THE ADHESION OF ODONTOBLAST TO TYPE I COLLAGEN

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Abstract

Odontoblasts are anchorage dependent cells adhering to a substrate via cell adhesive molecules. Receptor ligands such as integrins bind to these proteins and are known to function as signal transduction molecules in a series of critical recognition events of cell-substratum. The aim of this study is to examine the interaction of odontoblast (MDPC-23 cell) with type I Col and the effect of TGF-β1 and TNF-α on the expression of cell adhesion molecules.

In this study, MDPC-23 cells adhered to type I Col dose-dependently. Immunofluorescence data demonstrated that integrin α1, α2 and CD44 were expressed on cell surface, and FAK and paxillin were localized in focal adhesion plaques in MDPC-23 cells adhesion to Col. Cytokine TGF-β1 increased the adhesion of MDPC-23 cells to Col and the expression level of integrin α1, α2 and chondroitin sulfate on MDPC-23 cells. RT-PCR data demonstrated that cytokine TGF-β1 increased the amount of integrin α1 mRNA in MDPC-23 cells.

Therefore, MDPC-23 cells adhere to collagen type I Col and expressed a complex pattern of integrins and proteoglycans, including α1, α2, chondroitin sulfate and CD44 detected by immunoblotting and immunofluorescence assay. TGF-β1 treatment enhanced the expression of adhesion molecules such as integrin α1, α2 and chondroitin sulfate.

Key words : Odontoblast, Collagen type I, Cell adhesion

Ⅰ. Introduction

The development of tooth tissues requires reciprocal interactions between the oral epithelium and the ectomesenchyme and results in the differentiation of enamel-producing ameloblasts and dentin-producing odontoblasts. Odontoblasts are neural crest-derived mesenchymal cells organized as a densely packed layer at the dentin-pulp interface. Their main functions are synthesis and extracellular deposition of a type I collagen (Col)-rich matrix referred to as predentin, and the mineralization of this matrix to form dentin[1]. The odontoblasts secrete mainly type I Col, which makes up nearly 90% of the dentin, but also several non-collagenous glycoproteins, including dentin sialophosphoprotein, dentin matrix protein, bone sialoprotein, osteopontin, osteocalcin, osteonectin and matrix Gla protein[2]. The extracellular matrix (ECM) layer are both considered to be involved with cell proliferation and differentiations[3].

The interaction of cells with the ECM regulates a number of cellular processes such as growth, proliferation and differentiation. The adhesion of cells to the ECM is mediated by cell surface receptors such as those in integrin family of adhesion receptors[4]. The receptors interact with cytoskeletal proteins on the cytoplasmic side of the plasma membrane, providing a linkage between the ECM and cytoskeleton[5]. Increasing evidence

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indicates that binding of ECM molecules to their cognate cell surface receptors also gives rise to biochemical signals within the cells such as the activation of protein kinase C[6], tyrosine kinase[7] and mitogen-activated protein (MAP) kinase[8]. Integrin binding to the ECM and subsequent clustering directs the formation of specialized sites of contact between the cell and the ECM, termed focal adhesions[4]. These are concentrations of specific cytoskeletal proteins and sites of organization of the actin cytoskeleton and play a key role in cell adhesion, spreading and organization. They are also major sites of tyrosine phosphorylation and contain multiple signal transduction molecules, including receptors for soluble growth factors and many of their downstream effector molecules. Integrin stimulation initiates the association with the focal adhesion kinase (FAK) of Src (at phosphotyrosine 397) and paxillin, both required for integrin-mediatated activation of the MAP kinase pathway[8].

Integrin represents a major family of cell-surface receptors that facilitate the adhesion between cells and the surrounding ECM. Interactions between integrins and their ligands have been linked to many cellular processes including proliferation, differentiation, survival, motility, embryogenesis and apoptosis[9,10]. Many integrins bind to ECM proteins and thereby mediate cell-ECM interactions. The ECM ligands for integrins are fibronectin, laminin, various collagens, entactin, tenascin, thrombospondin, von Willebrand factor and vitronectin[11,12]. The process of odontoblast commitment and differentiation is regulated by signals between integrins and ECM. The $\alpha1\beta1$ and $\alpha2\beta1$ integrins are the major Col-binding integrins, with $\alpha1\beta1$ having a higher affinity for the basement membrane type IV Col and $\alpha2\beta1$ having a higher affinity for the fibrillar type I Col, the major protein constituent of bone[13,14].

