flow cytometry. For IF, cells were grown on coverslips or in 8-well Lab-Tek chamber slides(Nunc, Naperville, IL). The cells were washed in cold phosphate buffer saline(PBS) containing 0.9 mM $CaCl_2$ and 0.5 mM $MgCl_2$, incubated with 3% normal goat serum(Vector Laboratories, Brulingame, CA) for 45 minutes, washed three times, and incubated with primary antibodies(A9) for two hours. After washing, specimens were incubated for 45 minutes with rhodamine(TRITC)-conjugated affinity purified $F(Ab')_2$ fragment goat anti-mouse IgG (Accurate Chemicals & Scientific Co., Westbury, NY) diluted 1 : 200. All incubations were at 4℃ on a rotating platform. After fixing in cold acetone for three minutes, coverslips were mounted using a vectashield medium(Vector). For flow cytom-etry, cells were detached in trypsine/EDTA, collected in M10, counted, aliquoted at 1×10^6 cells per tube, and washed once in PBS containing 0.5% BSA(PBS-B). The cells were incubated with 100μl of primary antibody(A9) for 45 minutes on ice. After washing in PBS-B, 100μl of fluorescein(FITC)-conjugated affinity purified $F(Ab')_2$ fragment goat anti-mouse IgG (Accurate)(1 : 20) were added for 30 minutes on ice. Then cells were washed, resuspended in 12×75mm tube in 0.5ml of MEN with 10% FCS without phenol red and analyzed by flow cytometry(Coulter, Hialeah, FL) calibrated with standard be-ads at an excitement wave length of 488nm.

6. Immunoprecipitation

Cells were metabolically labeled for 4 hours with 100μCi/ml of S-35 labeled methionine(NEN, Dupont, Wilmington, DE) in methionione-free medium as described previously[19]. Labeled cells were rinsed with PBS and harvested by scraping in 200μl lysis buffer(1% NP-40 in PBS) containing protease inhibitor[16]. After 30 minutes on ice, the lysates were clarified by centrifugation for 5 minutes at 2000rpm at 4℃, mixed with 100μl PBS containing 1% BSA and 300μl radio-immunoprecipitation assay buffer (RIPA)(1% NP-40, 50 mM Tris-CL pH 8.0, 150 mM NaCl, 0.1 % sodium deoxycholate, 0.1% SDS, and 1mM PMSF), precleared twice with 30μl of protein A sepharose beads(Sigma), and incubated overnight at 4℃ with primary antibody A9. Antigen-antibody complexes were incubated for 2 hours at 4℃, washed three times in high salt RIPA(500 mM NaCl) and once in 150 mM saline RIPA. The samples were boiled for 3 minutes in reducing buffer(60μl containing 5% mercaptoethanol, 2% SDS, 10% glycerol, 0.0625 M Tris-Cl, and 0.005% bromophenol blue) , and the proteins were separated by electrophoresis in 7% polyacryamide.

7. Adhesion assay

Adhesion assays were performed in 24-well($2cm^2$) plates(Costar, Cambridge, MA). First, we coated a 24-well plate with PBS(0.4ml) containing 5μg/well of either fibronectin or laminin. After 4 hours of incubation, 1 ml of 0.5% BSA was added overnight at 4℃ to block nonspecific sites. 1×10^5 cells were seed into fibronectin/laminin-coated or plastic wells and the plates were then incubated at 37℃ for either 15, 30, 60, or 90 minutes. To examine the inhibition of adhesion to laminin, the cells were preincubated in suspension for 30 minutes at 4℃ with the following antibodies : UM-A9(20μg/ml), GoH3(5μg/ml), and Anti-β1(20μg/ml). We calculated the percent of attached cells under an inverted microscope. Next day the cells were washed with serum-free medium to remove unattached cells and adherent cells were trypsinized.

