Overexpression of integrin αv facilitates proliferation and invasion of oral squamous cell carcinoma cells via MEK/ERK signaling pathway that is activated by interaction of integrin αvβ8 with type I collagen

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Abstract. To examine the role of integrin αv subunit in the progression of squamous cell carcinoma (SCC), oral SCC cells were stably transfected with integrin αv cDNA. Integrin αv transfectants exhibited the enhancement of proliferation on type I collagen, and seemed to have a high ability to invade type I collagen gel. Overexpression of integrin αv led to rapid phosphorylation of focal adhesion kinase (FAK), mitogen-activated protein kinase kinase 1/2 (MEK1/2) and extracellular signal-regulated kinase 1/2 (ERK1/2) in SCC cells on type I collagen. The downregulation of integrin β8 in integrin αv transfectants by its specific antisense oligonucleotide led to a decrease in type I collagen-stimulated activation of FAK and the MEK/ERK signaling pathway, and also suppressed the proliferation on type I collagen and the invasiveness into type I collagen gel. Moreover, the expression of integrin β8 was induced following transfection with integrin αv cDNA. These results indicated that the overexpression of integrin αv induces integrin αvβ8 heterodimer formation and the binding of integrin αvβ8 to type I collagen might enhance the proliferation and invasion of SCC cells via the activation of the MEK/ERK signaling pathway.

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Introduction

The behavior of cancer cells such as invasion and metastasis has been proposed to be mediated by the surrounding microenviroment including extracellular matrix (ECM) proteins and stromal cells (1-5). Integrins are heterodimeric transmembrane receptors composed of α and β subunits, which bind a wide range of ligands such as ECM proteins and cell surface proteins. There are at least 18 α subunits and eight β subunits, forming 24 different integrin heterodimers (6,7). Most of α subunits dimerize with only one β subunit. In contrast, integrin αv is unique because αv subunit associates with β1, β3, β5, β6 or β8 subunit and forms five distinct heterodimers (7,8).

Integrin-ECM interaction leads to the activation of signal transduction pathways, which regulate various biological events including cell adhesion, migration, proliferation and differentiation (9,10). Integrins also contribute to tumor progression by facilitating the proliferation, migration and survival of cancer cells (11-13). Altered expression of integrins has been shown in malignant tumors compared to their normal counterparts (14-17). Especially, it has been shown that the overexpression of the integrin αv subfamily such as αvβ1, αvβ3, αvβ5 and αvβ6 correlates with poor prognosis in malignant tumors such as ovarian cancer (18,19), lung cancer (20), nasopharyngeal cancer (21), gastric cancer (22) and breast cancer (23). *In vitro* studies have also shown the participation of αv integrins in the proliferation (24), motility (25,26) and proteolysis (26-28) in various cancer cells. In addition, integrin αvβ3 is a cell-surface receptor for active matrix metalloproteinase (MMP-2), indicating that integrin αvβ3 regulates tumor invasion and metastasis by increasing pericellular proteolysis (29). However, the precise mechanism of the progression of squamous cell carcinoma (SCC) mediated by integrin αv is poorly documented.

In the present study, to elucidate the role of integrin αv in the progression of oral SCC, the effect of induction of integrin αv on the proliferation and invasion of oral SCC cells were examined. The signal transduction via integrin αv that

regulates the proliferation and invasiveness of oral SCC cells was also examined.

Materials and methods

Cells and culture. The oral SCC cell line, SCCKN (30) was grown in RD medium (a 1:1 mixture of RPMI-1640 and Dulbecco's Modified Eagle's Medium) supplemented with 5% fetal bovine serum (FBS) in 5% CO_2 at 37˚C. The proliferation of the cells on ECM proteins was estimated as follows: The wells of 24-well tissue culture plates were incubated with 100 μg/ml type I collagen, type IV collagen, fibronectin, laminin or vitronectin (all from Sigma, St. Louis, MO, USA) overnight at 4˚C. Poly-L-lysine (100 μg/ml; Sigma) was used as a non-integrin-dependent adhesion substrate. The cells ($2x10^4$) suspended in RD containing 10 μg/ml bovine insulin, 5 μg/ml human transferrin, 0.5 mg/ml fatty acid-free bovine serum albumin (BSA), 10 μM 2-mercaptoethanol, 10 μM 2-aminoethanol and 10 nM sodium selenite (all from Sigma) (31) were seeded in each well of the culture plates and cultured in 5% CO_2 for 6 days at 37˚C. The number of cells was measured by the Coulter counter (Beckman Coulter, Inc., Tokyo, Japan). The measurements were done in triplicate.

