Establishment of collagen gel-based co-culture models for invasive study with respect to cancer cell-fibroblast interaction

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감사의 글

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뒤돌아보니 모든 것이 하나님의 은혜였음을 고백합니다. 시작도 마침도 하나님의 은혜라고 생각합니다. 김삼환 목사님 감사드립니다. 목사님이 계셔서 오늘의 제가 있을 수 있었다고 생각합니다. 김 진 교수님 감사합니다. 제가 공부를 열심히 해서 행복하다고 말씀 하시던 모습이 오랫동안 제 기억에 남을 것입니다. 마지막 스승을 참으로 훌륭하고 좋으신 분을 만나게 해 주신 것에 대하여 하나님께 감사하고 있습니다. 아내인 영란이, 참으로 고생많았어요! 감사해요! 하나님, 영란이를 오늘 사랑 한 것 보다 내일 더 사랑하게 해주세요! 부족한 우리아들 동훈아 사랑한다, 그리고 예쁜 딸 민경아 사랑한다. 동훈아 민경아, 아빠가 드디어 박사가 되었단다!

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ABSTRACT

Establishment of collagen gel-based co-culture models for invasive study with respect to cancer cell-fibroblast interaction

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(Directed by Professor **Jin Kim**)

Epithelial and mesenchymal interaction(EMI) is well known to be essential in embryonic development, wound healing and carcinogenesis. This study was aimed to design *in vitro* model for the invasive study with respect to cancer cells and fibroblasts interaction.

This study used two oral squamous cell carcinoma cell lines YD-10B and YD-38. To establish *in vitro* system for invasion study, three different models were designed; Collagen gel-based cancer cell culture model devoid of Swiss 3T3 fibroblasts (C), Collagen gel-based indirect co-culture model (C-In) with intervening collagen layer between cancer cells and collagen gel layer with Swiss 3T3 fibroblasts, and Collagen gel-based direct co-culture model (C-Dr). C-In and C-Dr models were compared with separate co-culture and mixed co-culture models using transmembrane system. To evaluate whether these models are

appropriate for invasive study, the activity of matrix metalloproteases (MMP)-2 and MMP-9 was examined by gelatin RT-PCR and zymography because MMP family are well known to have an active role in cancer cell invasion and metastasis. Tissue from which YD-10B and YD-38 cell lines were established and used for immunohistochemical staining for the detection of MMP-2 and MMP-9.

As results, MMP-2 mRNA was expressed in both YD-10B and YD-38 cell lines, when cultured in the collagen gel without fibroblasts. On the other hand, MMP-9 was expressed faintly in YD-10B cells and was not detected in YD-38 cells. Direct co-culture of cancer cells and Swiss 3T3 fibroblasts in the collagen gel showed remarkable increase of MMP-2 and MMP-9 expression. In indirectly contacted co-culture models, both MMP-2 and MMP-9 were not increased. Gelatin zymography showed that the active form of MMP-2 was induced only by C-Dr model and mixed co-culture, suggesting that MMP-2 activity can be induced by direct contact between cancer cells and fibroblasts. In immunohistochemical staining of MMP-2 expression, multiple foci of strong positive reaction were found in the interface between cancer cell nests and surrounding stroma at the invasive front in both cell lines. The cancer cells also showed MMP-2 positive reaction in two cell lines. In MMP-9 expression, YD-10B cells showed diffuse positive reaction throughout cancer cells and surrounding stroma. MMP-9 expression showed diffuse positive reaction in the stroma of YD-38 cancer tissue, while cancer cells of YD-38 showed focal positive reaction in the infiltrating area into bony tissue.

From the results, MMP-2 may be activated by direct contact between cancer cells and fibroblasts, not by soluble factors generated by indirect co-culture of

two kinds of cells. As the techniques to isolate cancer cells of this model, the established C-In, C-Dr model can be useful to investigate the invasion study with respect to cellular interaction between cancer cells and fibroblasts.

Key words: cancer cell-fibroblast interaction, direct contact, indirect contact, collagen gel-based co-culture, matrix metalloproteinase

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I. INTRODUCTION

Epithelial and mesenchymal interaction (EMI) is well known to be essential in embryonic development and wound healing. In adult organs, extracellular matrix (ECM) and stromal cells have regulatory roles in epithelial cell growth and differentiation (Cunha, 1985). Moreover, recent cancer studies have begun to know that tumor stroma can play active roles in carcinogenesis (Radisky *et al.*, 2001; Tlsty, 2001). Carcinogenesis has been traditionally explained by activation of oncogene and inactivation of tumor suppressor gene. Recent research in carcinogenesis has been focused on the abnormal communication between the tumor cells and their microenvironment (Radisky *et al.*, 2001). Stromal

microenvironment may be associated with multistep process such as initiation, progression and invasion (Sympson *et al.*, 1995; Olumi *et al.*, 1999; Sternlicht *et al.*, 1999; Lynch *et al.*, 2002; van Kempen *et al.*, 2002).

Stromal microenvironment includes 1) ECM, 2) stroma consisting of fibroblast, adipose, vasculature and resident immune cells, 3) conventional milieu of cytokines and growth factors (Park *et al.*, 2000; Mueller and Fusenig, 2002). ECM provides both structural support and cell to cell and cell-matrix communication (Aumailley *et al.*, 1998; Howe *et al.*, 1998). For the investigation of the inter-relationship between tumor mesenchyme and parenchymal cancer cells, the mixed co-culture of two kinds of cells, the culture using conditioned medium and the separate co-culture by transmembrane device have been reported. However, ECM, one of the major factor of EMI, is evaded in these studies. Accordingly, it is necessary to establish *in vitro* experimental model including ECM akin to *in vivo* for the study of EMI.