While the specificity of the cell-matrix interactions seems to come from the integrins binding, most adhesive matrix molecules also contain sites that can interact with the glycosaminoglycan (GAG) component of proteoglycans. The binding of cell surface proteoglycans to such sites is likely to play an augmenting role in cell adhesion. Proteoglycans are found inside cells, on the cell surface and in the ECM. A cell surface proteoglycan appeared to be intercalated in plasma membrane. The polymorphic family of integral membrane glycoproteins CD44 is found on a wide variety of cells[15]. CD44, which exhibits significant sequence homology to the phylogenically conserved amino-terminal domain of cartilage link proteins, is an important cell surface adhesion molecule expressed on lymphoid cells, myeloid cells, fibroblasts, epithelial cells and endothelial cells[16].

It is known that cytokines such as transforming growth factor-β (TGF-β), interleukin-1β and tumor necrosis factor (TNF)-α can modulate the expression of matrix receptors of various cell lines[17,18]. These findings suggest that quantitative or qualitative changes in the level of expression of matrix receptors should be examined for the possible effects on the ability of cells to adhere to and migrate on ECM proteins.

The aim of this study is to examine the interaction of odontoblast with type I Col, which is a major component of dentin matrix, and the effect of TGF-β1 and TNF-α on the expression of cell attach molecules presumably dominating adhesion and migration to ECM.

Ⅱ. Materials and Methods

1. Antibodies

Monoclonal antibodies (MoAb) rat anti-mouse α1, α2 and CD44 were purchased from Pharmingen (Becton Dickinson). MoAb rat anti-chondroitin sulfate was purchased from Sigma (ST. Louis, MO). MoAb rat anti-focal adhesion kinase (p125FAK) and polyclonal rat anti-paxillin were purchase from Upstate Biotechnology (Lake Placid, NY). Peroxidase labelled anti-mouse and anti-rat IgG were purchased from Amersham Biosciences (UK).

2. Cytokines

TGF-β1 and TNF-α were purchased from R & D systems.

3. Cell culture

Mouse Dental Papilla Cell-23 (MDPC-23) cells were grown in Dulbecco's Modified Eagles Medium(DMEM) supplemented 25 mM Hepes, 100 ㎍/ml penicillin/streptomycin, 4 mM L-glutamine, 10% fetal bovine serum at 37℃ in a 5% CO_2 humidified air incubator.

4. Adhesion assay

Flat-bottomed wells in microtiter plates were coated overnight at 4℃ with 50 ㎕ of Col. Plates were rinsed

with PBS and binding sites were blocked by incubation with 100 ㎕ of 3% BSA in PBS for 1 hour at 37℃. The plates were rinsed again and a total of 4×10^5 cells in 100 ㎕ serum-free DMEM/1% BSA were plated in triplicate on each coated well and incubated for 60 minutes at 37℃. In order to determine nonspecific binding, cells were also added to wells that had been coated with 3% BSA in PBS alone. Unbound cells were removed by two gentle washes with PBS. Attached cells were quantified by MTT assay.

5. Fluorescence staining

Cells were plated onto Col-coated coverslips in DMEM including 1% calf serum, incubated for 60 minutes at 37℃ and then fixed in 4% paraformaldehyde for 10 minutes. Fixed cells were washed twice and were incubated in 0.1 M glycine in PBS at 4℃ for 30 minutes. Cells were permeabilized with 0.2% Triton X-100 in PBS for 3 minutes and incubated three times for 10 minutes in PBS containing 3% BSA to reduce nonspecific binding. Cells on coverslips were incubated with primary antibody in PBS containing 3% BSA for 1 hour. Cells were washed thoroughly and then incubated with secondary antibody for 1 hour. Cells were washed thoroughly in PBS containing 3% BSA for 10 minutes and then three times in PBS. Coverslips were mounted onto glass slides with 50% glycerol in PBS. Fluorescence microscopy was performed with a Carl Zeiss Axioskop fluorescence microscope.