Construction of integrin αv expression vector. The open reading frame of human integrin αv was amplified by PCR from plasmid CDM8 containing human αv cDNA, which was provided by Dr Joseph C. Loftus (Mayo Clinic Arizona, Scottsdale, AZ, USA). The forward primer (5'-CGGAATTCTTCGGCGATGGCTTTTCCGC-3') containing *Eco*RI site (underlined) and the reverse primer (5'-TCCCCCGGGTTAAGTTTCTGAGTTTCCTTCACCAT-3') containing *Sma*I site (underlined) were used for the amplification. The PCR products were double-digested with *Eco*RI (New England Bio Labs, Ipswich, MA, USA) and *Sma*I (New England Bio Labs) and then ligated into pCI-neo Mammalian Expression Vector (Promega Corporation, Madison, WI, USA) digested with both *Eco*RI and *Sma*I. The inserted cDNA sequences were all verified by DNA sequence analysis. The resultant plasmid was termed as pCI/neo-αv, and pCI/neo without insert was used as negative control.

Transfection and selection. SCCKN cells were transfected with pCI/neo-αv or pCI/neo (5 μg per 60-mm dish) using TransFast transfection reagent (Promega Corporation) according to the manufacturer's instruction. Selection was initiated 48 h after transfection by adding 600 μg/ml G418 (Geneticin; Invitrogen Life Technologies, San Diego, CA, USA) to the culture medium. The selection medium was changed every 4 days for 2 weeks until all non-transfected cells died. Resistant cell clones were isolated. Cell clones transfected with pCI/neo-αv or pCI/neo were termed as KNαv or KNmock, respectively.

Western blotting. To detect integrin αv and β8 proteins, SCCKN, KNmock and KNαv cells were lysed with lysis buffer [10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 1% protease inhibitor cocktail (Sigma)]. The samples containing 10 μg of total protein were electrophoresed on 10% SDS-polyacrylamide gels under reducing condition and transferred to polyvinylidene difluoride (PVDF) membrane filters (Millipore, Bedford, MA, USA). The filters were blocked in T-TBS (20 mM Tris-HCl, pH 7.5, 137 mM NaCl, 0.1% Tween-20) containing 5% skim milk for 1 h at room temperature and then incubated with rabbit anti-integrin αv polyclonal antibody (Santa Cruz Biotechnology, Inc., Dallas, TX, USA) or goat anti-integrin β8 polyclonal antibody (Santa Cruz Biotechnology, Inc.), followed with the incubation with horseradish peroxidase (HRP)-conjugated anti-rabbit IgG antibody (Cell Signaling Technology, Inc., Danvers, MA, USA) or HRP-conjugated anti-goat IgG antibody (KPL, Gaithersburg, MD, USA), respectively. Rabbit anti-β-actin polyclonal antibody (Cell Signaling Technology, Inc.) was used as internal control to confirm equal loading of total protein. Protein bands were visualized by enhanced chemiluminescence detection (ECL Plus System; GE Healthcare, Uppsala, Sweden).

Cell adhesion assay. The wells of 24-well culture plates were incubated with 100 μg/ml type I collagen, type IV collagen, fibronectin, laminin or vitronectin overnight at 4˚C. Poly-L-lysine (100 μg/ml) was used as a non-integrin-dependent adhesion substrate. The wells were washed five times with phosphate-buffered saline (PBS) and incubated with in PBS containing 1% BSA for 1 h at 37˚C to block non-specific binding. Subconfluent culture of cells was radiolabeled with 1 μCi/ml [methyl-^{3}H]-thymidine (Perkin-Elmer, Waltham, MA, USA) for 24 h. The labeled cells ($1x10^5$) suspended in RD medium containing 0.1% BSA were added to each well of the culture plates. After incubation for 30 min at 37 ˚C, the medium was aspirated, and the wells were gently rinsed twice with PBS. The cells adhering to the well were dissolved in 1 N NaOH, and the radioactivity was measured with a liquid scintillation counter (LSC903; Aloka, Co., Ltd., Tokyo, Japan). The adhesion capacity was determined relative to the radioactivity of seeded cells ($1x10^5$) that was considered to be 100%. Each assay was performed in triplicates and repeated three times.

Collagen gel culture. Five hundred-microliters of 0.21% type I collagen gel solution (Koken, Co., Ltd, Tokyo, Japan) in RD neutralized with reconstitution buffer (0.05 N NaOH, 2.2% $NaHCO_3$, 0.2 M HEPES) was pipetted into each well of 24-well culture plate and gelled as a basal layer with incubation for 1 h at 37˚C. Thereafter, 500 μl type collagen solution containing cells ($2.5x10^4$) was poured onto the basal layer and gelled, and received 1 ml RD containing 5% FBS. The cells in the gels were cultured for 12 days at 37˚C. The colonies that formed in the gel were fixed with phosphate-buffered 10% formalin. Sections were prepared and stained with hematoxylin and eosin.