The number of attached cells were calculated with either a Coulter Counter(Coulter, Hialeah, FL) or hemocytometer. In some experiments, 96-well plates were precoated by overnight incubation at 4℃ with 100μl of PBS containing 5μg/well of laminin. After washing and blocking with 0.5% BSA, 6×10^4 cells were preincubated in the presence or absence of the above antibodies. The cells were transferred to the laminin coated plates and incubated for an additional one hour at 37℃. The plates were washed twice with PBS to remove the nonattached cells. The attached cells were fixed in 10% formalin and stained with 0.5% toluidine blue in 10% formalin, destained in distilled water, and solubilized in 2% SDS. The adhesion was quantitated by measuring the absorbance at 630 nm using a Microplate Autoreader(EL 311, Bio-Tek Instruments, Winooski, VT). Values reported are the means of triplicate cultures.

8. Invasion assay

Invasion assays were performed in a 24-well BIOCOAT MATRIGEL invasion chamber(Becton Dickinson, Bedford, MA) consisting of cell culture inserts containing an 8-micron pore size PET membrane that had been treated with Matrigel basement membrane matrix. After adding laminin(20μg/ml) as a chemoattractant to the bottom well of the chamber, we transfered the chambers and control inserts to the wells. Subsequently, cells were suspended in culture medium at 1×10^5 cells/ml, and 0.5ml of cell suspension(5×10^4 cells) was added to the top well of the chambers. The chambers were incubated for 24 hours at 37℃ and then the non-invading cells were removed from the upper surface of the membrane by scrubbing with a cotton tipped swab. The cells on the lower surface of the membrane were fixed in 10% formaline and stained with 0.5% toluidine blue in 10% formalin. After removing the membrane from the insert housing using a sharp scalpel blade and placing it bottom side down on a microscope slide, invasion was quantitated by counting the cells in the central field of the membrane under a microscope at 100× magnification. Both membranes from each of the invasion chambers and control inserts were counted for each clone.

Results

1. Selection of clones expressing intgrin β4 subunit

Clones were selected for G418 resistance and tested for β4 surface expression using IF and flow cytometry and confirmed as positive clones by immunoprecipitation on unfixed cells. For IF, we tested UM-SCC-22B as a positive control(Fig. 2a) and parent UM-UC-2 as a negative control. Eleven G418 resistant clones were obtained from the pCMV/β4 FL transfectants. Of these, only one was positive for β4 by IF. Among fifty G418 resistant pCMV/β4 M clones, we randomly selected 43 clones. Of these, one was strongly positive, seven were weakly positive and one was faintly positive. Among 101 G418 resistant pCMV/β4 CD clones, 50 clones were randomly selected. Of these, eight were stained weakly and 10 were stained faintly on IF. From 60 pRc/CMV control transfectants, 11 were randomly selected and all were negative for β4 by IF. During the four consecutive IFs, one positive pCMV/β4 FL