Collagen gel-based organotypic co-cultures have been developed to provide the *in vitro* stromal microenvironment for the reconstruction of *in vivo* conditions (Rodriguez-Boulan and James Nelson, 1989). Additionally, the culture using basement membrane component or organotypic culture by type I collagen have been widely used (Weaver *et al.*, 1997; Olumi *et al.*, 1999; Spancake *et al.*, 1999; Vacariello *et al.*, 1999; Liotta and Kohn 2001).

Tumor cell invasion and metastasis are now regarded as multi-step phenomena, involving proteolytic dgradation of basement membranes and ECM, altered cell adhesion and physical movement of tumor cells. Angiogenesis, the formation of new blood vessels, is essential both for tumor growth and for successful tumor invasion and metastasis. Angiogenesis is complex and

dynamic, and requires proliferation of endothelial cells from pre-exsting blood vessels, breakdown of ECM and migration of endothelial cells. Thus, growth and development of blood vessels within tumors requires the same factors that are crucial to tumor cell invasion and the matrix metalloproteases (MMP) play a central role in all of these processes (Curran and Murray 2000). MMPs are one of the controling factors in ECM component and include about 20 different zinc-dependant proteinase depending on its molecular weight and function. It was first discovered as enzyme related to liquefaction of tadpole-tale (Gross and Lapiere 1962). MMPs have effect on ECM decomposition, wound healing, angiogenesis, embryogenesis, cancer invasion and metastasis (Olson *et al.*, 1998). The MMPs are responsible for degradation of the constituents of basement membranes and the ECM. Through interaction with an array of cell adhesion molecules, MMPs are implicated in altered adhesion between the tumor cell and its environment, and recently have been shown to play a role in the movement of cells through the ECM. In addition to their funtcion in the breakdown of the ECM, MMPs also have growth regulatory effects on both primary and secondary tumors (Curran and Murray 2000). Consequently, MMP-2 and MMP-9 have enormous influence on invasion and metastasis of tumor (Stamenkovic, 2003), becoming the subject of investigation to those who work on prevention, detection in early stage and treatment of cancer.

The present study was aimed at the establishment of *in vitro* model simulating *in vivo* for invasion study with respect to cancer cells and fibroblasts interaction. We developed modified collagen-gel based co-culture model useful for the study of the direct and indirect effects of cancer cells by fibroblasts stimulation. The gelatinolytic activity and expression of MMP-2 and MMP-9 are evaluated to confirm whether this model is appropriate for this study.

II. MATERIALS AND METHODS

1. Cell culture

YD-10B and YD-38 oral squamous cancer cells established in Yonsei University College of Dentistry were used for this study (Lee *et al.*, 2005). YD-10B expressed more tumorigenesity than YD-38 *in vivo* study. The cancer cells were fed with a mixture of Dulbecco's Modified Eagles Medium (DMEM; Gibco BRL, Grand Island, NY, USA) and Ham's nutrient mixture F12 (Gibco BRL, Grand Island, NY, USA) at a 3:1 ratio supplemented with 10% fetal bovine serum (Hyclone, Logan, UT, USA), 0.1 ㎍/㎖ cholera toxin, 0.4 ㎍/㎖ hydrocortisone, 5 ㎍/㎖ insulin, 5 ㎍/㎖ apo-transferrin, and 2 ㎍/㎖ 3'-5-triodo-l-thyronine (all purchased from Sigma St. Louis, MO, USA). Cells were cultured at 37℃ in an atmosphere containing 5% CO_2. The culture medium was changed every two or three days. The Swiss 3T3 fibroblasts were obtained from the ATCC and were grown in a mixture of DMEM and Ham's nutrient mixture F12 at a 3:1 ratio supplemented with 10% fetal bovine serum.

2. Three-dimensional culture

The cancer cells were cultured with dermal equivalents with or without fibroblasts. To produce demal equivalents, 300 ㎕ Type I-A collagen mixture (Nitta Gelatin Inc, Osaka, Japan) was generated by mixing 8 volumes of an

ice-cold collagen solution with 1 volume 10× reconstitution solution (0.022 g/mℓ $NaHCO_3$, 0.0477 g/mℓ HEPES, 0.05 N NaOH) and 1 volume 10× DMEM. Swiss 3T3 (1.5×10^5 cells in 30 μℓ) suspension in medium were added. Two hundred fifty microliters of this mixture were poured onto polycarbonate filter inserts (3.0 μm pore size, 12 ㎜ diameter; Millipore Corp, Bedford, MA, USA) and placed in 6-well plates (Becton Dickinson, Franklin Lakes, NJ, USA). After 1 day incubation at 37℃, 3.0 mℓ of the medium was added to 6-well plates. Oral squamous carcinoma cells (3×10^5 cells in 200 μℓ) were plated on to the dermal equivalents and after additional 2 days the media were changed in 6-well plates, and added 200 μℓ medium to the epidermal equivalents. After additional 2 days the cells were exposed to the air by removing all medium from surface and were cultured for 5 days. Medium was changed every 2 to 3 days. The culture tissue was fixed with 10% neutral formalin and embeded with paraffin. The paraffin section was stained by hematoxylin and eosin (H-E) to examine the histological findings of the three-dimensional culture tissue.