6. Staining of F-actin in MDPC-23 cells

Cells either plated on Col-coated coverslips were fixed for 10 minutes in 4% paraformaldehyde in PBS and rinsed cells three times for 5 minutes each in PBS. Fixed cells were permeabilized for 5 minutes in the 0.2% Triton X-100 solution and rinsed cells three times for 5 minutes each in PBS. Cells were labeled with the FITC-phalloidin at room temperature for 30 minutes and rinsed cells in PBS three times for 5 minutes each time and mounted in 50% glycerol in PBS and examined with a Carl Zeiss Axioskop fluorescence microscope.

7. Western blot analysis

For protein analysis, cells were lysed with RIPA buffer (10 mM Tris/HCl, pH 7.2, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 158 mM NaCl, 1 mM EGTA, 0.1 mM phenylmethylsulfonyl fluoride) on ice for 1 hour. Lysate were clarified by centrifugation at 12,000 rpm for 20 minutes at 4℃, and then the supernatant was obtained. The protein contents of the lysate were determined using the Bio-Rad Protein Assay (Bio-Rad laboratories Hercules, CA). The 20㎍ protein was mixed with equal volume of electrophoresis buffer (10 nM Tris/HCl, 200 mM DTT, 4% SDS, 0.2% bromophenol blue, 20% glycerol). After heating, the protein was resolved on polyacrylamide SDS gels and transferred to nitrocellulose membrane. After transfer, the membranes were blocked with blocking reagent (5% non-fat milk in distilled water) for 1 hour and then the membranes were incubated with primary antibody. The membranes were incubated for 1 hour with the corresponding secondary antibody, diluted in the above blocking reagent. After three final washes, the membranes were treated with chemiluminescence reagent. All the procedures were done at room temperature.

8. RT-PCR

Total RNA was purified by using commercial kit (komabiotech). For the cDNA synthesis, total RNA and primer were incubated 70℃, 5 minutes, 42℃, 60 minutes, 94℃, 5 minutes and then, in order to 20 ul PCR, the reaction mixture contained template cDNA 10 ng, each primer 20 pmol, Taq DNA polymerase 1 U, 250 mM dNTP, 1.5 mM $MgCl_2$, 40 mM KCl, 10 mM Tris-Cl (pH 8.0), and sterilized DW. Primer sequences were α1 upstream primer (5′-GCA-TCT-CAG-AAG-TCT-GTT-GCC-3′), α1 downstream primer (5′-CCT-GTT-GTT-ACC-TTC-AGG-GAG-3′). Thermocycling condition was one pre-reaction at 94℃, 5 minutes and 30 cycling reaction with 94℃, 30 second denaturation, 52℃, 30 seconds annealing, 72℃, 1 minute extension. After reaction, 15 ㎕ of RT-PCR products was analyzed on 1.5% agarose gel electrophoresis.

Ⅲ. Results

In an initial series of experiments, it was evaluated whether MDPC-23 cells adhere to Col. Plating MDPC-23 cells on different concentrations of Col-coated dishes resulted in marked cell adhesion and spreading. These cells displayed adhesion to Col that was optimal at the concentration of 50 ㎍/ml (Fig. 1).

To test whether cell adhesion to Col was mediated by

integrin and CD44 receptors, cells were treated with anti-integrin α1 and α2 and CD44 antibodies. Anti-α1 and α2 integrin and CD44 antibodies decreased cell adhesion significantly (Fig. 2).

The GAG side chains covalently bound to the core protein may be chondroitin sulfate or heparan sulfate and they dominate the physical properties of the protein. To investigate whether GAGs are involved in the adhesion of MDPC-23 cells to Col, MDPC-23 cells was treated with the chondroitinase ABC, which destroy GAGs. Treatment MDPC-23 cells with increasing amounts of chondroitinase ABC significantly decreased their adhesion to Col in dose-dependent manner (Fig. 3).

A wide range of cytokines including TGF-β1, and TNF-α were screened for their ability to promote MDPC-23 cells adhesion to Col in the absence of other stimuli. In order to investigate the effect of TGF-β1 on MDPC-23 cells adhesion to Col, treatment with TGF-β1 for 4 hours increased their adhesion to Col (Fig. 4). Treatment of MDPC-23 cells with increasing amounts of TNF-α slightly increased their adhesion to Col (Fig. 5).

