Soluble Factors released from Carcinoma-Associated Fibroblasts that induce the Invasion of Oral Squamous Cell Carcinoma

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Directed by Professor Jin Kim

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This certifies that the Doctoral Dissertation of Kyungshin Kim is approved.

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Twenty five years ago, when I was graduated in Mass Communication from my first university, a friend of mine gave me a congratulatory note. It read that ‘Be a Doktor!’ since I was planning to go to a graduate school in Germany at that time. I think that was a spell to me from that time on. Otherwise, it could not be explained why I tried to earn the doctor’s degree after all these years in which I attended four universities , changed my major dramatically from the social science to the dental one and went into practice.

Now, I am exonerated from the spell finally. And who set me free from that spell was not a prince Charming, but many people to whom I owed a great deal. Foremost among these is my supervisor, Prof. Jin Kim. She urged me to finish this work by giving assignments and setting deadlines every week. She knew exactly who I am who would not work without deadlines (a habit of once a reporter). Without her encouragement, enthusiasm and guidance, I could not have completed this work. I also would like to thank Prof. Jong In Yook who gave me deep and thoughtful considerations, Prof. In Ho Cha for his keen and constructive criticisms, Dr. Da-Woon Jung for offering her time and expertise in spite of her illness, and Prof. Do Jun Yoon for his comments and suggestions that corrected this treatise refinedly.

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Last but not least, I would like to express deepest gratitude for my family members who have constant confidence in me for all these years, and my son, Youngsuk, who willingly took trouble to find every reference that I asked while grumbling.

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ABSTRACT

Soluble Factors released from Carcinoma-Associated Fibroblasts that induce the Invasion of Oral Squamous Cell Carcinoma

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(Directed by Professor Jin Kim)

Cancer development is not merely a result of clonal expansion of mutant cells, but a product of the evolving interactions between cancer parenchymal cells and their surrounding stroma. Among the stromal cells, the fibroblasts play an active role in the process of cancer development such as initiation, progression and invasion which are changed morphologically and functionally by cancer cells.

Therefore, better understanding of the characteristics of carcinoma associated fibroblasts (CAF) and the soluble factors which are influencing on CAF and cancer cells in cancer invasion might offer a promising point of departure for the development of new therapies. With this hope, the purpose

of this study was to characterize CAF and to investigate the mediating soluble factors between cancer cells and fibroblasts in oral squamous cell carcinoma (OSCC).

From the immunofluorescence staining, the expression of vimentin and α-smooth muscle actin was found in both CAF and normal fibroblasts (NF), and CAF showed stronger expression pattern as compared to NF.

To determine whether fibroblasts facilitate OSCC cell invasion in vitro, a transwell invasion assay employing collagen type-I coated filters was established. OSCC cell invasion was significantly increased by co-culturing with fibroblasts as compared to that without co-culturing with fibroblasts. Also CAF enhanced cell invasion at significantly higher levels than NF.

To evaluate whether the chemokines released from fibroblasts increases by co-culturing with OSCC cells as compared to those from mono-cultured fibroblasts, sandwich ELISAs was established that is specific to IL-8, CXCL1, and CCL7. Co-culturing the OSCC cells and fibroblasts resulted in significant increase of the chemokine release into the culture media within 24 h of incubation. In addition, when comparing CAF to NF for their abilities to release the chemokines into the co-cultured media, chemokine release from the co-cultured CAF was significantly higher than that from the co-cultured NF, indicating that CAF were more responsive to OSCC cell stimulation for enhancing chemokine release than were NF.

To find the soluble factors released from OSCC cells that induce cytokine

secretion by CAF, antibody array using 36 cytokine detection kit was carried out. VEGF and IL-1α showed remarkable intensity difference between Ca9.22 and YD-38 cells. To confirm whether VEGF and IL-1α regulate cytokine secretion released from CAF, each cytokine such as CCL7, IL-8 and CXCL1 was measured by adding VEGF and/or IL-1α to CAF culture. Findings were that IL-1α induced CCL7, IL-8 and CXCL1 expression dose dependently in CAF, while VEGF failed to enhance CCL7 , IL-8 and CXCL1 expression. Interestingly, VEGF has a synergic effect on CCL7, IL-8 and CXCL1 secretion induced by IL-1α. This phenomenon was completely diminished by applying anti-IL-1α monoclonal antibody. When comparing the effect of invasiveness of IL-1α by neutralization, the invasive potential of OSCC cells by stimulation of CAF was markedly decreased by neutralization of IL-1α.

This study investigated whether IL-1RI on CAF plays an important role in releasing chemokines, such as IL-8, CXCL1, and CCL7, in response to paracrine IL-1α stimulation. To do so, IL-1RI siRNA into CAF was introduced to knockdown the protein expression. The down-regulated IL-1RI protein expression on CAF was confirmed by western blot analysis. The chemokine release from the transfected CAF stimulated by IL-1α was determined by ELISAs specific to IL-8, CXCL1, and CCL7. Inhibiting IL-1RI expression in CAF significantly reduced IL-8, CXCL1, and CCL7 secretion, when compared to control siRNA-transfected CAF. In addition, the invasion of OSCC cells co-cultured with siRNA- transfected CAF was

markedly reduced as compared to them co-cultured with control siRNA-transfected CAF. These observations indicate that IL-1α acts to enhance the chemokine release from CAF through the IL-1RI on fibroblasts.

To sum up, the present study attempted to identify the role of fibroblasts in cancer invasion by investigating the difference of cytokine expression between CAF and NF. And it was found that CAF secreted CCL7, IL-8 and CXCL1 more than NF did when co-cultured with cancer cells. In addition, the invasiveness of cancer cells was prominently increased when cancer cells were co-cultured with CAF than with NF. Since cytokine expression was increased when fibroblasts were co-cultured with cancer cells, this study tried to figure out which soluble factor secreted from the cancer cells stimulates fibroblasts, and found that IL-1α was the most prime candidate of cytokines.

The results of this study clarified the role of CAF by characterizing them morphologically and functionally and addressed cytokines that interact between CAF and cancer cells. Although this study may be an early step to understand the interaction process between cancer cells and fibroblasts, it could be a new branch point for research of cancer signaling and cancer development.

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Key words: carcinoma associated fibroblast, normal fibroblast, oral squamous cell carcinoma, invasiveness, soluble factors

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I. INTRODUCTION

Until recently, cancer research focused mainly on cancer cells themselves and specifically on their genetic changes that occur in epithelial cells as they progress from normal to malignant. For this reason, many of the tumor suppressor genes and potential oncogenes that are involved in cancer development have been successfully identified and characterized (Hanahan and Weinberg, 2000).

However, several lines of investigation suggest that concurrent changes

also occur in cells surrounding the epithelial malignancy (Ronnov-Jessen et al., 1996). That is, solid tumors are more than just a clonal expansion of mutant cells, but instead heterogeneous and structurally complex (Dvorak, 1984). And tumor progression can be considered a developmental process, in which a complex, multicellular organ forms in response to signaling between different cells (Radisky et al., 2001).