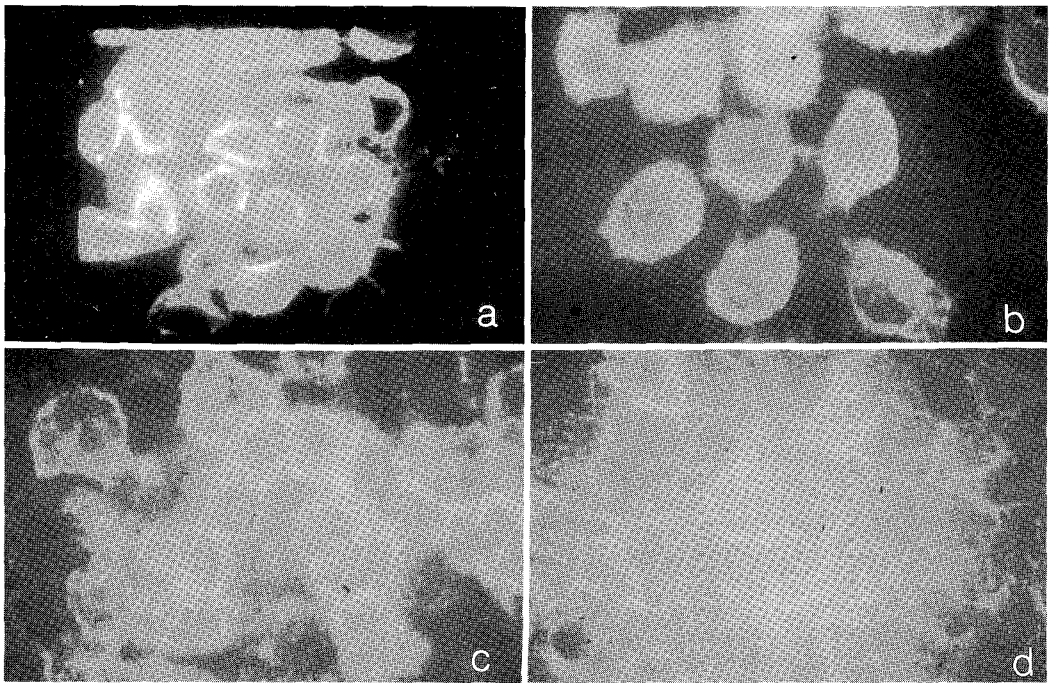

Fig. 2. Immunofluorescence findings expressing integrin β4 in SCC-22B(a), clone FL(b), Clone M(c), and Clone DC(d).

(Clone FL, Fig. 2b) and one positive pCMV/β4 M (Clone M, Fig. 2c) exhibited strong IF staining with UM-A9 antibody, and one positive-staining pCMV/β4 CD clone continued to be positive for β4, so we chose it as Clone CD(Fig. 2d).

2. Characterization of clones

For flow cytometry(Fig. 3), the above clones showed varying degrees of β4 expression. The intensity of expression was strongest in positive control UM-SCC-22B. Clone FL was second and Clone M and Clone CD were similar in their intensity. UC-2 and pRc/CMV were negative. The relative level of β4 expression of each clone was well correlated between IF and flow cytometry. By immunoprecipitation with UM-A9(Fig. 4) in Clone FL and Clone M, although the overall intensity was weaker than SCC-22B, the complex was detectable in positive control SCC-22B cells at 205 kD(intact β4), 185 and 155 kD(proteolytic breakdown products[16,19,21]. Clone CD exhibited the truncated 175 and 155 kD β4 protein bands. In addition, the 125 kD-processed α6 band was co-precipitated indicating that α6β4 heterodimers were being formed in this clone. Both parent UC-2 and control plasmid transfected pRc/CMV exhibited the absence of the β4 complex.

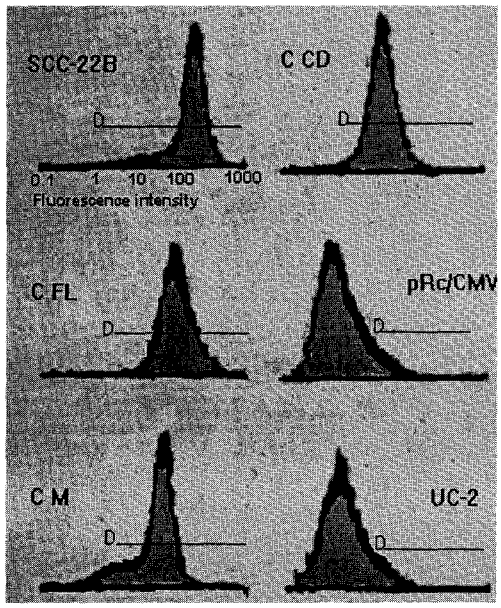

Fig. 3. Flow cytometric analysis of the expression of integrin β4 in four clones(C FL, CM, C CD, pRc/CMV) and two cell lines(SCC-22B, UC-2).