F-actin is a major cytoskeletal protein involved in cell motility. When MDPC-23 cells were stained with the F-actin-specific probe FITC-phalloidin, a diffuse pattern was observed with cells attached to BSA in control group. Cells adhered to Col radiated fluorescently la-

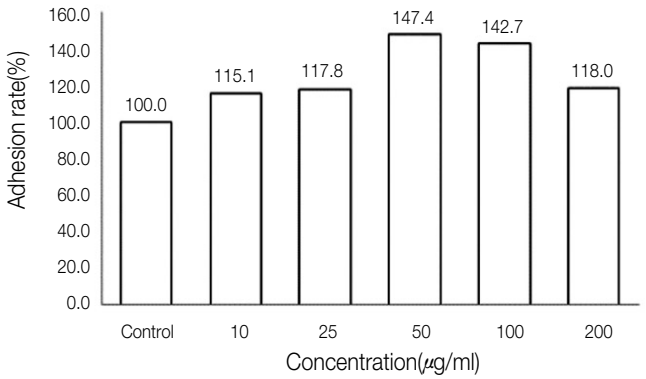

Fig. 1. Kinetics of MDPC-23 cells adhesion to Col. MDPC-23 cells were added to wells coated with different concentrations of Col. Values represent mean ± standard error(SE) for four experiments performed in quadruplicate.

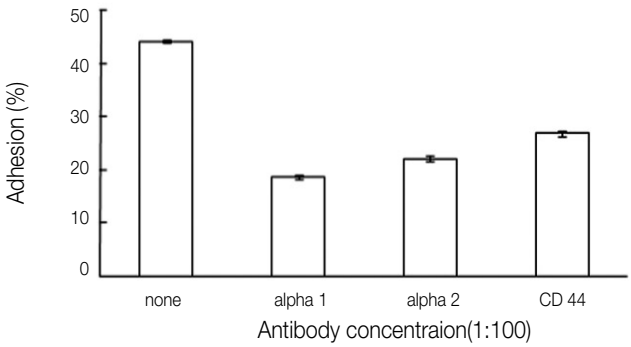

Fig. 2. MDPC-23 cells adhere to Col via integrin and CD44. MDPC-23 cells were treated with anti-integrin α1 and α2 and CD44 antibodies. Anti-α1 and α2 integrin and CD44 antibodies decreased cell adhesion significantly. The values represent the mean ± standard error (SE) of triplicate assays for one representative experiment of three independent experiments.

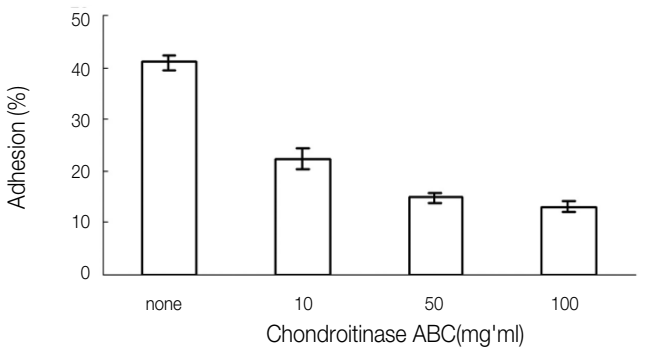

Fig. 3. Inhibition of MDPC-23 cells adhesion to Col by chondroitinase ABC. MDPC-23 cells was preincubated with different concentrations of chondroitinase ABC for 30 minutes at 37℃ and then the cells were plated on the 50 μg/ml Col-coated wells. Values represent mean ± standard error(SE) for four separate experiments performed in quadruplicate.

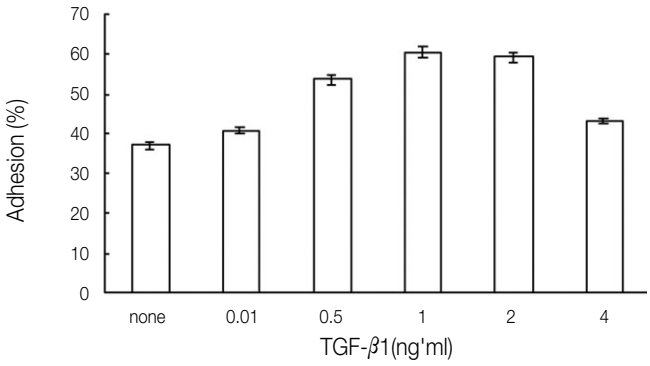

Fig. 4. Potentiation of MDPC-23 cells adhesion to Col after incubation with TGF-β1. MDPC-23 cells was cultured in the presence of different concentrations of TGF-β1 in 50 μg/ml Col-coated wells. Values represent mean ± standard error(SE) for four experiments performed in quadruplicate.