From this viewpoint, the hypothesis that tumor progression may be properly appreciated as a product of the evolving interactions between the different cell types within the tumor and the microenvironment was proposed (Radisky et al., 2001). In normal, healthy situation, the stromal components of tissues govern size, function of cells and their response to exogenous agents through elaboration and modification of the extracellular matrix (ECM), which transmits signals to the adjacent epithelial cells. These stromal elements include the vasculature, adipocytes, resident immune cells, and fibroblasts with their numerous cellular products, including various growth factors and ECM components (Sadlonova et al., 2005). During wounding and other pathological conditions, the stroma exhibits fundamental changes that are important on proper tissue response (Sappino et al., 1990).

ECM contains a mixture of glycoproteins, proteoglycans, cytokines and growth factors (Aumailley and Gayrand, 1998), and it provides both structural support and contextual information for cells to determine the correct response

to a given set of stimuli (Howe et al., 1998). The composition of ECM varies substantially both between and within different tissues (Stureuli, 1999) and ECM changes temporally, as an adaptation to changing signals during normal developmental processes (Huang and Ingber, 1999).

Each cell type displays a unique array of surface receptors tuned to its natural tissue environment. Ligation of cell surface receptors to ECM changes cell shape and behavior, alters the binding affinity or cellular distribution of various receptors, and deeply influences the response of the cell to soluble molecules such as growth factors (Aplin et al., 1999). Depending upon the ECM context, the same soluble factors may electively cause cells to functionally differentiate, to initiate proliferation, to arrest growth, or to cause apoptosis (Boudreau and Jones, 1999). Control of growth factor signaling by ECM-defined context makes sure that cells divide and differentiate only as needed by the organism (Radisky, et al., 2001).

Normal homeostasis is maintained by interpreting growth factor signaling in the ECM context. This control can be lost through the breakdown of communication mechanisms between the epithelium and the surrounding stroma (Radisky et al., 2001). For example, an epithelial cell might incorrectly initiate a signal to the stroma resulting in the stromal production of a growth factor that, in turn, can stimulate the abnormal proliferation of neighboring epithelial cells (Skobe and Fusening, 1998). On the other hand,

an aberrant matrix component produced by stromal cells in response to a local stress might be perceived by neighboring cells as a signal to grow or to enter a new developmental pathway (Coussens et al., 1999). Under normal homeostasis, these mistakes can be corrected by cell cycle arrest or apoptotic cell death. Occasionally if the abnormal signal persists, a tumor develops, creating a growing, interdependent, heterogeneous tissue, by its unresponsiveness to normal physiological controls (Pice et al., 1997).

As the tumor grows, the cells within continue to respond to their immediate environment (Iida et al., 1996), and since the tumor is itself in a state of flux, multiple and conflicting signals can lead to increased complexity and heterozygosity (Lukashev and Werb, 1998). Tumor remodeling can include interactions with various ECM components such as tenascin, fibronectin, elastin and laminin (Wenk et al., 2000), make alterations that can produce cellular proliferation, structural disruption (Shoenwaelder and Burridge, 1999), and circumvention of apoptosis (Sethi et al., 1999). Throughout the process of tumor development, the composition of ECM is controlled in a reciprocal manner between epithelial and stromal cells (Clezardin, 1998), and it is through these reciprocal interactions that the tumor creates a microenvironment that is favorable to its proliferation (Blobe et al., 2000), recruits new blood vessels (Hanahan and Folkman, 1996), and stimulates the production of metalloproteinase to invade adjacent tissues (Orr

et al., 2000).

The fibroblasts, the major cells in the stroma, are responsible for the elaboration of most of the connective tissue components such as the different collagens, proteolytic enzymes and their inhibitors, growth factors and determinants of intercellular adhesions (Hanahan and Weinberg, 2000). In response to different physiologic signals, whether they are normal or pathologic, the fibroblasts of the stroma changes accordingly. Depending on the characteristics of the cancer cell, the fibroblasts exerted either a positive or negative effect on tumor production (Tlsty, 2001).

Specifically, several characteristic changes have been acknowledged in the stroma surrounding cancer cells. First, collagen deposition and the number of fibroblasts are increased in the cancer tissue. This proliferation of fibroblasts, the novel expression of α-smooth muscle actin in the fibroblasts, and the increased presence of collagen in the vicinity of cancer cells indicated a change from the resting state and it was termed desmoplasia (Willis, 1967). Second, carcinoma-associated fibroblasts (CAF) exert unusual functional changes including abnormal migratory patterns on collagen gels (Schor et al., 1988), abnormal actin distribution (Antecol et al., 1986), decreased anchorage-dependent growth, and increased colony formation and loss of cell cycle controls (Azzarone et al., 1984). Here, CAF could be defined as the fibroblasts located around cancer cells and interact with cancer cells by soluble

factors. It has been elucidated that the morphological and functional changes of fibroblast around cancer cells are not the secondary changes by carcinoma, rather the fibroblasts play an active role in the whole process of cancer development such as initiation, progression, and invasion (Kunz-Schughart and Knuechel, 2002). Recently, several studies reported that fibroblasts play an essential role in the process of cancer cell invasion (Che et al., 2006; Yoon et al., 2005). Furthermore, there are some studies that compared CAF with NF. For example, fibroblasts from normal breasts have been found to inhibit the non-tumorigenic mammary epithelial cells, although fibroblasts from cancerous breasts stimulated proliferation (Dong-Le Bourhis et al., 1997; Van Roozendaal et al., 1996). Similar results have been found in coculture experiments with prostate cells (Grossfeld et al., 1998; Olumi et al., 1999). Also, according to Sadlonova et al. (2005), both normal breast-associated fibroblasts (NF) and CAF have the ability to inhibit epithelial cell proliferation and to induce glandular differentiation to a more normal phenotype, although CAF have less inhibitory capacity than NF. Transition to CAF from NF through carcinogen and/or transformed epithelial cells or cancer cells might play an active role in cancer development.

From the point of view that fibroblasts play a central role in cancer progression, the phenotypic and genotypic expression patterns of these CAF are under intense investigation. For example, Ronnov-Jessen et al. (1996)

demonstrated that the histology and growth characteristics of mammary CAF were different from those of fibroblasts associated with normal breast epithelial cells. They described the presence of activated or abnormal myofibroblasts associated with invasive breast carcinoma cells. Other phenotypic changes ascribed to CAF include abnormal migratory behavior in vitro (Schor, 1988) and altered expression of growth factors such as platelet-derived growth factor, insulin-like growth factor I and II, transforming growth factor-β1, hepatocyte growth factor/epithelial scatter factor and keratinocyte growth factor (Ellis et al.,1994; Kinzler and Vogelstein, 1998). Orimo et al. (2005) demonstrated that CAFs extracted from human breast carcinomas promote the growth of admixed breast carcinoma cells significantly more than do normal mammary fibroblasts derived from the same patients. The CAF, which exhibit the traits of myofibroblasts, play a central role in promoting the growth of tumor cells through their ability to secrete stromal cell-derived factors 1 (SDF-1); CAF promote angiogenesis by recruiting endothelial progenitor cells (EPCs) into carcinomas, an effect mediated in part by SDF-1. CAF-secreted SDF-1 also stimulate tumor growth directly, acting through the cognate receptor, CXCR-4, which is expressed by carcinoma cells. These findings indicated that fibroblasts within invasive breast carcinomas contribute to tumor promotion in large part through the secretion of SDF-1.