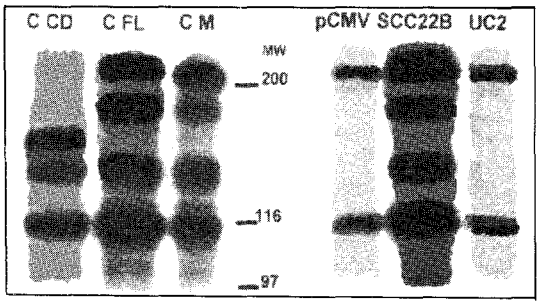

Fig. 4. Immunoprecipitation of α6β4 integrins from Clone CD, Clone FL, Clone M, pCMV, UM-SCC-22B and UM-UC-2 cells using UM-A9, anti-β4 antibody.

3. Adhesive properties of α6β4-expressing cells

The ability of α6β4-expressing cells(Clone FL, Clone M, Clone CD, SCC-22B) to adhere to laminin and fibronectin was compared with both the mock transfectant clone, Clone p/CMV, and UC-2 cell. As shown in Fig. 5, the high β4-expressing cells (Clone FL, SCC-22B) adhered much better to laminin than did the mock transfectants and UC-2, which lacked β4 expression. In contrast to the full-length β4 transfectant clone, the Clone CD did not exhibit a significant increase in its ability to adhere to laminin compared to the mock transfectants. This finding demonstrated that the marked increase in laminin adhesion observed in Clone FL is dependent on an intact β4 subunit. Clone M had an adhesive strength between Clone FL and Clone CD, which also suggested the role of the intact β4 cytoplasmic domain in adhesion(Fig. 6). Additional evidence that expression of α6β4, α3β1, and α6β1 increased laminin adhesion significantly is provided by the observation that the use of function-blocking mAbs(A9, GoH3, and Anti-β1) significantly inhibit laminin attachment on all the clones(Fig. 6). And the finding that the mock tansfectants and non-β4 expressing tranfectants did not differ from β4 expressing clones in their ability to adhere to fibronectin suggested the ligand-specficity for α6β4 in promotining cell adhesion(not shown).

4. Invasive properties of α6β4-expressing cells

We used the Matrigel assay to examine the invasiveness of our clones. Initially we compared the ability of β4 expressing clones(Clone FL, Clone M, Clone CD, SCC-22B) and non-β4 expressing clones (Clone p/CMV, UC-2). All of the β4 expressing