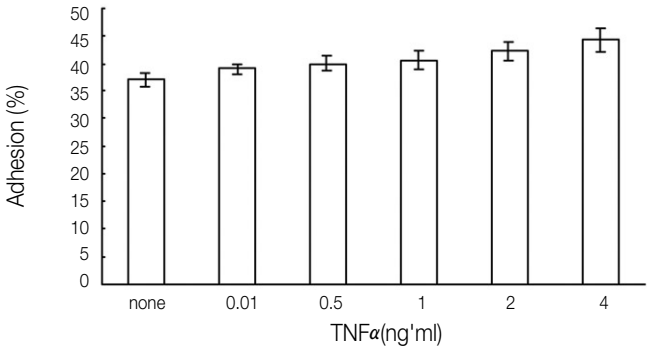

Fig. 5. Potentiation of MDPC-23 cells adhesion to Col after incubation with TNF-α. MDPC-23 cells was cultured in the presence of different concentrations of TNF-α in 50 μg/ml Col-coated wells. Values represent mean $\pm$ standard error(SE) for four experiments performed in quadruplicate.

beled F-actin bundles outward at edge of the cells in the process of spreading (Fig. 6). Many actin plaques were observed at 60 minutes.

To investigate the expression of integrin α1, α2 and proteoglycan CD44 on cells, MDPC-23 cells were cultured on Col-coated slide glasses for 60 minutes, and were stained with anti-α1, -α2 and anti-CD44 antibodies (Fig. 7, 8 and 9). MDPC-23 cells which were transferred to Col-coated slide glasses, usually constituted a variety of cell shapes from round to expanded with filopodia. Cell surface proteins α1, α2 and CD44 were located at the periphery.

To investigate the organization of FAK and paxillin during cell adhesion, the distribution of these two proteins was examined by immunofluorescent staining. FAK organization in confluent cells on Col was shown. FAK protein was expressed in outlining areas of cell (Fig. 10). Paxillin organization in confluent cells on Col was shown. In contrast to FAK staining, discrete paxillin staining was observed at lamellipodial edges in cells (Fig. 11).

MDPC-23 cells plated on Col-coated wells were incubated for 24 hours at 37℃. After washing the wells, medium with or without TGF-β1 and TNF-α was added to the adherent MDPC-23 cells and the wells were further incubated for 4 hours. Lysates were examined for expression of adhesive proteins by using Western blot. TGF-β1 treatment elevated the expression of integrin α1, α2 and chondroitin sulfate on cell surface (Fig. 12).

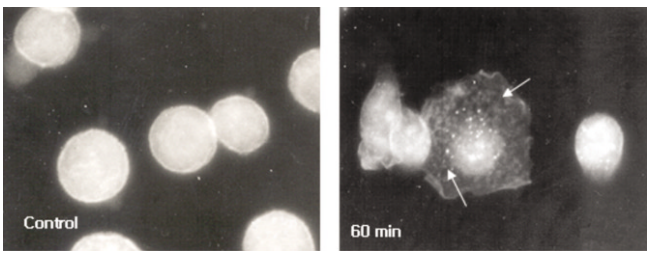

Fig. 6. F-actin organization during adhesion to Col. MDPC-23 cells to coverslips coated with 50 μg/ml Col were incubated for 60 minutes. Actin filaments are indicated with arrows.