To understand these changes in fibroblasts around cancer cells, the

mechanisms of interaction between cancer cells and stromal cells must be explained. There are two kinds of interaction mechanism. One is cell-cell contact (direct cell-cell interaction) and the other is by soluble factors such as cytokines and growth factors (indirect cell-cell interaction) (Löffek et al., 2006). Secretion of soluble factors by cancer cells activates surrounding cells, fibroblasts, and endothelial cells, and also recruits inflammatory cells. Among the most important growth factors and cytokines that play a role in the interaction of cancer cells and stromal cells are basic fibroblast growth factor (bFGF), IL-1(interleukin -1α and β) and vascular endothelial growth factor (VEGF).

The cytokine Interleukin-1 (IL-1) mainly affects inflammatory processes but also possesses various immune, degradative, and growth-promoting properties (Apte et al., 2006). IL-1 includes a family of closely related genes; the two major agonistic proteins, IL-1α and IL-1β, are pleiotropic and affect mainly inflammation, immunity and hemopoiesis. The IL-1 receptor antagonist (IL-1Ra) is a physiological inhibitor of pre-formed IL-1. IL-1 increases tumor invasiveness and metastasis. For example, IL-1expression at the site of tumor development enhances the expression of adhesion molecules on endothelial and malignant cells and facilitates the invasion of malignant cells into the circulation and their dissemination to remote tissues. Both IL-1α and IL-1β were shown to contribute to tumor angiogenesis and invasiveness,

but the role of IL-1β is more evident in these processes. This may be due to IL-1β being secreted into the microenvironment, thus activates cells in the tumor's stroma, including the malignant cells. In contrast, the effects of IL-1α are more limited, as this cytokine is largely cell-associated (Voronov et al., 2003). Human head and neck squamous cell carcinomas (HNSCC) constitutively express IL-1α and a repertoire of proinflammatory and proangiogenic cytokines that are potentially IL-1-inducible, including IL-6, IL-8, and GM-CSF. Coexpression of these cytokines correlated with activation of immediate-early transcription factors NFκB/Rel A and AP-1 and demonstrated that inactivation of NF-κB/Rel A inhibited the expression of this repertoire of cytokines as well as cell survival and growth. Exogenous and endogenous IL-1α contributes to the expression of IL-8 through the transcriptional activation of NFκB and AP-1 and also promotes cell survival and the growth of HNSCC cell lines in vitro (Wolf et al., 2001).

Chemokines are chemotactic cytokines that are classified onto four groups based on the position of the first two cysteine amino acid residues within the amino terminus: CXC, CC, C and CX_2C (Bacon et al, 2002). CXC chemokines act largely on neutrophils, lymphocytes and endothelial cells, whereas CC chemokines act on several cell types including monocytes, dendrite cells, basophils, eosinophils, and lymphocytes. Chemokines are important in directed cell migration, which is achieved through a seven

transmembrane chemokine receptor on cells upon activation and signaling of the chemokine receptor, cells then traffic in response to a chemokine ligand gradient. CXC chemokines function to enhance innate and adaptive immunity, regulate angiogenesis, prevent apoptosis, promote proliferation and mediate tumor cell metastases. The functions of CXC chemokines have a direct impact on both the biology of cancer and the host's response to the tumor (Balkwill, 2004). Over the last 20 years, it has been recognized that chemokines have an increasingly important role in mediating the trafficking of populations of leukocytes under both conditions of homeostasis and inflammatory immunological responses. In addition, numerous studies over the last decade have demonstrated that specific expression of chemokines and their receptors in the context of cancer are essential events that appear to be important in either for promoting tumor growth, tumor-associated angiogenesis, and metastasis or for inhibiting tumor growth via attenuation of tumor-associated angiogenesis (Gomperts and Strieter, 2006).

Therefore, better understanding of the characteristics of CAF and the soluble factors which are acting on CAF and cancer cells in cancer invasion might offer a promising point of departure for the development of new therapies. With this hope, the purpose of this study was to characterize CAF for their induction of oral squamous cell carcinoma invasion and to investigate

the mediating soluble factors between the cancer cells and the associated stromal fibroblasts.

II. MATERIALS AND METHODS

All studies involving human subjects were approved by the Institutional Review Board of Yonsei Dental Hospital, Yonsei University Health System, Seoul.

1. Chemokines and antibodies

Human recombinant monocyte chemotactic protein CCL7, Human recombinant IL-1α, and Human recombinant IL-8 were obtained from R&D Systems. Anti-human CCL7 monoclonal antibodies, anti-human IL-1α antibodies, and streptavidin-HRP conjugate were also purchased from R&D Systems. TMB solution was obtained from Sigma.

2. Fibroblast and oral cancer cell culture

CAF were derived from three oral squamous cell carcinoma (OSCC) patients and normal fibroblasts (NF) were from extracted healthy wisdom teeth of three patients. Cancer tissues obtained from the operation room suite were washed with Betadine three times, and after shorn with scissors, washed with 1X PBS and then incubated in a mixture of Dulbecco's Modified Eagles

Medium (DMEM; Gibco BRL, USA) and Ham's Nutrient Mixture -F12 (Gibco BRL, USA) culture media at a ratio of 3:1, supplemented with 10 % FBS and 1 % penicillin/streptomycin. Subsequently, CAF were selected by Versene solutions (0.1 g EDTA and 2 ml glucose in 500 ml 1x PBS buffer) from explanted cancer tissues. The normal fibroblasts were maintained in a mixture of Dulbecco's Modified Eagles Medium (DMEM; Gibco BRL, USA) and Ham's Nutrient Mixture -F12 (Gibco BRL, USA) culture media at a ratio of 3:1, supplemented with 10 % FBS and 1 % penicillin/streptomycin. The informed consent was given by six patients for this study. YD-10B and YD-38 OSCC cells were obtained from the Korean Cell Line Bank (Lee et al., 2005) and Ca9.22 cells were given by Dr. Takashi Muramatsu from Tokyo Dental College. OSCC cells were cultured with a mixture of Dulbecco's Modified Eagles 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 x 10^{-10}M cholera toxin, 0.4 mg/ml hydrocortisone, 5 μg/ml insulin, 5 μg/ml transferrin, and 2 x 10^{-11} M triiodotyronine (all purchased from Sigma, St. Louis, USA). All cells were cultured at 37 °C in an atmosphere containing 5 % CO_2.

