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Collagen-based co-culture for invasive study on cancer cells-fibroblasts interaction

Zhong Min Che [a], Tae Heob Jung [a], Jung Hee Choi [a], Do Jun Yoon [b], Hyun Jeong Jeong [b], Eun Ju Lee [a], Jin Kim [a,*]

[a] *Oral Cancer Research Institute, Department of Oral Pathology, Yonsei University College of Dentistry, Seoul, Republic of Korea*
[b] *Department of Biochemistry, Kwandong University College of Medicine, Kangnung, Republic of Korea*

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Abstract

The roles of tumor stroma in carcinogenesis are still unclear. This study was aimed at designing an in vitro model for investigating the effects of stromal fibroblasts in the invasive growth of squamous cell carcinoma. Using two cancer cell lines, we performed three-dimensional co-culture with dermal equivalents to evaluate the effects of fibroblasts in cancer invasion. In vitro models for cellular interaction study were designed as follows: a collagen gel-based direct co-culture model (C-Dr) and a collagen gel-based indirect co-culture model (C-In). The invasive growth was found only in the dermal equivalents with fibroblasts. MMP-2 activity could be induced by direct contact between cancer cells and stromal fibroblasts. Cathepsin D was also highly expressed when co-cultured with cancer cells and fibroblasts. The present study demonstrated that the presence of fibroblasts is essential in cancer invasion and that collagen gel-based co-culture models might be useful for invasive study.

Keywords: Cancer cells-fibroblasts interaction; Direct contact; Indirect contact; Collagen gel-based co-culture; Matrix metalloproteinase; Cathepsin D

Stromal microenvironments include extracellular matrix (ECM), stromal cells such as fibroblasts, adipose cells, resident immune cells, vasculature, cytokines, as well as growth factors, and have been shown to have regulatory roles in epithelial cell growth and differentiation [1,2]. As a component of stromal microenvironment, ECM provides cell-to-cell and cell-to-matrix communications as well as structural support [3,4].

Recently, cancer studies have acknowledged the active roles that tumor stroma can play in carcinogenesis, focusing on the abnormal communication between the tumor cells and their microenvironment [5,6]. Stromal microenvironment has been reported to be associated with a multistep process including initiation, progression, and invasion [7–12]. For instance, it has been previously demonstrated that carcinoma-derived fibroblasts stimulate tumor progression of initiated nontumorigenic prostatic epithelial cells [9]. It has also been found that radiation-induced changes in the stromal microenvironment can contribute to neoplastic progression in vivo [10]. Moreover, the MMP family that induces the invasion and metastasis are produced by cancer-derived fibroblasts [13,14].

The role of stromal microenvironment including ECM in carcinogenesis, however, has yet to be completely elucidated. In that cell-to-cell and cell-to-matrix interactions are extremely intricate, an understanding of the crosstalk between the tumor cells and their microenvironment may be beneficial to a more holistic view of carcinogenesis rather than depending on one facet of cellular or molecular change.

Invasion and metastasis are the hallmarks of malignant tumors [15]. For these steps to occur, the penetration of cancer cells through the basement membrane and ultimate destruction of ECM are required by enzymatic degradation. Although a variety of protease families have been

* Corresponding author. Fax: +82 2 392 2959.
E-mail address: jink@yumc.yonsei.ac.kr (J. Kim).

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implicated in this process, matrix metalloproteinases (MMPs) are one of the main controlling factors in the ECM component. In particular, MMP-2 and MMP-9 have enormous influence on tumor invasion and metastasis [16,17]. As another protease family, cathepsins are lysosomal endopeptidases and participate in the proteolysis of endocytosed proteins and proteolytic activation of secretory proteins in normal cells. Cathepsin D, in particular, is associated with the invasion and metastasis of several types of cancer [18–20].

While previous studies investigated the inter-relationship between tumor stroma and parenchymal cancer cells, reporting on the mixed or separate co-culture of cancer cells and fibroblasts [21,22], they did so without considering ECM, one of the major factors of stromal microenvironment. In prior studies, collagen gel-based organotypic co-cultures have been developed to provide the in vitro stromal microenvironment for cellular interaction or invasive studies [23,24]. Collagen was used because it may be conducive to the crosstalk between cell-to-cell and cell-to-matrix interaction as well as a supporting framework for tissue. Collagen induces MAPKinase phosphorylation which plays a major role in the mitogenic signal transduction pathway and is an essential component of both growth and differentiation, suggesting that collagen may be associated with carcinogenesis [25].