3. Collagen gel-based co-culture for direct and indirect contact between cancer cells and fibroblasts

Three different culture models were designed for the investigation of the direct and indirect effects of cancer cells by fibroblasts. The cancer cells were cultured on the type I collagen gel with fibroblasts or without fibroblasts. In indirect co-culture model (C-In), oral squamous carcinoma cells were separated from fibroblasts-embedded collagen matrix by acellular collagen layer, which model was designed for the examination of the indirect effect by fibroblasts. The

direct co-culture model (C-Dr) was designed that oral carcinoma cells could be directly contacted with Swiss 3T3 fibroblasts (Fig. 1). To prepare the collagen gel embedded with fibroblasts, 8 volume of ice-cold type I collagen was mixed with 1 volume of reconstitution buffer, 1 volume of 10× culture media and Swiss 3T3 fibroblasts (1.5×10^5 cells in a 60㎜ plate). The control (C) was established by the same components merely devoid of Swiss 3T3 fibroblasts. After the polymerization of collagen-fibroblast mixture for 1 hour and incubation overnight at 37℃, C-Dr model was designed that 3.0×10^5 cancer cells were added on the collagen matrix and cultured for 2 or 3 days as submerged in culture media. For C-In model, acellular collagen was applied on the top of fibroblasts-embedded collagen matrix.

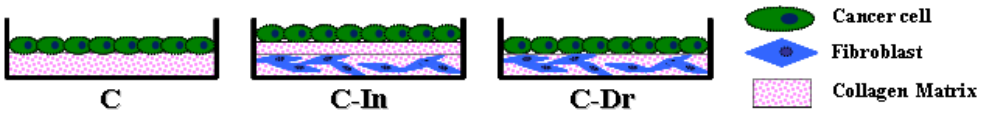

Fig. 1. Collagen gel-based cancer cell culture model devoid of fibroblasts (C), Collagen gel-based indirect co-culture model (C-In) with intervening collagen layer between cancer cells and fibroblasts and Collagen gel-based direct co-culture model (C-Dr).

4. Separate co-culture by transmembrane culture system

To perform separate co-culture for examining the effect of indirect contact between two kinds of cells, first, 1.5×10^5 cancer cells were seeded on millicell

(Millipore Corp, Bedford, MA, USA) with 0.4 ㎛ pore size membrane, then placed into a 60 ㎜ plate in which 1.5×10^5 Swiss 3T3 fibroblasts were seeded. This co-culture system was incubated with DMEM and Ham's nutrient mixture F12 at a 3:1 ratio supplemented with 10% fetal bovine serum at 37℃ for 24 hours (Fig. 2.A).

5. Mixed co-culture

To perform mixed co-culture for direct contact between cancer cells and fibroblasts, 1.5×10^5 number of each cells were cultured in a 60㎜ plate for 24 hours. The same media was used for this model (Fig. 2.B).

Fig. 2. Separate co-culture by transmembrane culture system (A) and mixed co-culture (B).

6. Immunohistochemistry (IHC)

To validate whether the collagen-gel based co-culture models were well designed to detect direct and indirect effects between two kinds of cells, we

examined the histological findings of these models. After fixation with 10% neutral formalin, the samples were embedded with paraffin, then paraffin-embedded tissues were used for immunohistochemical staining. After deparaffinization and dehydration, endogenous peroxidase was blocked with 3% H_2O_2 in methanol, and the sections were incubated with normal horse serum (GibcoBRL, Grand Island, NY, USA) at room temperature. Subsequently, anti-cytokeratin AE1/3 antibody diluted at 1:50 (DAKO, Glostrup, Denmark) was applied overnight at room temperature. The slides were then incubated with biotin-labeled horse anti-mouse/anti-rabbit IgG (Vector Lab, Burlingame, CA, USA) at room temperature for 30 min, and with horseradish peroxidase streptavidin (Vector Lab, Burlingame, CA, USA) at room temperature for 30 min. The visualization was performed by 3,3-diaminobenzidine tetrachloride (DAB kit, Vector, Burlingame, CA, USA) and the slides were counterstained with Meyer's hematoxylin. Tissue sections of YD-10B and YD-38 cell lines were used for immunohistochemical staining for the detection of MMP-2 and MMP-9 expression in the tissue. Primary antibodies against MMP-2 and MMP-9 diluted at 1:100 (Lab Vision, Fremont, CA, USA) were applied and avidin-biotin complex method was applied. Visualization was performed with 3,3-diaminobenzidine tetrachloride, and the slides were counterstained with Meyer's hematoxylin.

7. RT-PCR

Total RNA was isolated from cell lysates by RNeasy (Qiagen, Hilden, Germany) kit. The concentration of RNA was measured at a wavelength of 260

nm using a spectrophotometer (DU-70, Beckman, USA). Total RNA (1 ㎍) was reverse-transcribed with Moloney Murine Leukemia Virus (MMLV) reverse transcriptase (Promega, Madison, WI, USA) in 20 mM $MgCl_2$, 0.1 ㎎/㎖ bovine serum albumin, 10 mM dithiothreitol, and 0.5 mM deoxynucleotides. cDNAs were generated using random hexamers (Promega, Madison, WI, USA) at 37℃ for 60 min. PCR was carried out in a 20 ㎕ volume containing 20 ng of cDNA template, 10 pmol each of oligodeoxynucleotide primer, 200 mM each of deoxynucleotide triphosphate, 1.5 mM $MgCl_2$, 0.01% gelatin, and 1.5 U of Taq polymerase (Bioneer, Taejeon, Korea). RT-PCR analysis was performed under the following conditions: denaturation at 94℃ for 3 min, and then denaturation for 30 seconds at 95℃, annealing for 30 seconds at 53℃ for MMP-2, 58℃ for MMP-9 and GAPDH, and extension for 1 min at 72℃. The samples were processed through 30 cycles for MMP-2, 36 cycles for MMP-9 and 30cycles for GAPDH. The oligodeoxynucleotides were synthesized as specific primers in PCR (Table 1). Aliquots of the amplified DNA were electrophoresed on 2% agarous gel and stained with ethidium bromide. The signals were then semi-quantitated using an image analyzer (Bio-Imaging analyzer system 2500, Fuji, Japan). mRNA expression of each gene was normalized with that of GAPDH expression.