3. Immunofluorescence staining

The isolated fibroblasts were characterized by immunostaining with anti-vimentin and anti-α-smooth muscle actin. Established culture cells were seeded on glass-based dishes (Nunc, USA). After overnight growth, cells were washed with PBS and fixed in 4% formaldehyde/PBS for 10 minutes. Cells were incubated using 0.2% triton X-100 in PBS for 10 minutes on ice, and then incubated with 15% goat serum at room temperature for 10 minutes. Subsequently, primary mouse monoclonal anti-α-smooth muscle actin antibody (1:50, DAKO, Denmark) and goat polyclonal anti-vimentin antibody (1:50, R&D systems, Inc., USA) were applied at room temperature for one hour, respectively. Slides were then incubated with Alexa Fluor 594 donkey anti-goat immunoglobulin (1:100, Molecular Probes, Inc. Oregon, USA). After nuclear staining with 4,6-diamidinophenylindole (DAPI) (1:500, Sigma, USA) at room temperature for one hour, the slides were mounted with an anti-fade solution (Molecular Probes, Inc. Oregon, USA). Stained cells were observed using Zeiss Pascal laser-scanning microscopy.

4. Invasion assay

Invasion assay in 24-transwell plates (Corning) was carried out as

described in Youngs' study (1997). Inserts containing 8μm pore size filters were coated with collagen type I (1.5 μg/μl per well) for invasion assay. Briefly, 2×10^4 OSCC cells were placed in the transwell chambers with porous filters in the upper wells and 2.1×10^4 CAF or NF were added into the lower well. The cells that penetrated through the filter were fixed, stained with 0.25 % crystal violet and counted by light microscopy.

5. Conditioned medium

$1X10^6$ CAF and YD-10B OSCC cells were cultured for 2 days in 10 cm culture plates containing 5 ml DMEM: Hams-F12 (3:1). The supernatant was collected and used as conditioned media after filtration.

Media for ELISA were coating buffer (50mM carbonate/ bicarbonate buffer, pH 9.6), blocking buffer (PBS containing 1% BSA) and washing buffer (PBS containing 0.05% Tween 20)

2×10^4 cancer cells were cultured for 24 h in 24-transwell plates with or without fibroblasts (2.1×10^4). The supernatant was collected for ELISA. CXCL1 ELISA was performed as follows: each well of a 96-well plate was coated with 50 μl capture Ab diluted 1/250 in the coating buffer overnight at 4 °C. After washing three times with the washing buffer, the unbound sites of wells were blocked with 200 μl of the blocking buffer and incubated for 1 h at

37 °C. The wells were washed and then incubated with 50 μl standards or samples. After incubating for 2 h at 37 °C, the wells were washed and incubated with 50 μl detection antibody diluted 1/250 in the blocking buffer for 1 h at 37 °C. After washing, 50 μl streptavidin-HRP conjugate diluted 1/200 in the blocking buffer was added and incubated for 1 h at 37 °C. The wells were washed prior to incubation with 100 μl TMB for 30 min in the dark at RT. The reaction was stopped by adding 50 μL 2M H_2SO_4 to measure the activity of the enzymes bound to the solid phase at 450 nm using a microplate reader.

IL-8 and CCL7 ELISAs were also performed as described above with the exception that all incubation steps were carried out at room temperature and detection antibodies were diluted 1/4000.

6. Cytokine antibody array analysis

The human cytokine antibody array (Panomics, Inc.) detects 36 cytokines level. Two OSCC cells, Ca9.22 and YD-38 were selected for this study, which cells showed the highest expression difference of cytokines produced by CAF in the previous study (Che, 2007). The cells (2.5×10^6) were seeded in T75 tissue culture flasks overnight and the media were changed to 5 ml of

serum-free DMEM: Hams-F12 (3:1 ratio) after washing 3 times with PBS. After 24 h incubation, supernatants were collected and normalized for cell number between samples by dilution with serum-free media before binding to the antibodies immobilized on the array; the secondary biotin-labeled detection antibodies were then added to the array. This sandwich ELISA using the array membrane as a solid support allows the rapid and accurate profiling of multiple cytokines at a protein level of pg/ml. By comparing signal intensities, the relative expression level of the cytokines can be determined.

7. siRNA- Transfection

IL-1RI and control siRNAs were purchased from Santa Cruz Biotechnology, Inc, CA. siRNA mediated inhibition of gene expression was carried out according to manufacturer's instructions. CAF were seeded at 10^5 cells/well in a 6-well culture plate 24 hours before transfection.

The CAF were trypsinized 48 hours after transfection and then incubated with 50 pg/ml IL-1α in a 24-transwell plate. After 24 h incubation, the culture media were collected, centrifuged, and analyzed by IL-8, CXCL1, and CCL7-specific ELISAs.

8. Protein extraction and western blot

Protein was isolated from the CAF or NF, after 24 h incubation. Fibroblast were plated in 100 mm dishes (Falcon, USA) and cultured to confluence. Then IL-1α and YD-10B conditioned medium (0.5 mg/ml) were added to the dishes, which were cultured for 24 h. Cell lysate was obtained from RIPA (cell signaling, USA) with PMSF and protease inhibitor cocktail. The total protein concentration was quantified using the BCA protein assay reagent (Thermo, USA). The whole cell protein extract was resolved with SDS PAGE using a 10% polyacrylamide gel under reduced conditions. After transfer to NC membrane (Pall Gelman Lab, Ann Arbor, MI, USA), the protein stained with Ponceau staining to verify uniform loading and transfer. Membranes were blocked with 5% skim milk in Tris-buffered saline with tween 20 (Tris-HCL, NaCl, and 0.05 % tween 20, pH 7.4 ; TBS-T) for 1 h at room temperature and subsequently incubated with primary antibodies [IL-1RI (Millipore,Temecula, CA), NF-kB (Calbiochem, USA) and actin(Sigma, Saint Louis, MO, USA)] with 1:1000 dilution overnight at 4 ℃. The membrane was washed three times with TBS-T and incubated with the appropriate horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature. Subsequently, the membrane was washed three times with

TBS-T. ECL detection kit (Santa cruz, sc2048) was used for visualization.

For western blot analysis of IL-1RI expression, CAF protein was harvested 48 hours after transfection from triplicate wells of a 6-well culture plate. The western blot analysis was performed as previously described using anti- IL-1RI antibody (Millipore, Temecula, CA).

9. Statistical Analysis

One-way ANOVA and Mann-Whitney test were done using the SPSS statistical program (version 12.0K) to determine the statistical significance between measurements. P values of <0.05 were considered significant.

III. RESULTS

1. Immunofluorescence staining

The expression of vimentin and α-smooth muscle actin was found in both NF and CAF. NF were smaller than CAF, spindle shaped and had less cytoplasmic processes. CAF were larger than NF, polygonal shaped and had more irregular cytoplasmic processes. CAF showed stronger expression pattern of intensity as compared to NF (Figure 1).