Phosphorylation assay. SCCKN, KNmock and KNαv cells suspended in RD containing 2% BSA were seeded on culture dishes coated with 100 μg/ml type I collagen. At various incubation times, the cells were washed with ice-cold PBS containing 1 mM sodium vanadate and lysed with Laemmli sample buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 1% β-mercaptoethanol) supplemented with protease inhibitor cocktail and 1 mM sodium vanadate. The samples were separated on 10% SDS-polyacrylamide gels and transferred onto PVDF membrane filters. The immunoblot analysis was performed

using rabbit anti-phospho-focal adhesion kinase (FAK) monoclonal antibody, rabbit anti-phospho-mitogen-activated protein kinase kinase 1/2 (MEK1/2) monoclonal antibody and rabbit anti-phospho-extracellular signal-regulated kinase 1/2 (ERK1/2) monoclonal antibody. To detect total FAK, MEK1/2 and ERK1/2 proteins, rabbit anti-FAK monoclonal antibody, rabbit anti-MEK1/2 monoclonal antibody and rabbit anti-ERK1/2 monoclonal antibody were used, respectively. After incubation with primary antibodies, the membranes were incubated with HRP-conjugated secondary antibody, and protein bands were detected using an enhanced chemiluminescence reagent. All antibodies used for the phosphorylation assay were purchased from Cell Signaling Technology, Inc.

Integrin β8-specific morpholino antisense oligonucleotide. To downregulate integrin β8, a morpholino antisense oligonucleotide specific for integrin β8 obtained from GeneTools (Philomath, OR, USA) was used. The sequence of the antisense oligonucleotide is as follows: 5'-AAGCCAGGGC CGAGCCGCACATAAT-3'. A standard control morpholino oligonucleotide (5-CCTCTTACCTCAGTTACAATTTATA-3) was used as a negative control. Delivery of the oligonucleotides into the cells was performed according to the GeneTools protocol. Briefly, 80-100% confluent SCCKN, KNmock or KNαv cells were treated with 10 μM of the morpholino antisense oligonucleotide or the standard control oligonucleotide, and 6 μM of Endo-Porter reagent (GeneTools). After 24 h, the cells were used for the subsequent experiments.

Northern blot analysis. Total cytoplasmic RNA of SCCKN, KNmock or KNαv cells in confluent cultures was isolated with using TRIzol reagent (Life Technologies, Rockville, MD, USA) according to manufacture's instruction. Total RNA (20 μg) obtained from the cells were separated on a 1% agarose gels containing 2.2 M formaldehyde and transferred directly from the gel to a nylon membrane (Hybond-N+; GE Healthcare) in 10X SSC (1X SSC is 0.15 M NaCl plus 1.5 mM sodium citrate) overnight. After transfer, RNA was UV cross-linked (120,000 μJ of UV), and the membrane was prehybridized with Rapid-hyb buffer (GE Healthcare) for 15 min at 65˚C. The specific probe for integrin β8 was obtained by reverse transcriptase-PCR as follows: First-strand cDNA was synthesized from total RNA of SCCKN cells with ReverTra Ace (Toyobo, Osaka, Japan) and PCR amplification was performed using forward primer (5'-GATCAGACGTCTCATCTCGC-3') and reverse primer (5'-CTCTTCCACTGCACACTTGG-3'). The PCR products (961 bp) were subcloned into pGEM-T Easy Vector (Promega Corporation), and the inserted cDNA sequences were verified by DNA sequence analysis. The plasmid was digested with *Eco*RI, and the insert was gel-purified and radiolabeled with [α-^{32}P]-dCTP (Perkin-Elmer) using Rediprime II DNA Labeling System (GE Healthcare). Hybridization was carried out for 2 h at 65˚C in Rapid-hyb buffer containing 1x10^6 cpm/ml probe. The membrane was washed with 2X SSC/0.1% SDS for 20 min at room temperature and washed twice with 0.5X SSC/0.1% SDS for 15 min at 65˚C. Hybridization signals were detected by the BAS 2000 image analyzer (Fujifilm, Tokyo, Japan). Equivalent loading of ribosomal RNA was confirmed by methylene blue staining.

Results

Effect of overexpression of integrin αv on cell adhesion to ECM proteins. A small amount of integrin αv protein was observed in SCCKN and KNmock cells. In contrast, integrin αv transfectants in KNαv cells showed a large amount of integrin αv protein (Fig. 1A).

To examine the effect of integrin αv on cell adhesion to ECM proteins, SCCKN, KNmock and KNαv cells were seeded on various ECM protein-coated wells and incubated for 30 min. Only 5 or 10% of SCCKN and KNmock cells adhered to any ECM protein. In contrast, over 35% of KNαv cells adhered to type I collagen, type IV collagen, fibronectin and vitronectin after 30-min incubation (Fig. 1B).

Effect of overexpression of integrin αv on the proliferation of SCC cells on ECM proteins. To examine the participation of integrin αv in the proliferation of SCC cells, SCCKN, KNmock and KNαv cells were grown on various ECM proteins in the absence of serum for 6 days. Transfection with integrin αv cDNA led to a marked increase in cell proliferation on type I collagen. The number of KNαv cells grown on type I collagen was about 3-fold compared to the number of SCCKN and KNmock cells on type I collagen. In contrast, other ECM proteins exhibited no significant effect on the proliferation of KNαv cells (Fig. 2A).