Table 1. Primer sequences of MMP-2, MMP-9 and GAPDH genes

Primer Name		Sequence (5' → 3')	Anealing Temperature	Product Size
MMP-2	sense	GCG ACA AGA AGT ATG GCT TC	58 ℃	390 bp
	antisense	TGC CAA GGT CAA TGT CAG GA		
MMP-9	sense	CGC AGA CAT CGT CAT CCA GA	53 ℃	406 bp
	antisense	GGA TTG GCC TTG GAA GAT GA		
GAPDH	sense	ATC AAG AAG AGG GTG GTG AAG CAG G	58 ℃	320 bp
	antisense	GCG TGC CTT GGA GGC CAT GTA GG		

8. Zymography

The cancer cells and fibroblasts were cultured overnight using serum-containing media, and then washed with phosphate buffered saline twice. Subsequently, the cells were cultured using serum-free media for 24 hours. For the detection of gelatinolytic activity, the conditioned media were centrifuged for 10 minutes and were concentrated by centriprep centrifugal filter device (Vivascience, Hannover, Germany) and quantified protein content. Electrophoresis was done with 10% SDS-PAGE gel containing 0.2% gelatin. Then, the gels were washed in 2.5% Triton X-100 at room temperature for 1 hour, then incubated in reaction buffer containing 10 mM $CaCl_2$, 50 mM Tris, 0.15 M NaCl, 1% Triton X-100 at 37℃ for 18 hours. To detect MMP activity, the gels were stained by 0.05% Coomassie blue, 10% acetic acid, 10% methanol for 1 hour. After destaining with 10% methanol and 10% acetic acid for 2 hours, gelatinolytic activiy was examined as clear bands on a blue background. HT-1080 fibrosarcoma cells were used as a positive control.

III. RESULTS

1. Histological examination

(1) Three dimensional culture

Both YD-10B and YD-38 cells showed stratification of cancer cells in three dimensional culture. The cytological feature showed nuclear pleomorphism and hyperchromasia. Interestingly, the section from collagen gel devoid of fibroblasts revealed no infiltrative growth. In contrast, the section cultured with collagen gel embedded with fibroblasts showed definite infiltrative growth of cancer cells. Immunohistochemical staining for cytokeratin AE1/3 exhibited clearly infiltrating cancer cells into collagen gel (Fig. 3 A, B).

(2) Collagen gel-based co-culture models (C-Dr and C-In)

The collagen gel-based co-culture model, C-Dr showed a few foci of direct contact between two kinds of cells, while C-In model showed the thin collagen layer (103.0 ± 6.3㎛) between cancer cells and fibroblasts. Immunohistochemically cytokeratin AE 1/3 positive oral squamous cancer cells were directly contacted to the fibroblasts in C-Dr model (Fig. 4).

2. mRNA expression of MMP-2 and MMP-9

RT-PCR was done for observing mRNA expression of cancer cells when co-cultured with fibroblasts in C-Dr and C-In models. MMP-2 and MMP-9 mRNA were expressed in both YD-10B and YD-38 cell lines, when cultured in the collagen gel without fibroblasts. Direct co-culture of cancer cells and fibroblasts in the collagen gel showed remarkable increase of MMP-2 and MMP-9 mRNA expression. In indirectly contacted co-culture models, both MMP-2 and MMP-9 mRNA were not increased compare to the cancer cells cultured in the collagen gel without fibroblasts (Fig. 5).

3. Zymography

In monolayer culture, YD-10B and YD-38 oral squamous cancer cells exhibited MMP-2 and MMP-9 expression, but no active form of MMP-2 was found. Swiss 3T3 fibroblasts also showed abundant MMP-2 and MMP-9 expression. Mixed co-culture also showed the active form of MMP-2. No active MMP-2 form was found in the separate co-culture model (Fig. 6). When examined in the collagen gel-based C-Dr model, a clear band of active form of MMP-2 was shown, but no active form was shown in C-In model (Fig. 7).

4. Immunohistochemical findings of the surgical tissue from the patients

We examined IHC findings of MMP-2 and MMP-9 expression, to compare the results of culture tissue with the surgical tissue from the patients. In MMP-2 expression, multiple foci of strong positive reaction were found in the interface between cancer cell nests and surrounding stroma at the invasive front in both cell lines. The cancer cells also showed positive reaction in two cell lines (Fig. 8. A, B). In MMP-9 expression showed diffuse positive reaction in the stroma of YD-38 cancer tissue, while cancer cells of YD-38 showed focal positive reaction in the infiltrating area into bony tissue (Fig. 8. C). MMP-9 expression, YD-10B cells showed diffuse positive reaction throughout cancer cells and surrounding stroma (Fig. 8. D).

IV. DISCUSSION

There are many evidences that stromal fibroblasts have active roles in carcinogenesis. Olumi *et al.* (1999) demonstrated that carcinoma-derived fibroblasts stimulate tumor progression of initiated nontumorigenic prostatic epithelial cells. Barcellos-Hoff *et al.* (2000) found that radiation-induced changes in the stromal microenvironment can contribute to neoplastic progression *in vivo*. Ryan *et al.*(1993) and Shekhar *et al.*(2001) reported that stromal fibroblasts influence on the proliferation and differentiation of breast cancer cells. Moreover, MMP family that induce the invasion and metastasis is produced by cancer-derived fibroblasts (Basset *et al.*, 1990; Heppner *et al.*, 1996; Lynch and Matrisian, 2002; Singer *et al.*, 2002; Suzuki *et al.*, 2004).

The mixed co-culture and separate co-culture by transmembrane device have been widely used for the study of the interaction between tumor stroma and cancer cells (Suzuki *et al.*, 2004). We thought that ECM was evaded in those models, resulting in different results by different models. Accordingly we designed *in vitro* experimental model including ECM akin to *in vivo* for the study.