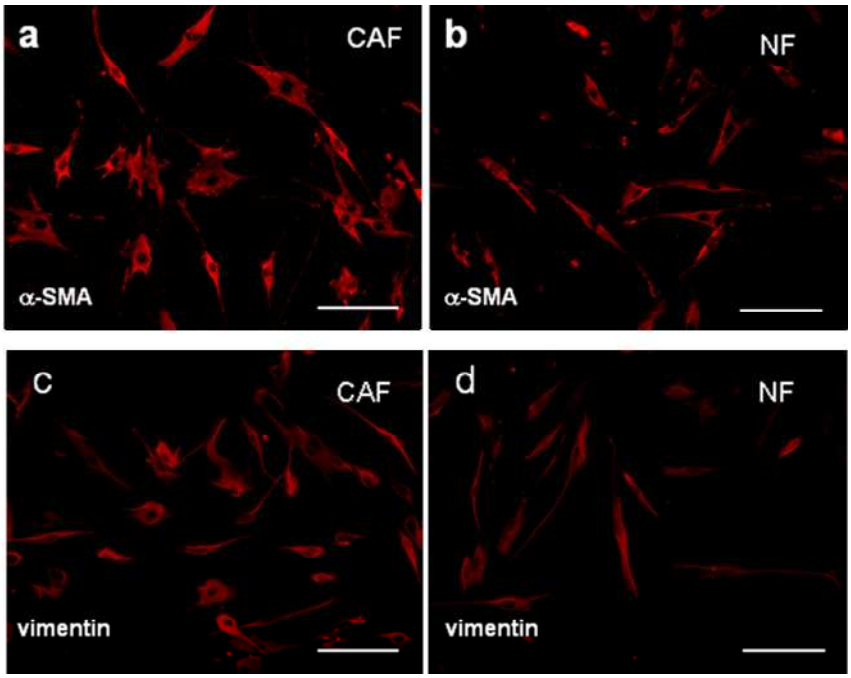


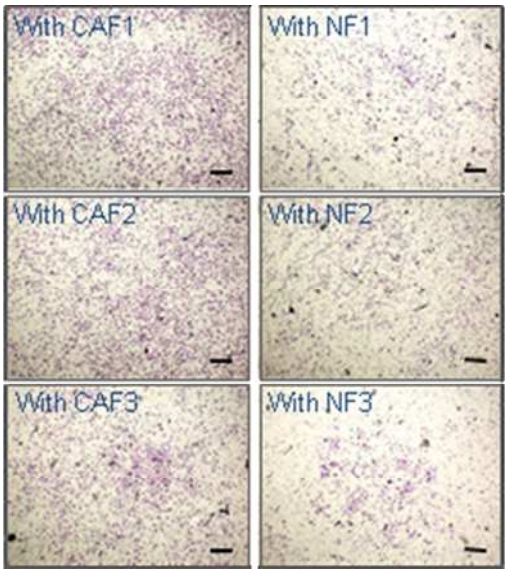
Fig. 1. Morphologic findings of NF and CAF by Immunofluorescence staining. CAF (a,c) and NF (b.d) cells were cultured in DMEM with 10% FBS. Immunostaining with anti-vimentin (c,d) and anti α-SMA antibody (a,b). NF showed spindle shaped cells, and CAF showed polygonal shaped cells, both with the expression of vimentin and α-smooth muscle actin. CAF showed more intense signals than NF. (CAF: cancer associated fibroblasts. NF: normal fibroblasts. α-SMA: α-smooth muscle actin. Laser-scanning microscopy. Scale bar, 150 μm.)

2. Comparison of invasiveness between CAF and NF.

To determine whether fibroblasts facilitates OSCC cells invasion in vitro, a transwell invasion assay employing collagen type I-coated filters was established. It was found that the invasion of OSCC cells was significantly increased by co-culturing with fibroblasts when compared to that without co-culturing with fibroblasts (Figure 2).

CAF was also compared to NF for enhancing cancer cell invasion. All three CAF populations tested enhanced OSCC cell invasion at significantly higher levels than did NF populations.

a.


With CAF1
With NF1
With CAF2
With NF2
With CAF3
With NF3

b.

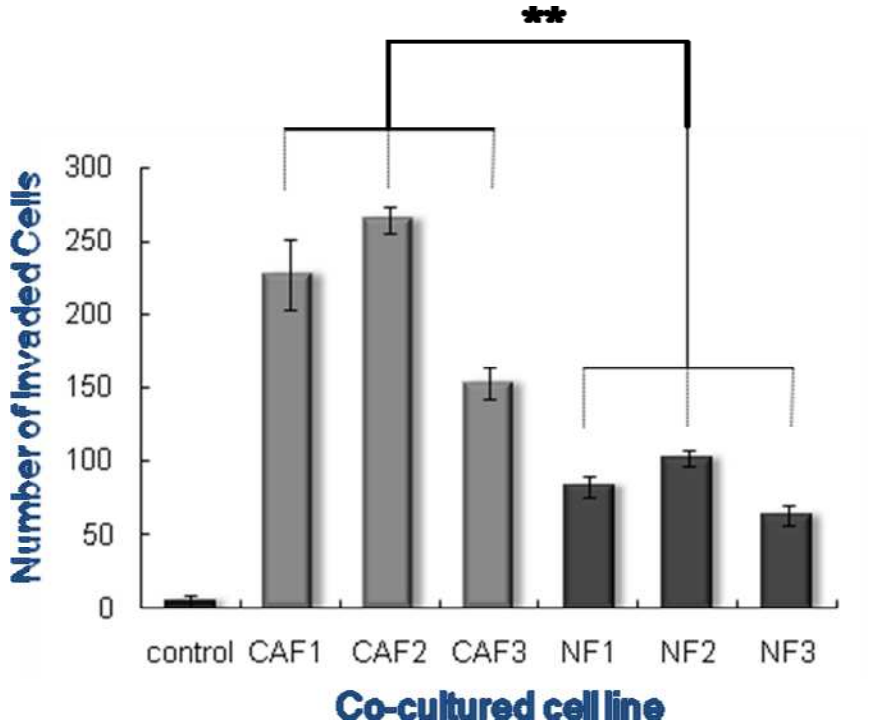

**
Number of Invaded Cells
300
250
200
150
100
50
0
control
CAF1
CAF2
CAF3
NF1
NF2
NF3
Co-cultured cell line

Fig. 2. Invasion of OSCC cells was facilitated by co-culturing with fibroblasts. In vitro transwell invasion assay was carried out with inserting filters with 8 μm pore size coated with collagen type I. 2x 10^4 OSCC cells were placed in the upper wells and 2.1x10^4 CAF or NF were added into the lower well. The cells that penetrated through the filter were fixed, stained with 0.25% crystal violet. CAF were significantly more potent than NF in promoting the invasion of OSCC cells. To measure cell invasion, the number of cells that penetrated the transwell membrane were stained and counted under light microscopy. Results were reported as the mean± s.d. of triplicate assays (Scale bar, 450 μm. ** designates a P value of <0.01).