Accordingly, our study aimed at establishing an in vitro model containing collagen, simulating in vivo for invasive study with respect to cancer cells-fibroblasts interaction. To evaluate the usefulness of this model, we analyzed the expressions of MMP-2, MMP-9, and cathepsin D in the collagen gel-based co-culture models, and then compared them with conventional mixed co-culture models.

Materials and methods

Materials. YD-10B and YD-38 oral epithelial cancer cells from Yonsei University College of Dentistry were used for this study [26]. The cancer cells were fed with a mixture of Dulbecco's modified Eagle's medium (DMEM; Gibco BRL, USA) and Ham's nutrient mixture F12 (Gibco BRL, USA) at a 3:1 ratio supplemented with 10% fetal bovine serum (Hyclone, Logan, UT), 1×10^{-10} M cholera toxin, 0.4 mg/ml hydrocortisone, 5 μg/ml insulin, 5 μg/ml transferrin, and 2×10^{-11} M triiodotyronine (all purchased from Sigma, St. Louis, USA). Cells were cultured at 37 °C in an atmosphere containing 5% CO_2. The culture medium was changed every 2 or 3 days. The Swiss 3T3 fibroblasts were obtained from the American Type Culture Collection (Manassas, VA, USA) 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.

Three-dimensional culture and histological examination. For three-dimensional culture, the dermal equivalent was prepared, as described by Asselineau and Prunieras [27]. The cancer cells were cultured on the dermal equivalents intermixed with and without fibroblasts. To produce dermal equivalents, 300 μl Type I-A collagen mixture (Nitta Gelatin Inc., Osaka, Japan) was generated by mixing eight volumes of an ice-cold collagen solution with one volume 10× reconstitution solution (0.022 g/μl $NaHCO_3$, 0.0477 g/μl Hepes, and 0.05 N NaOH) and one volume 10× DMEM. Swiss 3T3 fibroblasts (1.5×10^5 cells in 30 μl) suspension in medium were added. The type I collagen mixture was dispensed into a 12 mm Millicell (Millipore Co., Bedford, MA, USA), which was placed in six-well plates (Becton–Dickinson, Franklin Lakes, NJ, USA). After 1 day of incubation at 37 °C for collagen polymerization, oral squamous cancer cells (3×10^5 cells in 200 μl) were plated onto the dermal equivalents and submerged into the culture medium for 4 days. The cells were then exposed to the air by removing the medium from the surface and were cultured for another 4 days. The medium was changed every 2–3 days. The culture tissue was fixed in 10% neutral formalin and embedded with paraffin. For histological examination, the tissue was sectioned and stained with hematoxylin and eosin.

Collagen gel-based co-culture for cancer cells-fibroblasts interaction. The three-dimensional culture model was modified for the investigation of the direct and indirect effects of cancer cells by fibroblasts. The cancer cells both with fibroblasts and without fibroblasts were cultured on the type I collagen gel. The indirect co-culture model (C-In) was designed for the examination of the indirect effect by fibroblasts in which oral squamous carcinoma cells were separated from the fibroblasts-embedded collagen matrix by an acellular collagen layer (Fig. 1B-1, 2). The direct co-culture model (C-Dr), on the other hand, was designed to allow cancer cells to have direct contact with fibroblasts (Fig. 1C-1, 2). The control (Fig. 1A) was established by the same components merely devoid of Swiss 3T3 fibroblasts. To prepare the collagen gel embedded with fibroblasts, the type I collagen mixture was made by the same method as the three-dimensional culture. Swiss 3T3 fibroblasts (3×10^5 cells) suspension in medium was added to the type I collagen mixture and then dispensed into a 60 mm plate. In the C-Dr model, 3.0×10^5 cancer cells were added onto the collagen matrix and cultured for 1 day, being submerged in the culture medium. For the C-In model, 500 μl of the type I collagen mixture was applied to the top of the fibroblasts-embedded collagen matrix before the seeding of cancer cells.

Separate and mixed co-culture models. Separate co-culture studies for examining the effects of indirect contact between cancer cells and fibroblasts were performed. First, 1.5×10^5 cancer cells were seeded on Millicell (Millipore Co., Bedford, MA, USA) with 0.4 μm pore size membrane. Then, they were placed onto a 60-mm plate in which 1.5×10^5 Swiss 3T3 fibroblasts were seeded. They were incubated with DMEM and Ham's nutrient mixture F12 at a 3:1 ratio supplemented with 10% fetal bovine serum at 37 °C for 24 h (Fig. 2A). To examine the direct contact between cancer cells and fibroblasts for mixed co-culture studies, 1.5×10^5 the number of each cell were cultured in a 60-mm plate for 24 h (Fig. 2B). The same medium was used for this model.