When tumor cells and fibroblast contact in co-culture media, they form tumor-stroma complex, and then activated, destorying stroma and ultimately causing the metastasis. This tumor-stroma complex increases activation of NF-κB or adds NF-κB in squamous cell carcinoma. NF-κB is a transcription factor which activates the genes involved in tumor growth, invasion and inhibition of tumor apoptosis (Bhowmick and Moses, 2005). Co-cultured fibroblast enhanced

the induction of an active form of MMP-9, cell motility, and the activation of a transcription factor NF-κB in tumor cells. Stromal fibroblast may induce NF-κB activation and promote the invasion of oral squamous cell carcinoma (Ikebe *et al.*, 2004). Fibroblasts, taking an important role in these process, are surrounded by a complex, three-dimensional extracellular matrix. In particular, collagen has a role inducing mitogen-activated protein kinase (MAPK) phosphorylation as a fibroblasts-collagen interaction as well as having a function of structural support (Langholz *et al.*, 1997). MAPKs play a major role in the mitogenic signal transduction pathway and are essential components of both growth and differentiation. Constituitive activation of the MAPK cascade is associated with the carcinogenesis and metastasis of human breast and renal cell carcinoma (Reddy *et al.*, 1999).

Based on those experiments above, cancer cells might be greatly affected by surrounding environment and this molecular biological change would be the main target for cancer research (Park *et al*, 2000). .Consequently, we applied collagen gel-based co-culture for the detection of cancer cells-stromal fibroblasts interaction in terms of invasiveness.

Both YD-10B and YD-38 cells showed stratification of cancer cells in three dimensional culture. Interestingly, the section from collagen gel devoid of fibroblasts revealed no infiltrative growth. In contrast, the section cultured with collagen gel embedded with fibroblasts showed definite infiltrative growth of cancer cells. This result suggests that fibroblasts play an essential role in cancer cell invasion.

Consequently, the present study established the *in vitro* models to investigate direct and indirect effects of cancer cells by fibroblasts stimulation. In collagen

gel-based co-culture models using RT-PCR. mRNA expression of MMP-2 and MMP-9 were augmented when fibroblasts was directly contacted to cancer cells in both YD-10B and YD-38 cancer cells. Compared to MMP activities between collagen gel-based direct and indirect models, MMP-2 active form was found only in direct co-culture model, suggesting that MMP-2 activity may be induced only by direct contact between two cells. Moreover, the mixed co-culture system showed the same result with collagen gel-based direct co-culture model. In immunohistochemical results, MMP-2 expression was also found in the surrounding stroma at the invasion front of the cancer cells. These results suggest that invasive growth of cancer cells may be influenced by fibroblasts stimulation, in particulary by direct contact. Thus collagen gel-based co-culture models may be useful for these study.

In many solid tumors, MMPs are indeed produced by tumor stromal cells. This expression pattern is, at least in part, regulated by tumor-stroma interaction via tumor cell associated extracellular matrix metalloproteinase inducer (EMMPRIN) (Tang *et al.*, 2004). When cancer cells and fibroblasts contact directly, fibroblast cells were exposed to EMMPRIN stimulus of cancer cells, moreover expression is up-regulated in fibroblasts. Eventually, when tumor cells and fibroblast contact via EMMPRIN, then MMP and EMMPRIN are up-regulated.

However, the cell to cell contact seems to be complicate. The interactions may largely depend on cancer cell type and experimental model (Bartsch *et al.*, 2003, Trudel *et al.*, 2003). In the prestent study, YD-38 and YD-10B cell expressed both MMP-2 and MMP-9, and active form of MMP-2 was found in both cell type by fibroblasts stimulus. Collagen gel-based model showed the same pattern of MMP-2 and MMP-9 expresion, when compared to mixed and separated

co-culture model. But the gelatinolytic bands of gelatin zymography was less prominent than those of two models, suggesting that the collagen gel might have a role of buffering gelatinolytic enzyme or work as a reservoir.

Taken all these results together, MMP-2 may be activated by direct contact between fibroblasts and cancer cells, not by soluble factors generated by indirect co-culture of two kinds of cells. As the techniques to isolate each cell type of this model, the established C-In and C-Dr model can be useful to investigate the cellular interaction between stroma fibroblasts and cancer cells.

IV. CONCLUSION

This study was aimed to design *in vitro* model for the invasive study with respect to cancer cells and fibroblasts interaction. The results are summarized as:

1. MMP-2 and MMP-9 mRNA were expressed in both YD-10B and YD-38 cell lines, when cultured in the collagen gel without fibroblasts. Direct co-culture of cancer cells and fibroblasts in the collagen gel showed remarkable increase of MMP-2 and MMP-9 expression. When indirectly contacted co-culture models, both MMP-2 and MMP-9 were not increased.

2. Gelatin zymography showed that the active form of MMP-2 was induced only by collagen gel-based direct co-culture model (C-Dr) and mixed co-culture model, suggesting that MMP-2 activity can be induced by direct contact between cancer cells and stromal fibroblast.

3. In immunohistochemical staining MMP-2 expression, multiple foci of strong positive reaction were found in the interface between cancer cell nests and surrounding stroma at the invasive front of cancer tissue.

From the results, MMP-2 may be activated by direct contact between fibroblasts and cancer cells, not by soluble factors generated by indirect co-culture of two kinds of cells. In conclusion, the established collagen gel-based indirect/direct co-culture models can be useful to investigate the cellular interaction between stroma fibroblasts and cancer cells with respect to invasion growth of cancer cells.