3. Comparison of chemokines release between CAF and NF in response to co-cultured OSCC.

To determine whether the chemokines released from fibroblasts increases by co-culturing with OSCC cells as compared to those from mono-cultured fibroblasts, sandwich ELISAs was established that is specific to IL-8, CXCL1, and CCL7. In the microarray data and real-time PCR data, pro-inflammatory cytokines and chemokines, such as IL-8, CXCL1, CXCL2, CXCL3, and CCL7 were up-regulated in CAF by co-culturing with OSCC cells, implicating that they may play a central role in tumor progression by promoting angiogenesis and chemotaxis (Che, 2007, Table 1). For this reason, IL-8, CXCL1, and CCL7 were selected for analyzing chemokines released from the co-cultured CAF.

OSCC cells were cultured with or without fibroblasts in a 24-transwell plate for 24 h and the culture media were collected for the ELISAs (Figure 3a, b and c). NF co-cultured with OSCC cells released significantly higher levels of CCL7, IL-8 and CXCL1 compared to mono-cultured NF ($P < 0.01$ by Mann- Whitney test). CAF co-cultured with OSCC cells released significantly higher levels of CCL7, IL-8 and CXCL1 compared to mono-cultured CAF ($P < 0.01$ by Mann- Whitney test). In addition, CAF and NF,

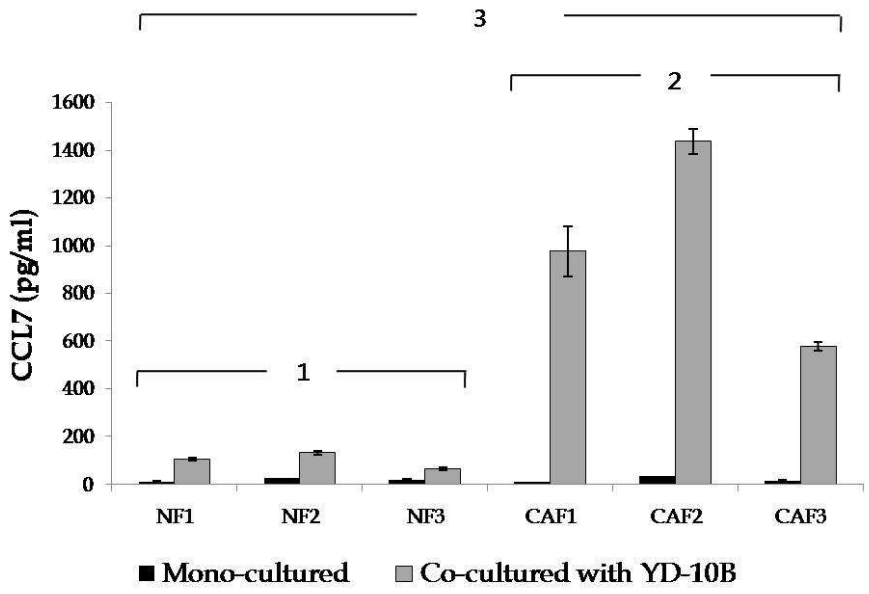
both co-cultured with cancer cells, were compared for their abilities to release the chemokines into the co-culture media. Chemokine release from the co-cultured CAF was significantly higher than that from the co-cultured NF, indicating that CAF were more responsive to OSCC cell stimulation for enhancing chemokine release than were NF ($P < 0.01$ by Mann- Whitney test). However, there was no significance in releasing cytokines between mono-cultured NF and mono-cultured CAF ($P > 0.05$ by Mann-Whitney test).

Table 1. Profiling cytokine expression in CAF using a co-culture system (Che, 2007)

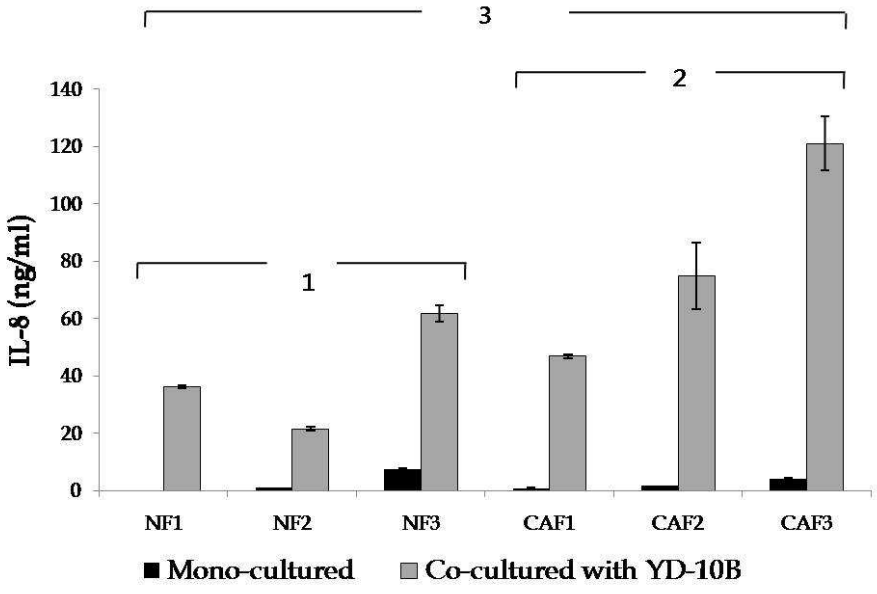
Validation of microarray data by real-time PCR

	GENE	Fold change	
		Microarray	Real time PCR
Fibroblast (CAF)	CCL7/MCP-1	2.08	55.55(n=6)
	CXCL1/GRO-α	3.35	26.25(n=6)
	CXCL2/GRO-β	2.67	4.04(n=3)
	CXCL3/GRO-γ	3.19	7.36(n=6)
	CXCL8/IL-8	2.07	9.32(n=3)

a.


3
2
1
1600
1400
1200
1000
800
600
400
200
0
CCL7 (pg/ml)
NF1
NF2
NF3
CAF1
CAF2
CAF3
Mono-cultured
Co-cultured with YD-10B

b.