Immunohistochemistry. To validate whether the collagen gel-based co-culture models were appropriately designed to detect direct and indirect effects between cancer cells and fibroblasts, we examined the histological findings of these models. After fixation with 10% neutral formalin, the samples were embedded with paraffin. The paraffin-embedded tissues were then used for immunohistochemical staining. After deparaffinization and dehydration, endogenous peroxidase was blocked with 3% H_2O_2 in methanol, and sections were incubated with normal horse serum at room temperature. Subsequently, anti-cytokeratin AE1/3 antibody diluted at 1:50 (DAKO, Glostrup, Denmark) was applied overnight at room temperature for the differentiation of cancer cells from fibroblasts in the co-culture models. 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. Visualization was performed by 3′,3-diaminobenzidine tetrachloride (DAB kit, Vector Lab, Burlingame, CA, USA) and the slides were counterstained with Meyer's hematoxylin. The three-dimensional culture tissues were also immunohistochemically stained with anti-cytokeratin AE1/3 antibody by the same procedure to observe whether cancer cells invade into dermal equivalents.

Reverse transcriptase-polymerase chain reaction (RT-PCR). Cancer cells were isolated while examining them under an inverted microscope to prevent fibroblast contamination. Total RNA was isolated from cell lysates using the RNeasy (Qiagen, Hilden, Germany) kit. The concentration of RNA was measured at a wavelength of 260 nm using a spectrophotometer (DU-70, Beckman, CA). Total RNA (1 μg) was reverse-transcribed with Moloney Murine Leukemia Virus reverse transcriptase (Promega, Madison, WI, USA) in 20 mM $MgCl_2$, 0.1 mg/ml bovine serum

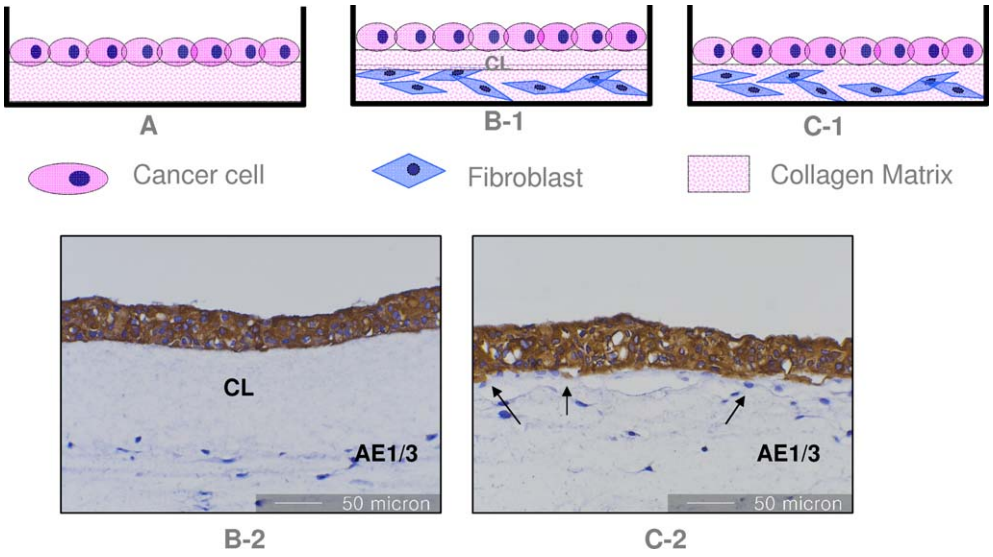

Fig. 1. Collagen gel-based co-culture models for direct and indirect contact between cancer cells and fibroblasts. (A) Control showing that cancer cells were plated onto the collagen gel without fibroblasts. (B-1) C-In model showing that cancer cells were seeded onto collagen layer (CL) applied to the top of the fibroblasts-embedded collagen matrix. (B-2) The cancer cells were separated from fibroblasts by intervening CL. (C-1) C-Dr model designed for the direct contact between cancer cells and fibroblasts. (C-2) The cytokeratin-positive cancer cells (arrows) directly contacted to fibroblasts. (B-2,C-2) Immunohistochemical staining with anti-cytokeratin AE1/3 antibody.