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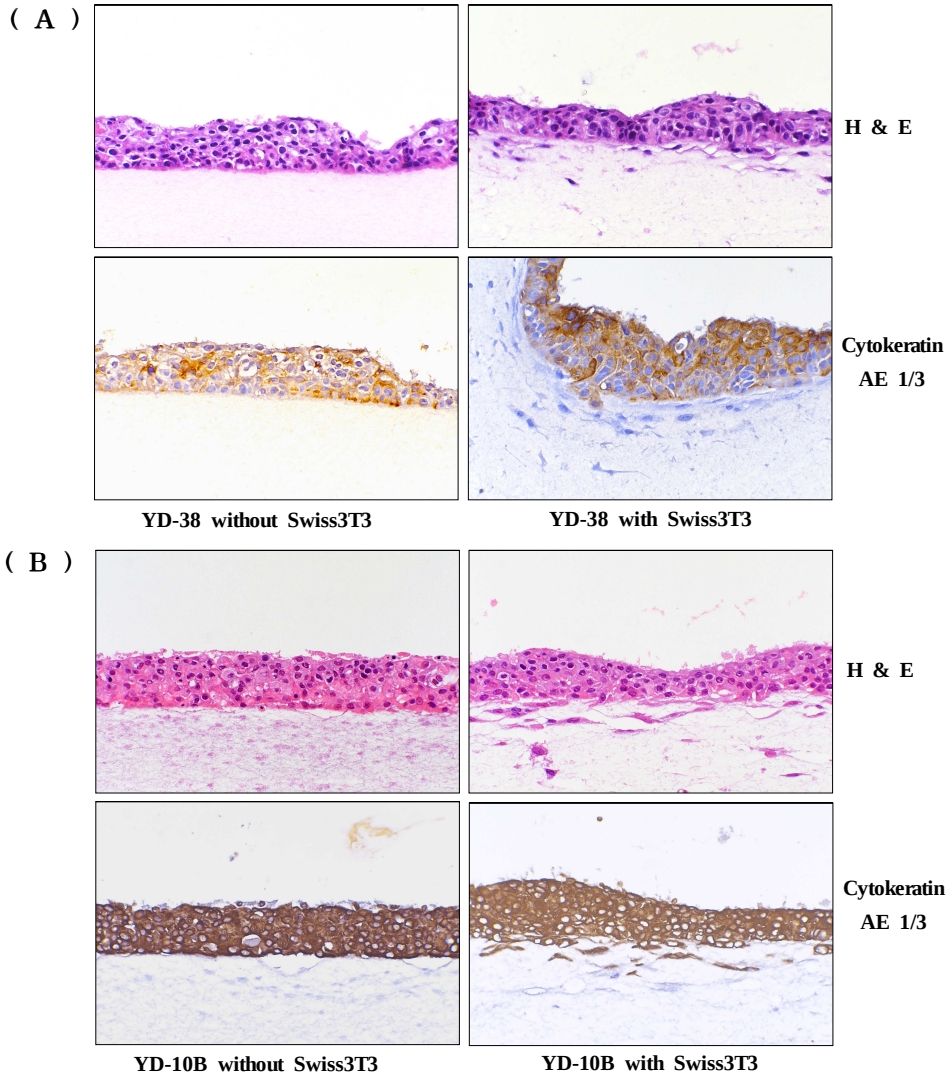

Fig. 3. The histological analysis of three-dimensional culture. Three-dimensional culture of YD-38 (A) and YD-10B (B) cells on collagen gel embedded with Swiss 3T3 fibroblasts showed the invasive growth of cancer cells. (H & E, × 200; cytokeratin AE1/3, × 200)

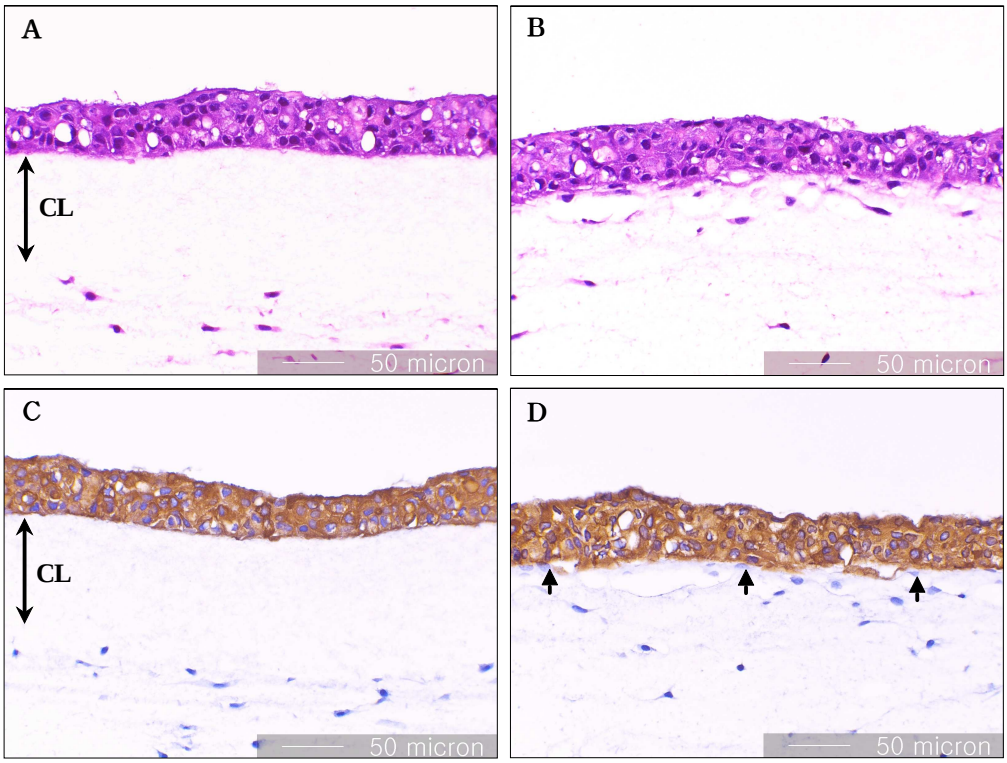

Fig. 4. H & E stain (A, B) and Immunohistochemical staining of cytokeratin AE1/3 (C, D) in the collagen gel-based co-culture model. C-Dr showed a few foci of direct contact between two kinds of cells, while C-In model showed the thin collagen layer (CL) between cancer cells and fibroblasts. (H & E, × 200; cytokeratin AE1/3, × 200)