3
2
1
140
120
100
80
60
40
20
0
IL-8 (ng/ml)
NF1
NF2
NF3
CAF1
CAF2
CAF3
Mono-cultured
Co-cultured with YD-10B

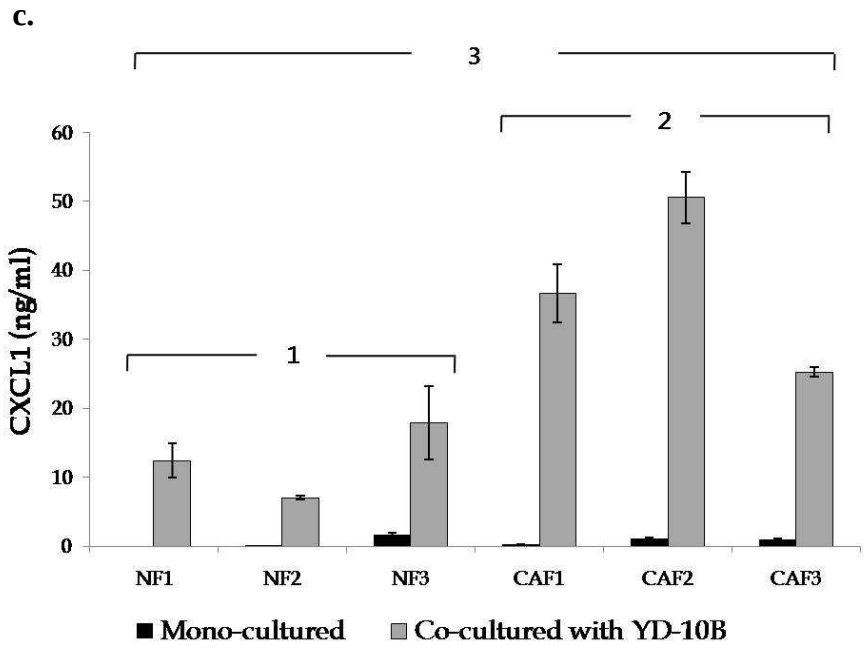


Fig. 3. Comparison of chemokines release between CAF and NF in response to co-cultured with OSCC. There were significant differences in releasing chemokines between mono-cultured NF and co-cultured NF (1 designates a P value of <0.01), mono-cultured CAF and co-cultured CAF (2 designates a P value of <0.01) and co-cultured NF and co-cultured CAF (3 designates a P value of <0.01 by Mann- Whitney test). a, b, and c showed CCL7, IL-8 and CXCL1 secretion were preferentially increased in CAF compared to NF.

4. Evaluation of cytokine release from OSCC for stimulation of fibroblasts

To find the soluble factor(s) secreted by OSCC cells that increases cytokines secretion by CAF, culture media collected from OSCC cell lines were analyzed and screened changes in the expression levels of 36 cytokines (Figure 4). It was speculated that VEGF and/or IL-1α might stimulate CAF to increase cytokines secretion, because the expression levels of these cytokines were higher in Ca9.22 cells, which induced higher CCL7 secretion by CAF compared to YD-38 as shown in the previous study (Che, 2007). Alternatively, the cytokines (IL-4, IL-12, RANTES) that showed higher expression levels in the YD-38 control cell line might attenuate the effect of CCL7-inducing soluble factor(s).

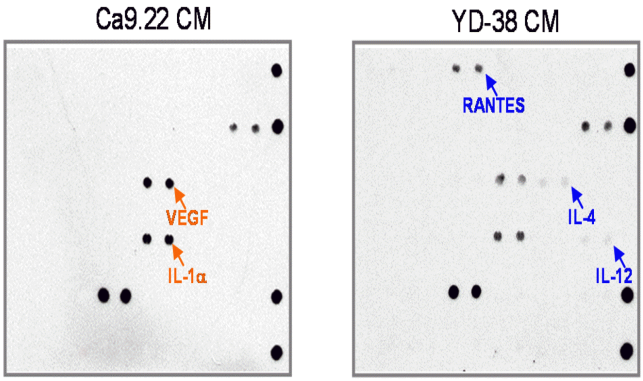
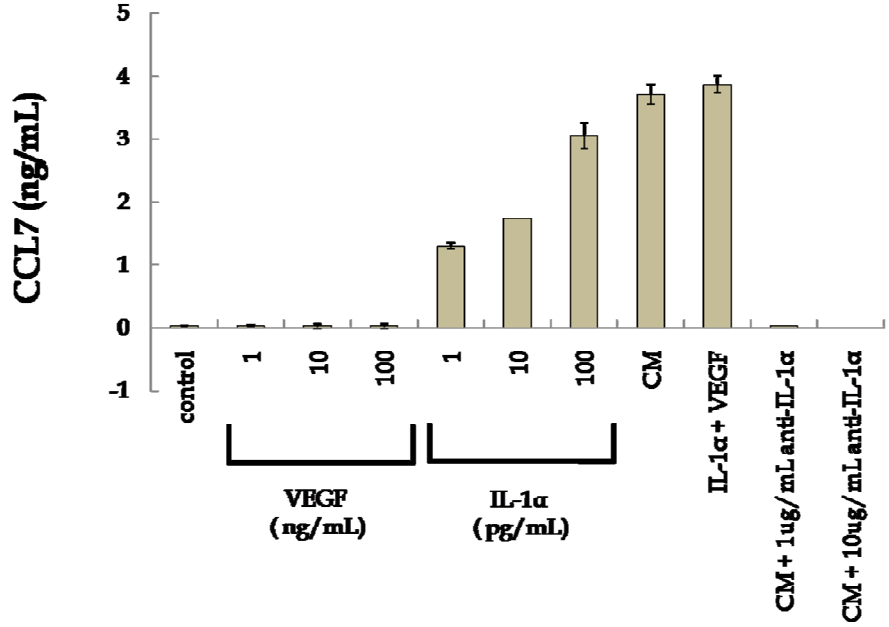


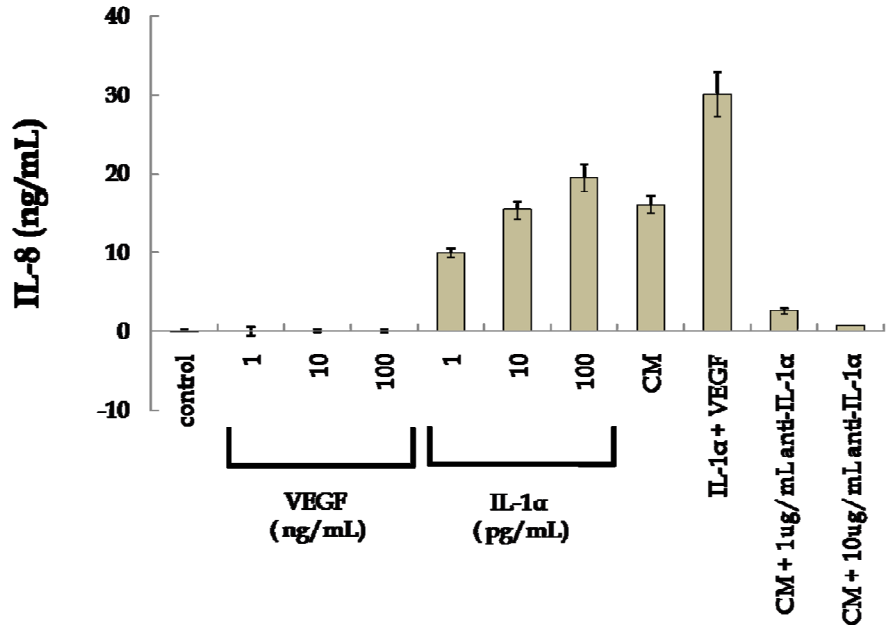
Fig. 4. VEGF and IL-1α showed remarkable intensity difference between Ca9.22 and YD-38 cells. Cytokine array revealed that VEGF and IL-1α showed comparable signals between two cancer cells among various cytokines expressed in them by co-culturing with CAF .