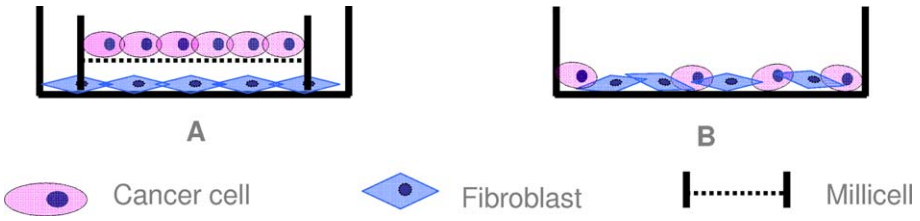

Fig. 2. Separate and mixed co-culture models. (A) Separate co-culture model showing that cancer cells placed in the Millicell were separated from fibroblasts in a 60-mm plate. (B) Mixed co-culture model showing that both cancer cells and fibroblasts were seeded together in a plate.

albumin, 10 mM dithiothreitol, and 0.5 mM deoxynucleotides. cDNAs were generated using random hexamers (Promega, Madison, WI, USA) at 37 °C for 60 min. PCR was carried out in a 20-μl volume containing 20 ng cDNA template, 10 pmol oligodeoxynucleotide primer, 200 mM deoxynucleotide triphosphate, 1.5 mM $MgCl_2$, 0.01% gelatin, and 1.5 U Taq polymerase (Bioneer, Taejeon, Korea). The cDNAs were processed for 28 cycles, each cycle consisting of denaturation for 30 s at 95 °C, annealing for 30 s at 53 °C for MMP-2 and at 58 °C for MMP-9, and extension for 1 min at 72 °C. Twenty-eight cycles for GAPDH were carried out as follows: 1 min at 94 °C, 1 min at 55 °C, and 1 min at 72 °C. The primer sequences are shown in Table 1. Aliquots of the amplified DNA were electrophoresed in 2% agarose 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 standardized with that of GAPDH expression.

Table 1
Primer sequences of MMP-2 and 9 mRNA used in RT-PCR

Primer name	Sequence (5′–3′)
MMP-2 sense	GCG ACA AGA AGT ATG GCT TC
MMP-2 antisense	TGC CAA GGT CAA TGT CAG GA
MMP-9 sense	GAG ACA GCA TGG CCA AAT TA
MMP-9 antisense	CTC TAG AAA CTG CTG AGG GC
GAPDH sense	ATC AAG AAG AGG GTG GTG AAG CAG G
GAPDH antisense	GCG TGC CTT GGA GGC CAT GTA GG

Zymography. The cancer cells and fibroblasts were cultured overnight using serum-containing medium and then washed twice with phosphate-buffered saline. Subsequently, the cells were cultured using serum-free medium for 24 h. For the detection of gelatinolytic activity, the conditioned medium was centrifuged for 10 min and was 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. The gel was then washed in 2.5% Triton X-100 at room temperature for 1 h, then incubated in reaction buffer containing 10 mM $CaCl_2$, 50 mM Tris, 0.15 M NaCl, and 1% Triton X-100 at 37 °C for 18 h. To detect MMP activity, the gel was stained by 0.05% Coomassie blue, 10% acetic acid, and 10% methanol for 1 h. After destaining with 10% methanol and 10% acetic acid for 2 h, gelatinolytic activity was examined as clear bands on a blue background. HT-1080 fibrosarcoma cells were used as a positive control.

Western blot. Cathepsin D expression was examined to evaluate the usefulness of the collagen gel-based co-culture models. Cancer cells were isolated while examining them under an inverted microscope to prevent fibroblast contamination. Cell lysates obtained from cancer cells were applied to the 8% SDS–PAGE and blotted to the BioTrace NT membrane (Pall Gelman Lab, Ann Arbor, MI, USA). The blot was incubated with anti-cathepsin D antibody (Santa Cruz Inc., Santa Cruz, CA, USA) diluted at 1:500 and then with horseradish peroxidase-conjugated anti-rabbit antibody (Cell Signaling, Beverly, MA, USA) at room temperature for 1 h. Vimentin expression was examined to evaluate whether fibroblasts were accidentally included during the removal of cancer cells from the collagen gel. Anti-vimentin antibody (Lab Vision, Fremont, CA, USA)

diluted at 1:1000 was incubated at room temperature for 2 h. ECL detection kit (Amersham Bioscience, Piscataway, NJ, USA) was used for visualization of cathepsin D and vimentin. An anti-actin antibody (Sigma, Saint Louis, MO, USA) was used as a loading control. MCF-7 cells were used as a positive control of cathepsin D and Swiss 3T3 fibroblasts were used as a positive control of vimentin.