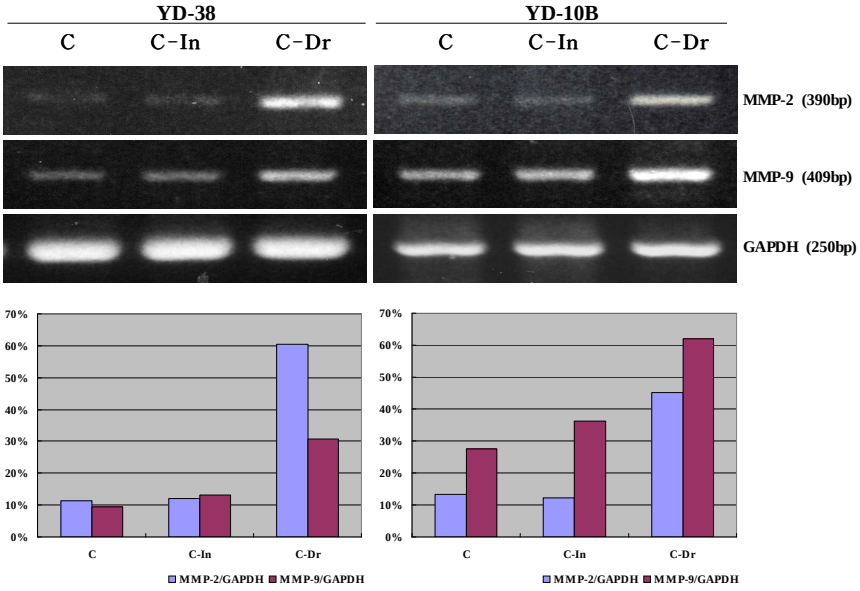

Fig. 5. mRNA expression analysis by RT-PCR and Densitrometric measurements for MMP-2 and MMP-9 in cancer cell culture model C-In, C-Dr and Control. The expression of MMP-2 and MMP-9 was upregulated by C-Dr model.

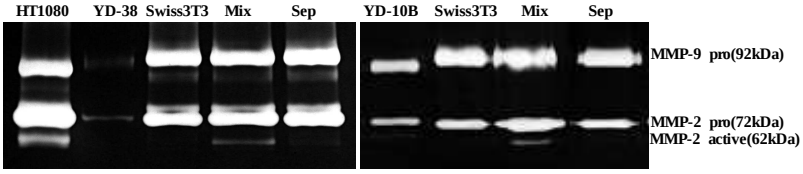

Fig. 6. Zymography in YD-38, YD 10B, Swiss 3T3 control, mixed co-culture (Mix) and separate co-culture model (Sep). Mix model showed the active form of MMP-2. But active form MMP-2 was not found in the Sep model. HT1080 human fibrosarcoma cells were used as a positive control.

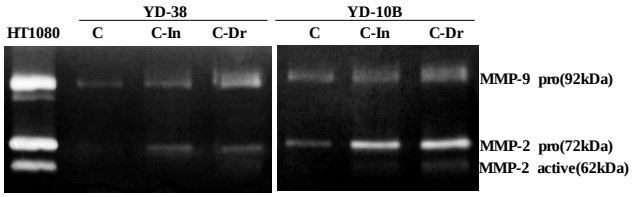

Fig. 7. Zymography in control (C), Indirect co-culture model (C-In) and direct co-culture model (C-Dr). Directe contact of cancer cells and Swiss 3T3 fibroblast (C-Dr) induced the upregulation of MMP-2 and MMP-9. Active form of MMP-2 was induced only by C-Dr model.