Effect of overexpression of integrin αv on the morphology of colonies of SCC cells in three-dimensional type I collagen gels. The behavior of SCCKN, KNmock and KNαv cells was examined by three-dimensional culture using type I collagen gel. The cells embedded in type I collagen gel were cultured for 12 days. SCCKN and KNmock cells formed small and spherical colonies in the gel. In contrast, KNαv cells formed dilated colonies with irregular margins, and some cells migrated into the surrounding collagen gel, suggesting that transfection with integrin αv cDNA led to the enhancement of invasiveness of SCC cells (Fig. 2B).

Activation of FAK and the MEK/ERK signaling pathway by type I collagen. Type I collagen enhanced the proliferation of KNαv cells, and KNαv cells exhibited the enhanced invasiveness into type I collagen gel compared to SCCKN and KNmock cells. Several studies have shown that the MEK/ERK signaling pathway via integrin αv regulates cell proliferation (32-34). To clarify the participation of the MEK/ERK signaling pathway in type I collagen-induced proliferation and invasion of KNαv cells, SCCKN, KNmock and KNαv cells were detached and replated onto type I collagen, and the phosphorylation of FAK, MEK1/2 and ERK1/2 in the cells was investigated after cultivation for various periods. The phosphorylation of FAK, MEK1/2 and ERK1/2 in KNαv cells was observed at 2, 6 h and 6 h after replating on type I collagen, respectively. In contrast, the phosphorylation of FAK, MEK1/2 and ERK1/2 in SCCKN and KNmock cells was observed at 12, 24 h and 24 h after replating, respectively (Fig. 3).

Participation of integrin β8 in integrin αv-mediated cell adhesion of SCC cells. To examine the effect of the suppression of integrin β8 on the adhesion of SCC cells to ECM proteins,

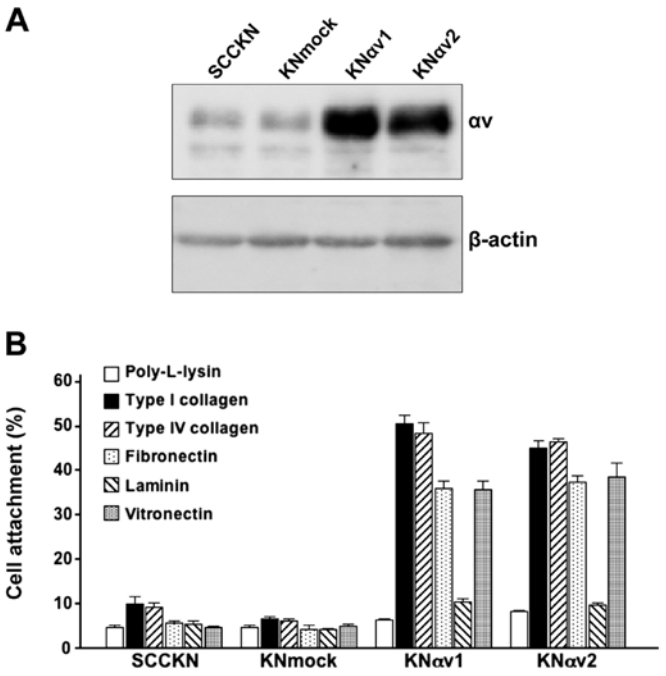

Figure 1. Effect of integrin αv on the cell adhesion to extracellular matrix (ECM) proteins. (A) The expression level of integrin αv protein in SCCKN, KNmock and KNαv cells was analysed by western blotting. The cell lysates were electrophoresed on a 10% SDS polyacrylamide gel and transferred to a polyvinylidene difluoride (PVDF) membrane. The expression of integrin αv protein was detected by anti-integrin αv antibody, followed by enhanced chemiluminescence detection reagents as described in Materials and methods. (B) [Methyl-^{3}H]-thymidine-labeled cells ($1x10^5$) suspended in RD containing 0.1% bovine serum albumin (BSA) were added to 24-well culture plates coated with indicated ECM proteins. After incubation for 30 min at 37˚C, the medium was aspirated, and the wells were gently rinsed twice with phosphate-buffered saline (PBS). The radioactivity of cells adhering to the well was measured. The adhesion capacity was determined relative to the radioactivity of seeded cells ($1x10^5$), which was considered to be 100%. The results are represented the means ± SD of triplicate determinations.