Results

Histological features of C-Dr and C-In models

The C-Dr model showed a few foci of direct contact between two kinds of cells, while the C-In model showed a thin collagen layer (103.0 ± 6.3 μm) between cancer cells and fibroblasts (Fig. 1B-2). Immunohistochemically, cytokeratin AE1/3 positive cancer cells had direct contact with the fibroblasts in the C-Dr model (Fig. 1C-2).

Three-dimensional culture confirms that the presence of fibroblasts is essential in cancer invasion

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

mRNA expressions of MMP-2 and MMP-9 are increased in the C-Dr models

RT-PCR was done to observe mRNA expression of cancer cells when co-cultured with fibroblasts in the C-Dr and C-In models. MMP-2 mRNA was expressed in both the YD-10B and YD-38 cell lines when cultured alone in the collagen gel (Fig. 4, control). Likewise, MMP-9 was also expressed in the YD-10B and YD-38 cells (Fig. 4, control). Direct co-culture of cancer cells and fibroblasts in the collagen gel showed a fourfold increase of MMP-2 and MMP-9 expressions in both cells. However, neither MMP-2 nor MMP-9 mRNA was increased when having no contact in the co-culture models (Fig. 4).

MMP-2 activities are increased in the C-Dr and mixed co-culture models

HT-1080 fibrosarcoma cells showed two clear bands of 72 kDa MMP-2 and 92 kDa MMP-9. The Swiss 3T3 fibroblasts also showed abundant MMP-2 and 9 expressions. The YD-10B and YD-38 oral squamous carcinoma cells exhibited MMP-2 and 9 expressions, when grown on the collagen gel which had no fibroblasts (Fig. 5A, control). The monolayer culture of two cancer cells also showed definite MMP-2 and MMP-9 expressions (Fig. 5B, YD-38 and YD-10B lanes). However, their expressions in the YD-38 cells were not as clear as those in the YD-10B cells. Upon examining the C-Dr model, a clear band of the active form of MMP-2 was shown in both the YD-10B and YD-38 cells (Fig. 5A). A weak band of the active form was seen in the C-In model of the YD-10B cells, while the YD-38 cancer cells showed no detectable band in the C-In model. Mixed co-culture also showed the active form of MMP-2 in both the YD-10B and 38 cells. On the other hand, the active form of MMP-2 was insignificant in the separate co-culture model (Fig. 5B).

Cathepsin D expression is increased in both C-Dr and C-In models

Cathepsin D was expressed in both the YD-10B and 38 cells grown on the collagen gel. Its expression increased twofold when the cancer cells were stimulated by fibroblasts, regardless of whether or not their relationship was direct or indirect, suggesting that cathepsin D expression may be induced by fibroblast stimulation (Fig. 6). We examined vimentin expression to evaluate whether the increased expression in the C-Dr model might be due to fibroblast contamination. No vimentin expression was found in the three collagen gel-based co-culture models except for a positive control, confirming that no fibroblasts were included in the cancer cell lysates (data not shown).

Discussion

To provide a more holistic view for understanding cancer cells-fibroblasts interaction, the present study attempted to develop an experimental model akin to in vivo by creating a collagen matrix intermixed with fibroblasts as a dermal equivalent. From the present study, we discovered that when the cancer cells were grown in the dermal equivalents, the invasive growth was found only in the collagen gel intermixed with fibroblasts, supporting our premise that fibroblasts have an essential role in cancer invasion.