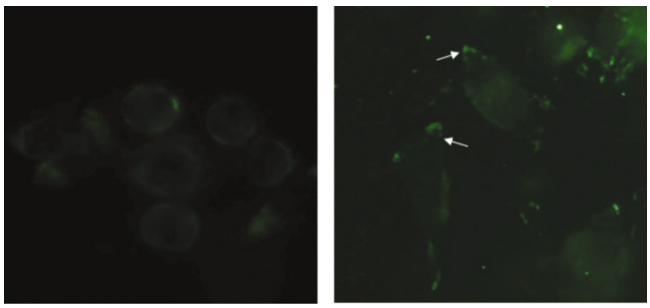

Fig. 7. Immunofluorescent detection of integrin α1 antibody of MDPC-23 cells. MDPC-23 cells plated on Col-coated coverslip were incubated for 60 minutes.

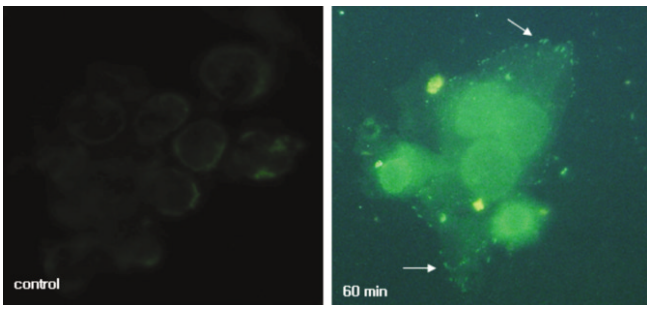

Fig. 8. Immunofluorescent detection of integrin α2 antibody of MDPC-23 cells. MDPC-23 cells plated on Col-coated coverslip were incubated for 60 minutes.

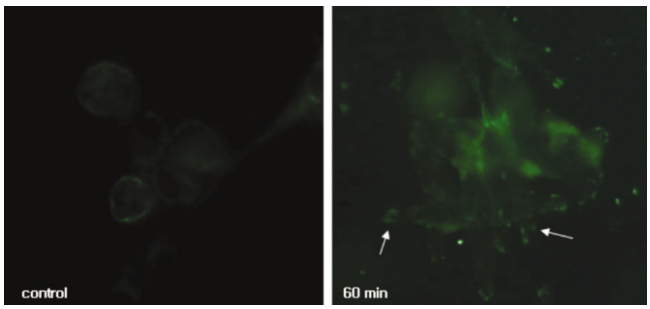

Fig. 9. Immunofluorescent detection of proteoglycan CD44 antibody of MDPC-23 cells. MDPC-23 cells plated on Col-coated coverslip were incubated for 60 minutes.

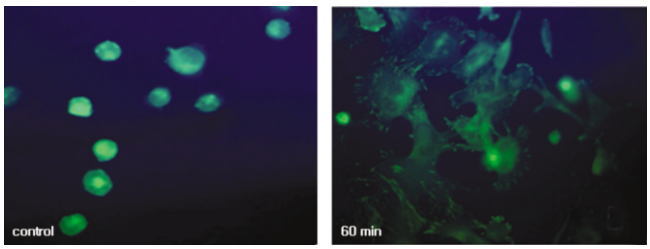

Fig. 10. Localization of FAK in MDPC-23 cells plated on Col-coated coverslips. MDPC-23 cells plated on Col-coated coverslip were incubated for 60 minutes.

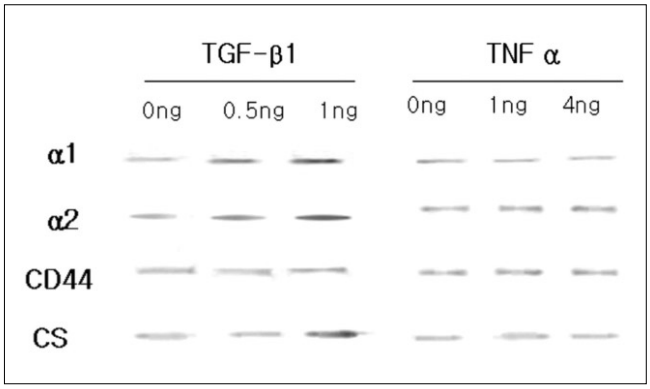

Fig. 12. The effect of TGF-β1 and TNF-α on expression of integrin α1, α2, CD44 and chondroitin sulfate. MDPC-23 cells plated on Col-coated wells were incubated for 60 minutes at 37℃. Wells were washed twice with DMEM. Midium with TGF-β1 and TNF-α or without TGF-β1 and TNF-α was added and the wells were further incubated for 4 hours. Cell lysates containing the same amount of protein were analyzed by 7.5 % SDS-PAGE and immunoblotted with anti-integrin α1, α2 CD44 and chondroitin sulfate.