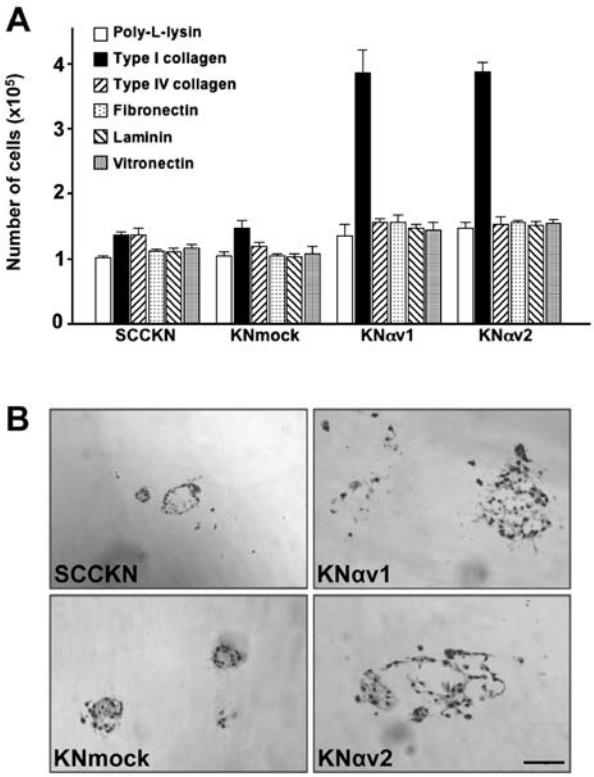

Figure 2. Effect of αv integrin on the growth of squamous cell carcinoma (SCC) cells. (A) SCCKN, KNmock and KNαv cells ($2x10^4$) suspended in RD containing 10 μg/ml bovine insulin, 5 μg/ml human transferrin, 0.5 mg/ml fatty acid-free bovine serum albumin (BSA), 10 μM 2-mercaptoethanol, 10 μM 2-aminoethanol and 10 nM sodium selenite were seeded in each well of 24-well tissue culture plates coated with indicated extracellular matrix (ECM) proteins. After cultivation for 6 days, the number of cells was measured. The results are the means of triplicate determinations ± SD. (B) Morphology of SCCKN, KNmock and KNαv cells in type I collagen gel. The cells ($2.5x10^4$) embedded in type I collagen gel were cultured for 12 days. The colonies were fixed in buffered formalin and embedded in paraffin, and the sections stained with hematoxylin and eosin. Scale bar, 250 μm.

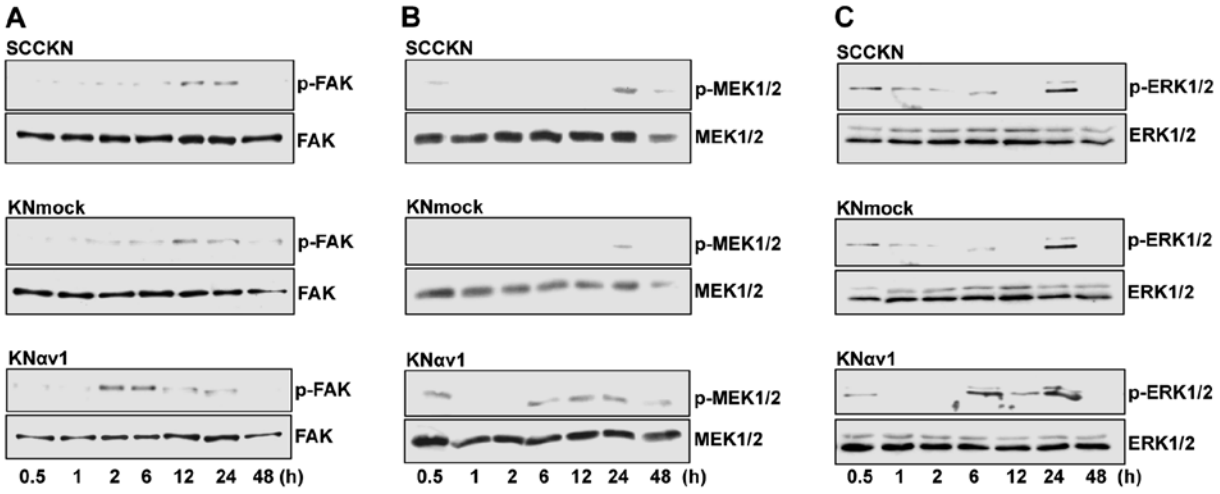

Figure 3. Phosphorylation of focal adhesion kinase (FAK), mitogen-activated protein kinase kinase 1/2 (MEK1/2) and extracellular signal-regulated kinase 1/2 (ERK1/2) in squamous cell carcinoma (SCC) cells on type I collagen. SCCKN, KNmock and KNαv cells were detached and replated onto type I collagen. The cells were lysed at the indicated times after replating, and the phosphorylation of (A) FAK, (B) MEK1/2 and (C) ERK1/2 was analysed by western blotting.

the cells were transfected with a morpholino antisense oligonucleotide targeting integrin β8 subunit (Fig. 4A) or a control oligonucleotide (Fig. 4B). The adhesion of SCCKN and KNmock cells to ECM proteins were not greatly affected