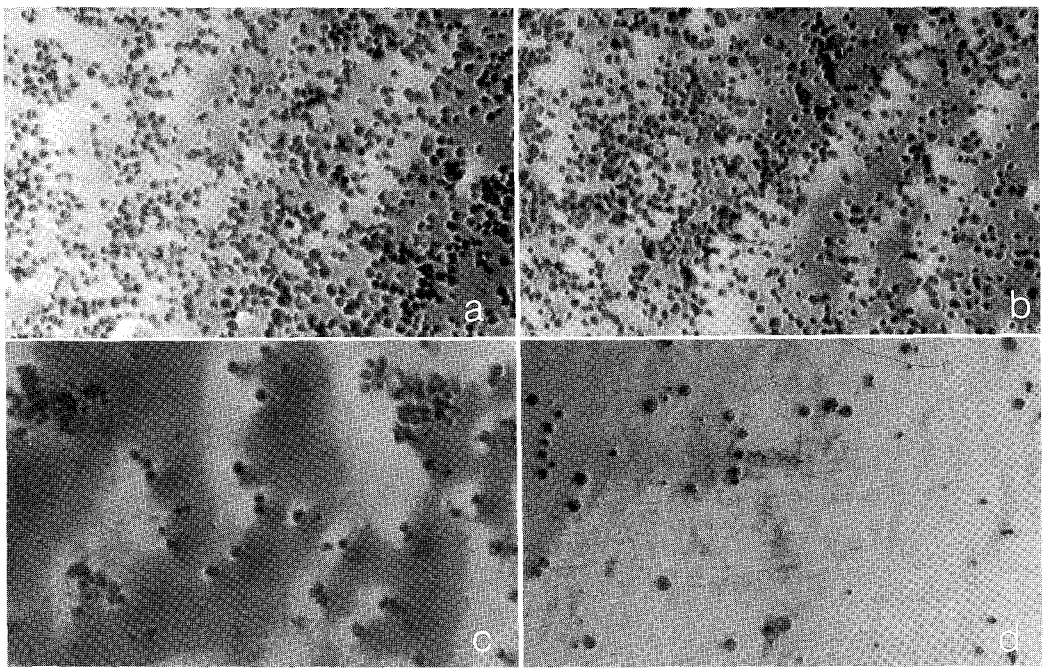

Fig. 5. The adhesion of the high β4 integrin expressing cells, SCC-22B(a), Clone FL(b), to laminin shows marked difference in the number of attached cells from β4 integrin expression-deficient cells, pRc/CMV(c) and UC-2(d).

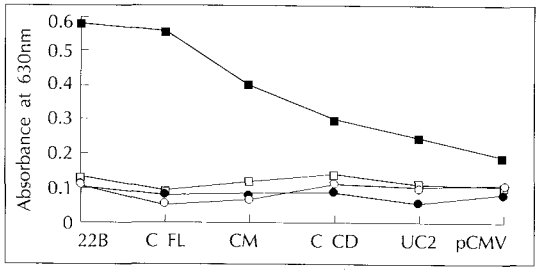

Fig. 6. Adhesion assay with laminin and function-blocking anti-bodies(A9, GoH3, anti-β1).
■ Laminin ● A9 ○ GoH □ Anti-β1

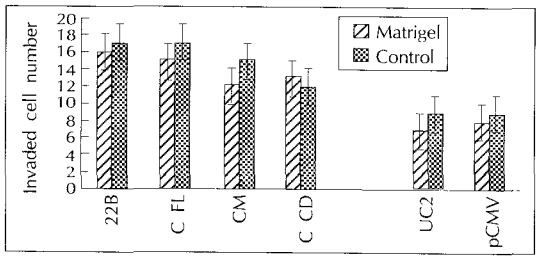

Fig. 7. Invsion assay with Matrigel & Control membrane.

clones invaded Matrigel significantly better than the mock transfectant and UC-2 cell in a 24 hour assay (Fig. 7). However, the number of invaded cell didn't show any significant difference between Clone FL, Clone M, Clone CD, and SCC-22B, suggesting the intactness of β4 cytoplasmic domain was not required in promoting cell invasion. In addition, when we used a control insert which didn't have an extracellular matrix, the extent of invasion did not show any significant difference from that of Matrigel, indicating ligand-independence for the α6β4 integrin in facilitating cell invasion(Fig. 7).

Discussion

For some time, we have focused our efforts on understanding the behavior of squamous cell carcinomas of the head and neck, because these tumors are known to be extremely aggressive and are usually associated with a low rate of patient survival. Although it was known that the expression of α6β4 is associated with the progression of head and neck squamous cell carcinoma[19], evidence for the direct involvement of α6β4 in the aggressiveness of these tumor cells was lacking.

In normal epithelia, the α6β4 integrin is found within the hemidesmosome, an organell involved anchoring the epithelium to the basement membrane. We postulated that the disrupted function of hemidesmosome is a common characteristic of tumors. Indeed, the structure and putative signaling properties of the unusually large(1000-amino acid) β4 cytoplasmic domain are distinct from other integrin subunits[22-24]. Tyrosine phosphorylation of this motif in β4 occurs in response to α6β4 clustering in A431 cells and this phosphorylation has been shown to be necessary for α6β4 to mediate hemidesmosome formation[6,14]. And the cytoplasmic deletion mutant of β4 could act as an inhibitor of α6β1 function in breast carcinoma cells by competing for the α6 subunits and blocking α6β1 mediated attachment and migration[25].