TNF-α treatment did not elevate the expression of integrin α1, α2, CD44 and chondroitin sulfate.

For RT-PCR, total cytoplasmic RNA was isolated. MDPC-23 cells plated on Col-coated wells were incubated for 24 hours at 37℃. After washing the wells, medium with or without TGF-β1 and TNF-α was added to the adherent MDPC-23 cells and the wells were further incubated for 4 hours (Fig. 13). The expression of integrin α1 mRNA was increased by TGF-β1 (1 ng/ml).

Ⅳ. Discussion

The dentinogenesis process is very similar to that of osteogenesis. Indeed, odontoblasts and osteoblasts synthesize an extracellular matrix that mineralizes to form dentin and bone, respectively. These tissues share many similarities, both morphologically and in the composition of the extracellular matrix formed. The odontoblasts se-

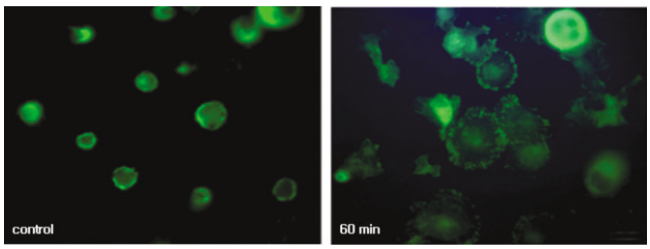

Fig. 11. Paxillin reorganization during MDPC-23 cells adhesion to Col. MDPC-23 cells plated on Col-coated coverslip were incubated for 60 minutes.

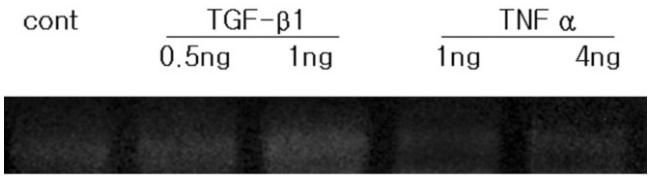

Fig. 13. Results of RT-PCR of integrin α1 expressions. MDPC-23 cells plated on Col-coated wells were incubated for 24 hours at 37℃. After washing the wells, medium with or without TGF-β1 (0.5 and 1 ng/ml) and TNF-α (1 and 4 ng/ml) was added to the adherent MDPC-23 cells and the wells were further incubated for 4 hours.

crete a collagenous extracellular matrix which subsequently mineralizes into dentin, a bone-like hard tissue[1]. Tooth development involves a series of interactions between the oral epithelium and cranial neural crest-derived mesenchymal cells. These interactions progressively transform the tooth primordia into complex mineralized structures. During advanced stages of odontogenesis, mesenchymal cells differentiate into odontoblasts that synthesize the dentin matrix, whereas epithelial cells differentiate into ameloblasts that are responsible for enamel matrix formation. These epithelial-mesenchymal interactions have been extensively investigated in relation to regulatory mechanisms by transcription factors, growth factors, extracellular matrix molecules, and cell adhesion molecules[19]. Cell adhesion molecules are cell surface glycoproteins involved in diverse biological processes such as cell adhesion, cell recognition, control of cell division, and migration, differentiation, and morphogenesis[20]. In this study, the molecular interactions were examined and the expression of proteins that are involved when odontoblasts initially adhere to type I Col, which is a major component of dentin, were determined .

Cell adhesion to ECM involves a complex set of binding interactions between cell adhesion receptors and

components of the matrix. The adhesive behavior of a particular cell depends on the repertoire of cell adhesion receptors expressed on its surface, the composition of the ECM that surrounds the cell and the adhesion proteins that the cell produces and incorporates into the surrounding matrix. Integrins are heterodimeric membrane glycoproteins that consist of one 130~200 kD α-subunit and one 90~130 kD β-subunit. Both subunits span the membrane and have a relatively small, well-conserved cytoplasmic domain that can interact with cytoskeletal components[21]. In this study, monoclonal antibodies to α1 and α2 prevented MDPC-23 cells adhesion to Col. These present that the adhesion of MDPC-23 cells to Col is mediated by integrin α1 and α2.