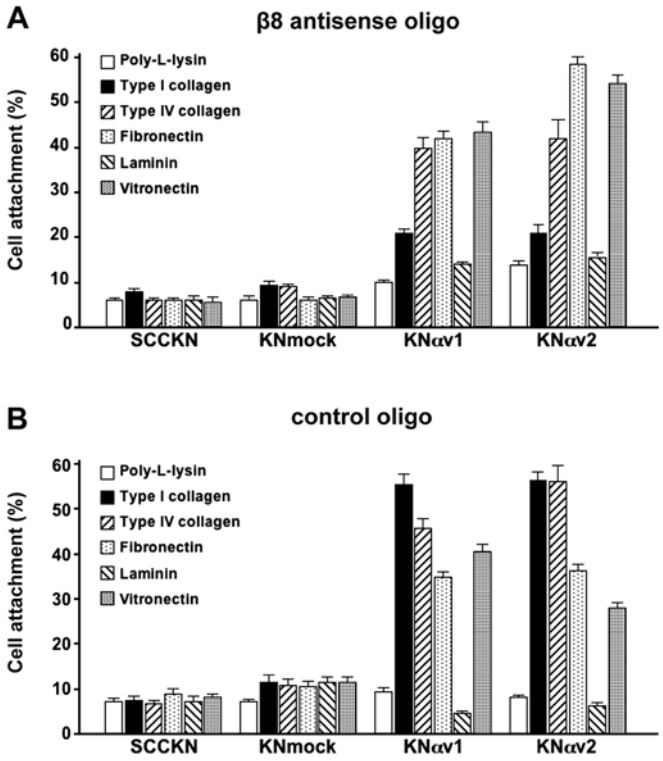

Figure 4. Participation of integrin β8 in integrin αv-mediated adhesion of squamous cell carcinoma (SCC) cells to extracellular matrix (ECM) proteins. SCCKN, KNmock and KNαv cells were transfected with (A) an antisense oligonucleotide targeting integrin β8 (β8 antisense oligo) or (B) a control oligonucleotide (control oligo). The cells were radiolabeled with [methyl-^{3}H]-thymidine. The cells ($1x10^5$) suspended in RD containing 0.1% bovine serum albumin (BSA) were added to 24-well culture plates coated with indicated ECM proteins. The adhesion capacity of the cells was determined as described in the legend of Figure 1. The results are presented the means ± SD of triplicate determinations.

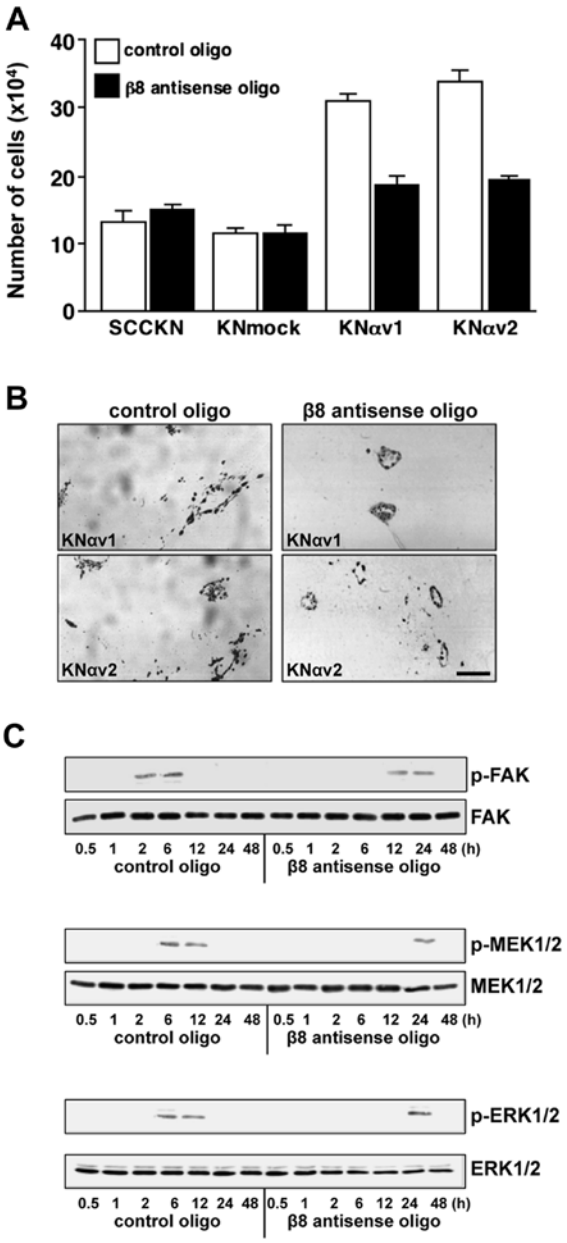

Figure 5. Suppression of integrin β8 reduces type I collagen-induced growth and phosphorylation of focal adhesion kinase (FAK), mitogen-activated protein kinase kinase 1/2 (MEK1/2) and extracellular signal-regulated kinase 1/2 (ERK1/2) of squamous cell carcinoma (SCC) cells. (A) SCCKN, KNmock and KNαv cells ($2x10^4$) transfected with β8 antisense oligo or control oligo were suspended in RD containing 10 μg/ml bovine insulin, 5 μg/ml human transferrin, 0.5 mg/ml fatty acid-free bovine serum albumin (BSA), 10 μM 2-mercaptoethanol, 10 μM 2-aminoethanol and 10 nM sodium selenite, and were seeded in each well of 24-well tissue culture plates coated with type I collagen. After cultivation for 6 days, the number of cells was measured. The results are the means of triplicated determinations ± SD. (B) KNαv cells transfected with β8 antisense oligo or control oligo were embedded in type I collagen gel and cultured for 12 days. The cells were fixed in buffered formalin and embedded in paraffin, and the sections stained with hematoxylin and eosin. Scale bar, 250 μm. (C) KNαv cells transfected with the β8 antisense oligo or control oligo were detached and replated onto type I collagen. The cells were lysed at the indicated times after replating, and the phosphorylation of FAK, MEK1/2 and ERK1/2 was analysed by western blotting.

by the suppression of integrin β8. In contrast, the suppression of integrin β8 by the antisense oligonucleotide led to the remarkable decrease in the attachment of KNαv cells to type I collagen compare to the attachment of KNαv cells transfected with the control oligonucleotide. However, the suppression of integrin β8 did not reduce the adhesion of KNαv cells to any ECM protein except type I collagen.