In these contexts, we transfected β4 into the β4-deficient UM-UC-2 bladder carcinoma cells and observed behavioral changes induced by cDNA expression of β4 subunits in UC-2 cells(Clone FL, Clone M, Clone CD) and compared the changes with UM-SCC-22B cells which express high levels of α6β4 and exhibit the phenotype of rapid adhesion and high invasive potential. Collectively, these results and observations implicate a key role for the α6β4 integrin in head and neck squamous cell carcinoma progression. This study provided evidence to support the involvement of the integrin α6β4 in the aggressive behavior of squamous cell carcinoma.

The importance of laminin-mediated adhesive events in the biology of carcinoma has been appreciated for some time. Early studies emphasized the need for the invasive carcinoma cell to transgress the epithelial membrane[26]. A more recent review concerning the role of the basement membrane in carcinoma progression argues that the basement membrane is a dynamic structure that participates actively in the invasion process and that it is not simply a passive barrier that impedes stromal invasion[27,28]. Indeed, much attention has been focused on identifying laminin receptors on carcinoma cells and on assessing

their potential importance in tumor progression. The best studied integrins that function as laminin receptors on carcinoma cells are α2β1, α3β1, α6β1, and α6β4[9)12)29)]. In squamous cell carcinoma, α2β1 and α6β1 are readily detected by immunopurification methods[19)]. The identification of ligands for the α6β4 integrin was a source of debate. Now it has been confirmed that this integrin is a receptor for several laminin isoforms such as laminin-1 and laminin-5[2)5)9)]. Our study showed that α6β4 expressing clones revealed strong adhesiveness to laminin-1 and suggested the involvement of the β4 cytoplasmic domain in the association of α6β4 integrin with laminin.

We observed that the expression of the α6β4 integrin in UC-2 cells, a β4-deficient urinary bladder cell line, significantly increases the ability of these cells to invade Matrigel. These findings have been confirmed using RKO cells, a β4-deficient rectal carcinoma[30)]. The validity of the behavioral changes induced by cDNA expression of the β4 subunit in UC-2 cells is strengthened by the finding that SCC-22B cells which express relatively high levels of α6β4 exhibit the same phenotype of high invasive potential as did the β4 transfectants. The same result was also obtained using the Clone A cell which is a colorectal carcinoma expressing a high level of α6β4[30)]. These results implicate a key role for the α6β4 integrin in carcinoma invasion. A recent study which observed that the expression of the α6β4 integrin is enriched at the invasive fronts of gastric carcinomas[31)] supports our data. Our data also showed the role of the β4 cytoplasmic domain in carcinoma adhesion and invasion. In adhesion, intactness of this domain seemed to be required in promoting cell adhesion, however, in invasion, the wholeness of this domain might not be so important as in adhesion.

The putative signaling properties of this unusually large domain can have a profound effect on modulating the behavior of carcinoma cells. The expression of the α6β4 integrin in UC-2 cells might substantially increase their ability to stimulate protein tyrosine phosphorylation in response to matrix attachment or receptor ligation. Interestingly, this induction of tyrosine phosphorylation is observed even when cells are plated on substrates that are not ligands for the α6β4 integrin, such as fibronectin[30)], which is consistent with our adhesion assay done on fibronectin. And expression of the α6β4 integrin in carcinoma cells might promote the formation of a substate important for tyrosine phosphorylation[30)]. These findings also agree with our functional data that expression of the α6β4 integrin can promote invasion on the control insert membrane which have no extracellular matices.

Conclusion

Using the gene transfection method, we made clones which contained specific expression constructs of cDNA, and using these clones we performed functional assays to study the role of integrin β4 in cell adhesion and invasion. The β4 integrin, especially the cytoplasmic domain, played an important role in cell adhesion. But in invasion, this integrin domain was not so important as in adhesion. The specific binding of the β4 integrin with an extracellular ligand such as laminin was important in promoting cell adhesion, however, this binding was not required in increasing cell invasion. Future studies should focus on establishing links between α6β4-mediated signaling pathways and carcinoma progression.

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