Next, the present study established the in vitro models to investigate direct and indirect cellular interactions between cancer cells and fibroblasts. In the collagen gel-base, mRNA expression of MMP-2 and 9 was augmented when fibroblasts were in direct contact with the YD-10B and YD-38 cancer cells. The increased expressions of MMP-2 and MMP-9 were recapitulated in zymography. Moreover, the MMP-2 active form was found in the C-Dr model, suggesting that MMP-2 activity may be induced only by direct contact between cancer cells and fibroblasts. These results indicate that the direct contact between cancer cells and fibroblasts may aggravate the invasive growth of cancer cells, as the cancer cells meet fibroblasts after they penetrate the basement membrane and destroy the ECM. The same results of MMP-2 and MMP-9 expressions in the C-In and C-Dr models with those in the mixed and separate co-culture models indicate that the established

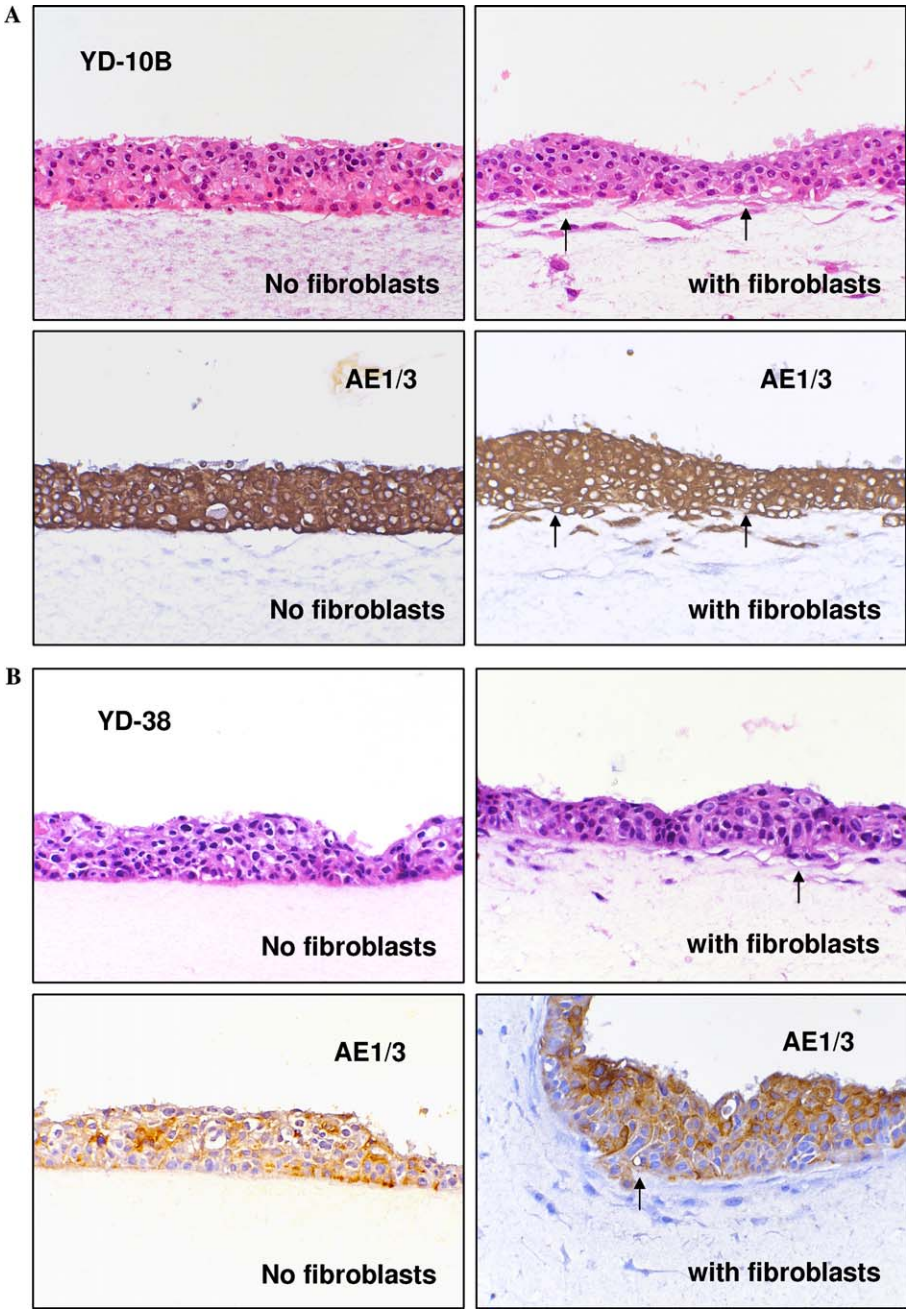

Fig. 3. Invasive growth of cancer cells was found in the dermal equivalents with fibroblasts. (A,B) YD-10B (A) and YD-38 (B) cancer cells were seeded onto the dermal equivalents with and without fibroblasts and submerged in the culture medium for 4 days. The cells were then exposed to the air by removing the medium from the surface and were cultured for another 4 days. Both cancer cells showed invasive growth (arrows) when cultured on the dermal equivalent with fibroblasts, whereas cancer cells showed no invasion in the collagen gel without fibroblasts. Immunohistochemical staining with cytokeratin antibody (AE1/3) showed the invasion of the cytokeratin-positive cancer cells.

models from our research can be useful for cancer cells-fibroblasts interaction study.