Proteoglycans that composed of core protein and GAGs are produced by most eukaryotic cells and are versatile components of pericellular and ECM. CD44s have emerged as a broad class of integral membrane glycoproteins expressed on the surface of many cell types including hemopoietic and epithelial cells, fibroblasts and brain tissue[22]. In this study, MDPC-23 cells adhesion to Col involved in the expression of CD44, indicating that these adhesion molecule plays a role in the adhesion of MDPC-23 cells to Col. Because CD44 has GAGs as side chains, ongoing studies were performed to determine the participation of GAGs. MDPC-23 cells were treated with chondroitinase to destroy GAGs on cell surface. The adhesion of MDPC-23 cells was partially decreased by chondroitinase. These present that GAGs on cell surface, especially chondroitin sulfate participate in MDPC-23 cells adhesion to Col.

The receptors interact with cytoskeletal proteins on the cytoplasmic side of the plasma membrane, providing a linkage between the ECM and cytoskeleton[5]. Binding of ECM molecules to their cognate cell surface receptors also gives rise to biochemical signals within the cells such as the activation of protein kinase C[6], tyrosine kinase[7] and MAP kinase[8]. FAK and paxillin are the major tyrosine phosphorylated protein present in cells upon activation of the integrin receptors[8]. Immunofluorescence data showed that FAK and paxillin are localized in focal adhesion plaques in MDPC-23 cells adhesion to Col.

Cytokines may modulate integrin-mediated cell adhesion[14]. Prolonged incubation of human cell lines with cytokines such as 1L-1β, TNF-α, or TGF-β1 results in the increased expression of integrin receptors[23,24]. Another effect of cytokines is represented by a sudden T cell adhesion to Fn after exposure to macrophage inflammatory protein-1β[25,26]. The nature of the signal provided by cytokines and mechanisms leading to this change in cell adhesion are not known. In this study, TGF-β1 treatment enhanced the expression of integrin α1, α2 and chondroitin sulfate in contrast to TNF-α treatment. This result suggests that TGF-β1 play an important role in expression of cell adhesion molecules on odontoblast cells.

Taken together, MDPC-23 cells adhere to type I Col and expressed a complex pattern of integrins and proteoglycans, including α1, α2, chondroitin sulfate and CD44 detected by immunoblotting and immunofluorescence assay. TGF-β1 treatment enhanced the expression of adhesion molecules such as integrin α1, α2 and chondroitin sulfate.

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국문초록

상아모세포의 Ⅰ형 아교질에 대한 부착

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상아모세포는 부착분자들을 이용하여 기질에 부착하는 세포이며, 인테그린과 같은 부착분자들이 일련의 세포와 세포외기질을 인지하는 신호전달분자로 알려져 있다. 본 연구의 목적은 상아모세포(MDPC-23 세포)와 I형 아교질과의 상호작용과 TGF-β1과 TNF-α가 세포부착분자의 발현에 미치는 영향을 알아보기 위해 시행하였다.

본 연구에서 MDPC-23 세포는 농도의존적으로 I형 아교질에 부착했으며, 면역형광염색법에서 MDPC-23 세포가 아교질에 부착할 때, 국소부착점에서 인테그린 α1, α2, CD44, FAK 그리고 paxillin의 발현양상을 관찰할 수 있었다. 싸이토카인 TGF-β1은 MDPC-23 세포의 아교질에 대한 부착성 및 인테그린 α1, α2와 chondroitin sulfate의 발현을 증가시켰으며, RT-PCR의 결과에서는 인테그린 α1의 mRNA의 양이 TGF-β1에 의해서 증가되었음을 확인하였다.

결론적으로 MDPC-23 세포는 아교질에 부착 친화성을 갖고 있으며, 부착 시에 인테그린 α1, α2 와 CD44 그리고 chondroitin sulfate와 같은 부착분자들이 관여한다. 그리고 TGF-β1은 인테그린 α1, α2 그리고 chondroitin sulfate와 같은 부착분자의 발현을 증가시켰다.

주요어: 상아모세포, I형 아교질, 세포부착