Participation of integrin αvβ8 on the proliferation of SCC cells via the MEK/ERK signaling pathway. We examined the participation of integrin αvβ8 in type I collagen-induced proliferation of KNαv cells. The suppression of integrin β8 by a morpholino antisense oligonucleotide targeting integrin β8 led to remarkable decrease in the proliferation of KNαv cells cultured on type I collagen. However, transfection with the antisense oligonucleotide did not have strong effect on the proliferation of SCCKN and KNmock cells on type I collagen (Fig. 5A).

We next examined the effect of the suppression of integrin β8 on the morphology of colonies of KNαv cells in type I collagen gels. KNαv cells transfected with the control oligonucleotide formed dilated colonies with irregular margin, and some cells migrated into the surrounding collagen gel. In contrast, the colonies of KNαv cells transfected with the antisense oligonucleotide were small and spherical colonies (Fig. 5B).

The phosphorylation of FAK, MEK1/2 and ERK1/2 in KNαv cells transfected with the control oligonucleotide was observed at 2, 6 h and 6 h after replating on type I collagen,

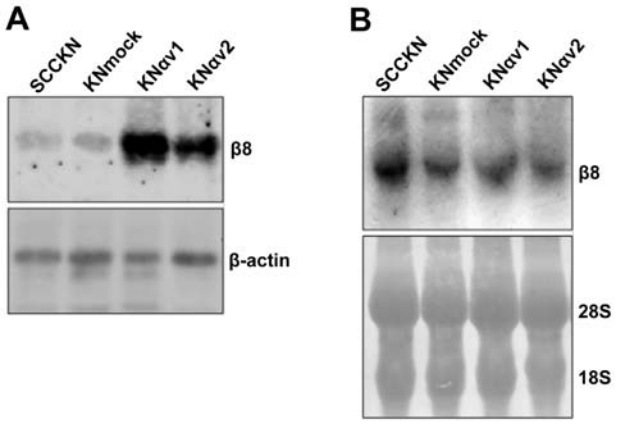

Figure 6. The expression level of integrin β8 protein and mRNA in SCCKN, KNmock and KNαv cells. (A) The expression of integrin β8 protein in SCCKN, KNmock and KNαv cells was analysed by western blotting. The cell lysates were electrophoresed on a 10% SDS polyacrylamide gel and transferred to a polyvinylidene difluoride (PVDF) membrane. The integrin β8 protein was detected by anti-integrin β8 antibody, followed by enhanced chemiluminescence detection reagents as described in Materials and methods. (B) The expression of integrin β8 mRNA in SCCKN, KNmock and KNαv cells was analysed by Northern blotting. Total RNAs obtained from the cells were separated on a 1% agarose gels containing 2.2 M formaldehyde and transferred directly from the gel to a nylon membrane. The immobilized RNAs were hybridized with the radiolabeled specific probe for integrin β8. The membrane was stained in methylene blue to visualize the relative amounts of 28S and 18S rRNA present in each lane (lower panel).

respectively. In contrast, the phosphorylation of FAK, MEK1/2 and ERK1/2 in KNαv cells transfected with the antisense oligonucleotide was observed at 12, 24 h and 24 h after replating, respectively (Fig. 5C).

Expression of integrin β8 in integrin αv transfectants. The proliferation of KNαv cells on type I collagen was reduced by the suppression of integrin β8. Moreover, type I collagen-stimulated phosphorylation of FAK, MEK1/2 and ERK1/2 in KNαv cells was also inhibited by the suppression of integrin β8. These findings suggest that the interaction of integrin αvβ8 with type I collagen might activate FAK and the MEK/ERK signaling pathway and induce the proliferation of SCC cells. Therefore, the expression of integrin β8 protein and mRNA in SCCKN, KNmock and KNαv cells was examined by western blotting and northern blotting, respectively. The expression of integrin β8 protein in KNαv cells was enhanced compared to SCCKN and KNmock cells. In contrast, there was no remarkable difference in the expression of integrin β8 mRNA between SCCKN, KNmock and KNαv cells (Fig. 6).

Discussion

Integrin αv, which heterodimerizes with β1, β3, β5, β6 or β8, regulates several biological events such as cell adhesion, proliferation and differentiation (7,8). Several studies have shown that integrin αvβ3, αvβ5 and αvβ6 are implicated in carcinogenesis, tumor invasion and metastasis (34-37). Our previous study has also shown that integrin αv mediates the proteolytic activity of SCC cells by anchoring active MMP-2 on the cell surfaces, suggesting that integrin αv might promote the progression of oral SCC (38).