A previous study provided evidence that MMP-9 expression in tumor stroma is dependent upon direct cell-to-cell interaction in breast cancer cells, whereas MMP-2 production can be induced by soluble factors via paracrine interactions [21]. In another study, MMP-2 expression was dependent on direct contact between cancer cells and fibroblasts, when breast cancer cells were grown with bone marrow fibroblasts [28]. Moreover, it has been suggested that pro-MMP-2 production and activation are induced by the direct cell–cell interaction rather than humoral factors in laryngeal cancer [22]. These varying results show that the cellular interaction may largely depend on the cancer cell type and experimental model [23,29,30].

Cathepsin D is another active participant promoting cancer invasion. Its active role in carcinogenesis was previously reported to affect the multiple steps of tumor

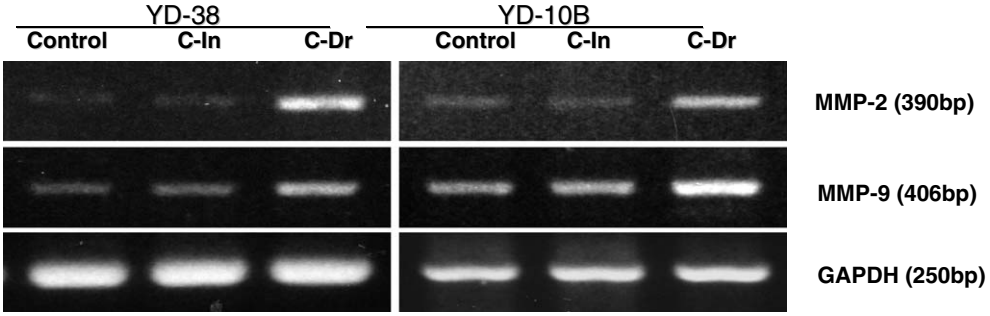

Fig. 4. MMP-2 and MMP-9 mRNA expressions were increased in C-Dr models. MMP-2 and MMP-9 mRNA expressions were examined by RT-PCR from the cancer cell isolated from the collagen gel-based co-culture models. Both YD-10B and YD-38 cancer cells showed the expression of MMP-2 and MMP-9 mRNAs in the control which the cancer cells were cultured alone in the collagen gel. The expression of MMP-2 and MMP-9 mRNAs was fourfold increased only in the C-Dr models of YD-10B and YD-38 cancer cells.