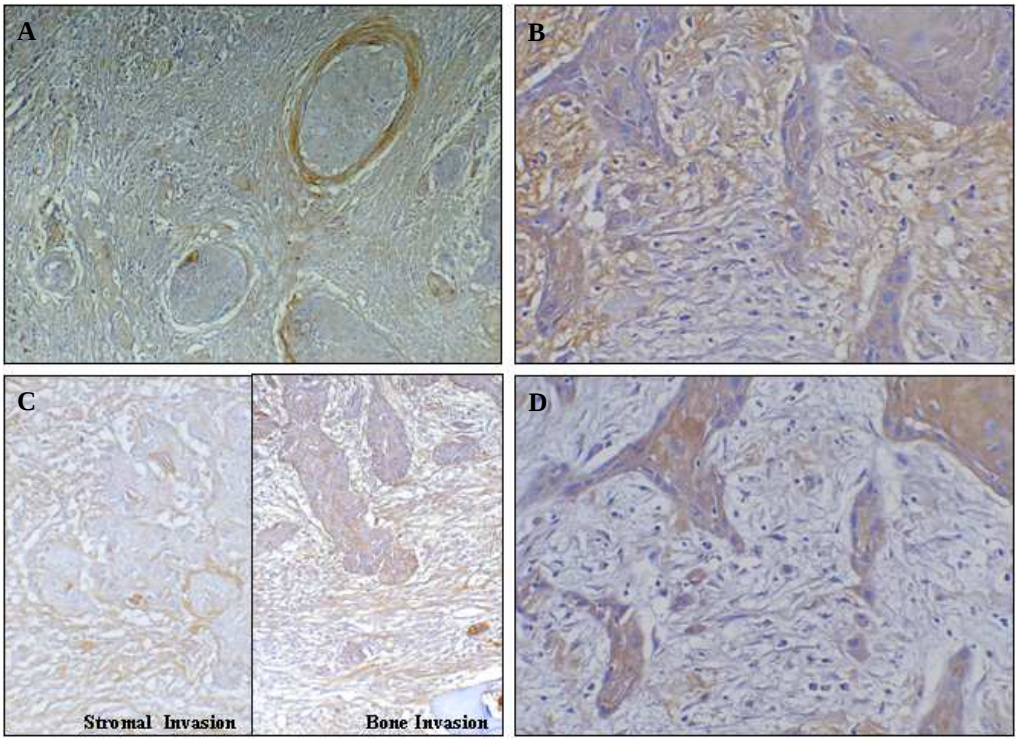

Fig. 8. Immunohistochemical staining of YD-10B and YD-38 squamous cell carcinoma tissue. Multiple foci of strong positive reaction were found in the interface between cancer cell nests and surrounding stroma at the invasive front in both cell lines (A, B, MMP-2, × 200). YD-38 cancer tissue Showed diffuse positive reaction in the stroma, while cancer cells of YD-38 showed focal positive reaction in the infiltrating area into bony tissue (C, MMP-9, × 200). YD-10B cells showed diffuse positive reaction throughout cancer cells and surrounding stroma (D, MMP-9, × 200).

국문요약

침윤성 성장 연구를 위한 암세포-섬유아세포의 Collagen gel을 이용한 혼합배양 모델 확립

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지도교수: 김 진

상피와 결체 조직사이의 상호작용 (epithelial mesenchymal interaction, EMI)은 배아의 발육, 상처의 치유, 암의 발생에 있어서 중요하다. 본 실험에서 EMI에 초점을 맞추어 침윤성 성장 연구를 위하여 생체 조건과 가장 유사한 체외실험 모델을 디자인하였고 이 모델이 생체 조건과 유사하며 상피와 결체 조직간의 상호작용에 대한 연구에 적합하다는 것을 증명하고자 하였다.

본 연구에서는 암세포로 두 종류의 구강 편평세포암종 세포주 YD-10B와 YD-38을 이용하였고, 섬유아세포로는 Swiss 3T3를 사용하였다. 우선 collagen gel을 이용한 입체배양을 하여 collagen gel에 섬유아세포 유무에 따른 침윤 효과를 관찰하였다. 상피와 결체조직사이의 상호작용에 대한 연구를 하기 위한 생체와 유사한 환경을 만들어 주기위해 다음과 같이 3종류의 모델을 고안하였다. Type I collagen gel을 이용하여 섬유아세포를 사용하지 않고 암세포를 입체 배양한 모델 (C), type I collagen gel으로 중간층을 만들어 섬유아세포와 암세포를 분리하여 입체 배양한 모델 (C-In), 섬유아세포가 포함된 type I collagen gel을 이용하여 배양한 모델 (C-Dr)로 진행하였다. 또한 섬유아세포와 암세포를 type I collagen 없이 혼합 배양한 모델과 transmembrane system을 이용하여 두 종류의 세포를 분리하여 배양한 모델과 비교하였다. 본 연구에서 제안된 모델이 유용한지를 알아보기 위해 암세포의

침윤과 전이에 중요한 역할을 하는 MMP-2와 MMP-9의 발현 및 활성도를 RT-PCR과 zymography로 분석하였으며, 실험에 사용된 두 종류의 세포주가 유래된 환자 조직에 대한 면역화학염색을 통하여 생체 조건과의 유사성과 암의 침윤과정에서 MMP-2와 MMP-9의 발현을 비교하였다.

입체 배양에서 두 세포주는 섬유아세포가 collagen gel에 포함된 경우에만 침윤성 성장을 보였다. YD-38 세포주에 비하여 YD-10B 세포주가 더욱 뚜렷한 침윤성 성장을 보였다. C, C-In, C-Dr모델에서 암세포를 분리하여 MMP-2와 MMP-9 mRNA 발현을 본 결과 C-Dr 모델에서 MMP-2와 MMP-9의 발현증가를 보였다. 이와 마찬가지로 C, C-In, C-Dr모델에서 MMP-2와 MMP-9의 활성도를 비교한 결과에서도, C-Dr모델에서만 active form MMP-2가 발현되었다. 암세포와 섬유아세포의 혼합배양한 모델과 transmembrane system을 이용한 간접 배양 모델에서 MMP-2와 MMP-9의 활성도를 비교한 결과, 두 세포의 혼합배양 모델에서만 active form MMP-2가 발현됨으로서, C, C-In, C-Dr모델에서와 같은 연구 결과를 보였다. 두 종류의 암세포주가 유래한 환자 조직의 면역화학염색에서 MMP-2의 발현은 침윤하고 있는 암세포를 둘러싸고 있는 섬유아세포와 기질에서 강양성 반응을 보였다.

이상의 연구결과에서 MMP-2의 활성도는 섬유아세포와 상피세포의 직접적인 접촉에 의해 활성화 되는 것을 알 수 있었다. 이 연구 결과는 혼합배양 모델과 collgen gel 이용한 모델에서 같은 결과를 보였고, 면역조직화학 염색에서도 이를 뒷받침하는 결과를 보였다. 이로서 제안된 실험모델이 상피와 결체조직사이의 상호작용 연구를 위한 적절한 모델임을 확인하였다.

Key words: 암세포-섬유아세포의 상호작용, collagen gel-based co-culture, matrix metalloproteinase