In this study, the role of integrin αv in the progression of SCC cells was examined. Induction of integrin αv expression led to the enhancement of cell attachment of SCCKN cells to any ECM proteins used in the present study. Especially, integrin αv transfectants, KNαv cells had a high ability to bind type I collagen, type IV collagen and fibronectin. We next examined the proliferation of SCC cells on ECM proteins. The proliferation of SCCKN and KNmock cells was not greatly influenced by any ECM proteins. In contrast, the proliferation of KNαv cells on type I collagen was significantly enhanced. Moreover, the effect of integrin αv on the behavior of SCC cells using three-dimensional type I collagen gel culture system was examined. SCCKN and KNmock cells embedded in type I collagen gel formed small and spherical colonies. In contrast, KNαv cells in type I collagen gel formed dilated colonies with irregular margins, and some cells migrated into the surrounding gel. These findings suggest that the binding of αv integrins to type I collagen activates the signaling pathway involved in the proliferation and invasion of SCC cells.

The binding of integrins to ECM proteins leads to the activation of FAK, and results in the activation of several signaling pathways such as Akt/PI3 kinase signaling, mitogen-activated protein (MAP) kinase signaling and Rho family GTPase signaling (39-42). It is well known that cell proliferation via the integrin αv subfamily such as αvβ3 and αvβ5 is mediated by the MEK/ERK signaling pathway, which is characterized firstly in MAP kinase cascades (32-34). Therefore, the participation of the MEK/ERK signaling pathway in type I collagen-induced growth of integrin αv transfectants was examined. Rapid phosphorylation of FAK, MEK1/2 and ERK1/2 was observed in KNαv cells cultured on type I collagen. In contrast, the phosphorylation of these molecules was delayed in SCCKN and KNmock cells on type I collagen. These findings indicate that type I collagen induces the activation of FAK and the MEK/ERK signaling pathway via αv integrins.

Formation of α/β heterodimer is essential for the expression and function of integrins. Integrin αv subunit associates with β1, β3, β5, β6 or β8 subunit and forms five distinct heterodimers (7,8). Integrin αv transfectants, KNαv cells had a high ability to bind type I collagen, and exhibited a remarkable proliferative response to type I collagen. These findings suggest that some of the five αv integrins of KNαv cells interact with type I collagen and regulate cell proliferation as well as cell adhesion. The five αv integrins bind various ECM proteins such as fibronectin, laminin, vitronectin, fibrinogen and osteopontin. The binding of the αv integrins to ECM proteins is dependent upon the β subunit counterpart. Some studies have shown that only integrin αvβ8 in the integrin αv subfamily is a potential receptor for collagens (43,44). We therefore examined the participation of integrin αvβ8 in type I collagen-induced proliferation of KNαv cells. The suppression of integrin β8 by its antisense oligonucleotide led to a remarkable decrease in the adhesion of KNαv cells to type I collagen. The treatment of integrin β8 antisense oligonucleotide also reduced the proliferation of KNαv cells on type I collagen and the invasiveness of KNαv cells into type I collagen gel. In addition, the activation of FAK and the MEK/ERK signaling pathway of KNαv cells on

type I collagen was inhibited by the treatment of integrin β8 antisense oligonucleotide. These findings indicate that the binding of integrin αvβ8 to type I collagen might induce the proliferation and invasion of SCC cells via the MEK/ERK signaling pathway.

We next examined the expression of integrin β8 in SCCKN, KNmock and KNαv cells. Interestingly, KNαv cells expressed a large amount of integrin β8 protein compared to SCCKN and KNmock cells, whereas there is no significant difference in the expression of integrin β8 mRNA between SCCKN, KNmock and KNαv cells. At present, the mechanism of the enhanced expression of integrin β8 protein following transfection with integrin αv cDNA is unclear. A previous study showed that the expression of mouse integrin β1 and β7 was induced following transfection with human integrin α4 subunit in mouse fibroblasts (45). The possibility should be considered that integrin β8 subunit dimerizes with integrin αv subunit expressed abundantly in KNαv cells, and integrin β8 dimerized with integrin αv might be stable compared to integrin β8 monomer. Most of integrin β8 subunits in SCCKN and KNmock might exist as monomer because of the insufficiency of integrin αv subunits available for forming αvβ8 heterodimers.

In conclusion, the overexpression of integrin αv led to the enhancement of the proliferation of oral SCC cells via interaction with type I collagen. The expression of integrin β8 subunit is induced following transfection with integrin αv subunit in SCC cells. Interaction of integrin αvβ8 with type I collagen activates the MEK/ERK signaling pathway in SCC cells, resulting in the enhancement of the proliferation and invasiveness. These findings suggest that integrin αvβ8 might be a prognostic factor for oral SCC and could serve as a therapeutic target to prevent the progression of oral SCC.

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