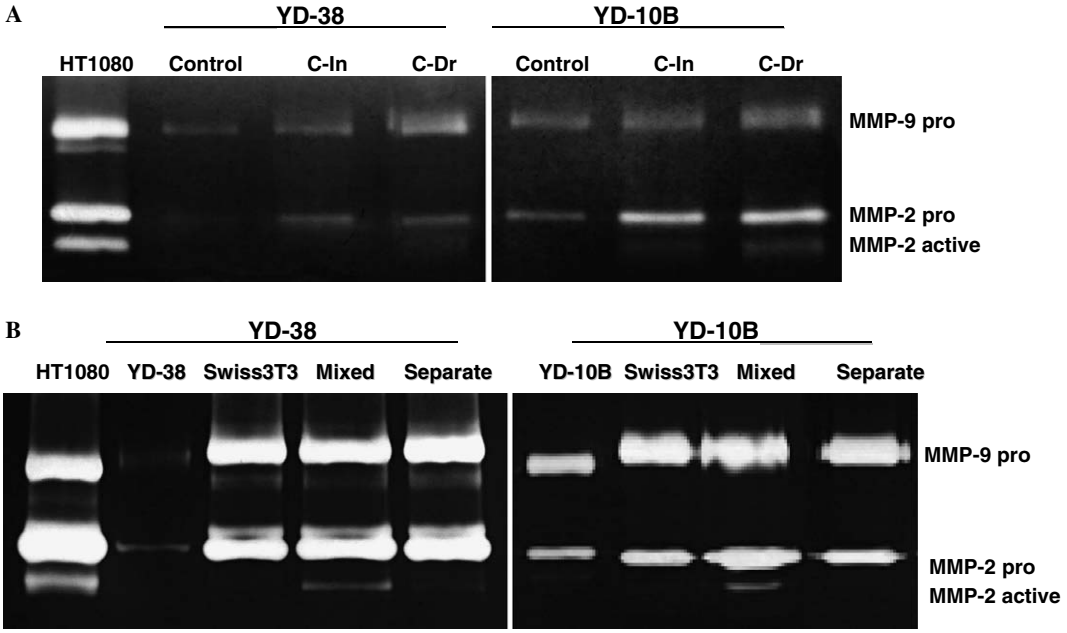

Fig. 5. Activities of MMP-2 and MMP-9 were increased in the direct co-culture conditions. To observe MMP activities, gelatin zymography was done in collagen gel-based co-culture models (A) and separate and mixed co-culture models (B). (A) The YD-10B and YD-38 oral squamous carcinoma cells exhibited MMP-2 and 9 expressions, when grown on the collagen gel which had no fibroblasts (control). The active form of MMP-2 was insignificant in YD-10B cells and was not detectable in YD-38 cells, when they were cultured alone in the collagen gel. A clear band of the active form of MMP-2 was shown in both YD-10B and 38 cells, when they grew in the C-Dr models. A weak band of the active form was seen in the C-In model of YD-10B cells, while YD-38 cancer cells showed no detectable band in the C-In model. (B) Monolayer culture of two cancer cells also showed definite MMP-2 and MMP-9 expressions (YD-38 and YD-10B lanes). The expression of MMP-2 and MMP-9 was stronger in YD-10B cells than in YD-38 cells. Mixed co-culture also showed the active form of MMP-2 in both YD-10B and YD-38 cancer cells.

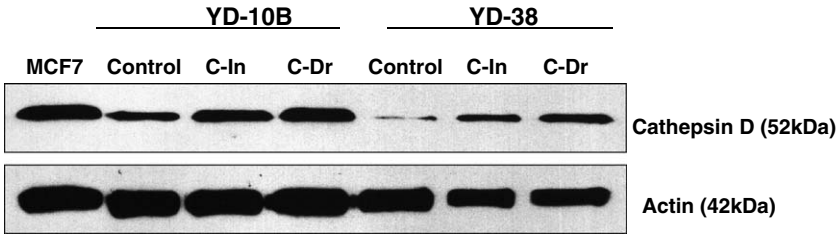

Fig. 6. Cathepsin D expression was increased in the collagen gel-based co-culture models cathepsin D expression was examined by Western blotting from cancer cell lysates isolated from the collagen gel-based co-culture models. YD-10B and YD-38 cancer cells expressed cathepsin D in control and its expression was doubled when seeded in the co-culture conditions. The difference of cathepsin D expression between C-Dr and C-In models was not significant.

progression, thus, to be a predictor for lymph node metastasis in head and neck squamous cell carcinomas [31,32]. However, its role in the crosstalk between cancer cells and fibroblasts has been relatively unknown. As expected, the present study demonstrated that cathepsin D expression increased when co-cultured with fibroblasts, suggesting that cathepsin D expression may be induced by fibroblasts stimulation.

These results suggest that the C-Dr and C-In models developed in the present study are valid for utilization towards cancer cells-fibroblasts interaction studies with respect to cancer invasion. The two new models are thought to have several advantages compared to the conventional mixed co-culture models. First, these models more closely simulate in vivo conditions in that they included collagen. Second, unlike mixed co-culture models, the cancer cells can be separated from collagen embedded fibroblasts, eventually enabling us to extract cell lysates for molecular study. Third, the inter-relationship between cancer cells and fibroblasts can be examined histologically.

Cancer cells cannot invade into a dermal equivalent unless stromal fibroblasts exist, even though cancer cells can express MMP-2, MMP-9, and cathepsin D, confirming that fibroblasts are essential in cancer invasion. Additionally, upon invading the basement membrane, the cancer cells have direct interaction with fibroblasts, stimulating the active form of MMP-2. From these results, we can presume that the initial step of cancer invasion should be influenced at least by other soluble factors secreted by cancer cells-fibroblasts interaction. The development of an improved in vitro model with an intervening basement membrane would be encouraged to explain the detailed mechanism of cancer cells-fibroblasts interaction and to emerge new stromal therapy for cancer prevention and intervention.

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