5. Secretion pattern of CCL7, IL-8, CXCL1 from CAF by IL-1α stimulation and neutralization

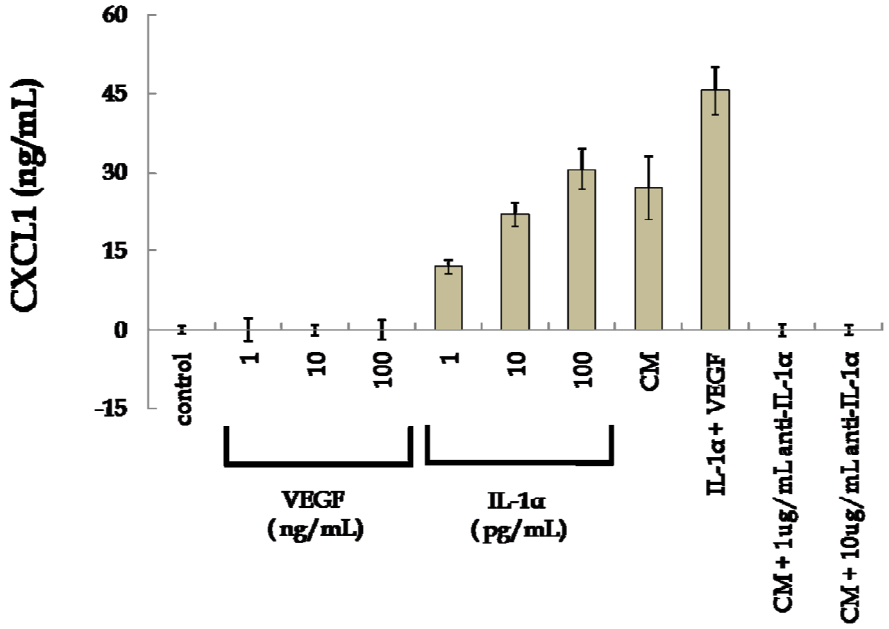
To confirm whether VEGF and IL-1α regulate cytokine secretion released from CAF, each cytokine, CCL7, IL-8, CXCL1 was measured after adding VEGF and/or IL-1α to CAF culture. Findings were that IL-1α induced CCL7, IL-8, CXCL1 expression (pg/ml) dose dependently in CAF, while VEGF failed to enhance CCL7 expression. Interestingly, 1 ng/ml of VEGF had a synergic effect on CCL7 secretion induced by IL-1α (1 ng/ml) (Figure 5a, b and c), increasing the levels of chemokine secretion as high as those stimulated by YD-10B-CM. Furthermore, it was noted that pre-incubation with anti-IL-1α monoclonal antibody almost completely abolished the secretion of CCL7, CXCL1, and IL-8 induced by YD-10B-CM.



a. CCL7 secretion decreased by neutralizing IL-1α antibody



b. IL-8 secretion decreased by neutralizing IL-1α antibody



c. CXCL1 secretion decreased by neutralizing IL-1α antibody

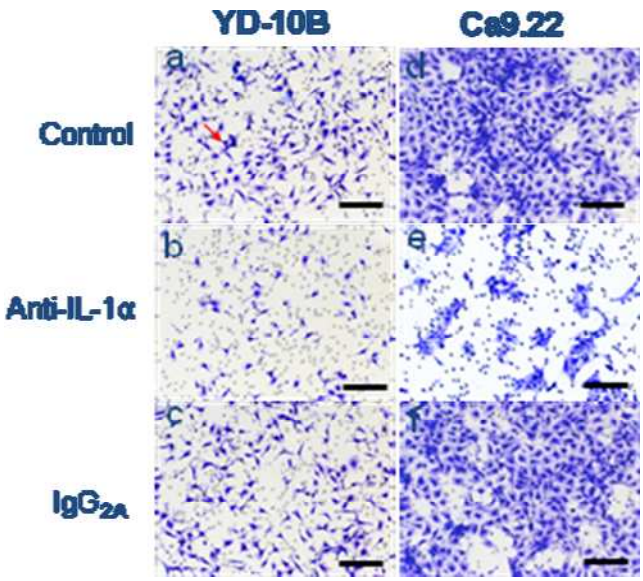
Fig. 5. IL-1α stimulates cytokine secretion from CAF

a,b,c, The release of IL-8, CXCL1, and CCL7 from CAF stimulated by IL-1α was analyzed by IL-8, CXCL1, and CCL7-specific ELISAs. CCL7, IL-8, CXCL1 secretion by CAF increased dose-dependently by IL-1α and decreased by neutralizing IL-1α antibody. VEGF failed to enhance the secretion of IL-8, CXCL1, and CCL7, but had a synergic effect on cytokines secretion induced by IL-1α (1 ng/ml of VEGF and 1 ng/ml of IL-1α).

6. Changes in the invasion of OSCC cells by anti-IL-1α neutralizing antibody

When comparing the effect of invasiveness of IL-1α by neutralization, the invasive potential of OSCC cells by stimulation of CAF was markedly decreased by neutralization of IL-1α (Figure 6 a, b). In this experiment, YD-10B and Ca9.22 cells were used since they have similar potential of invasiveness (Che, 2007)

a.



b.

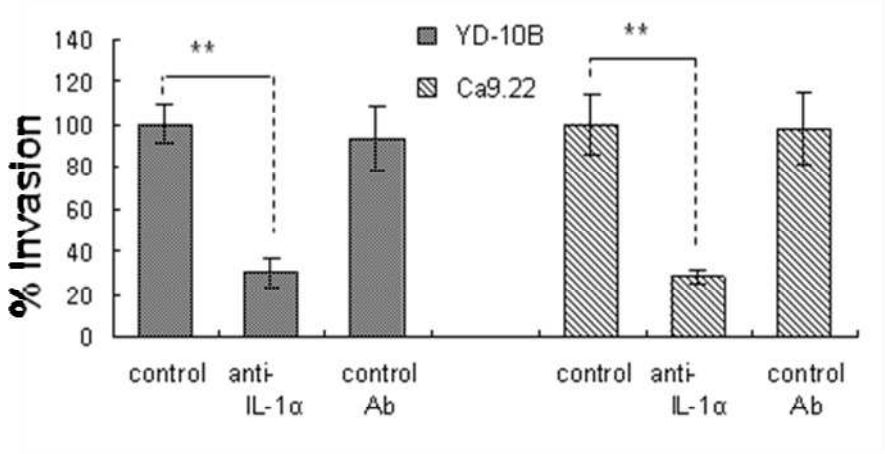
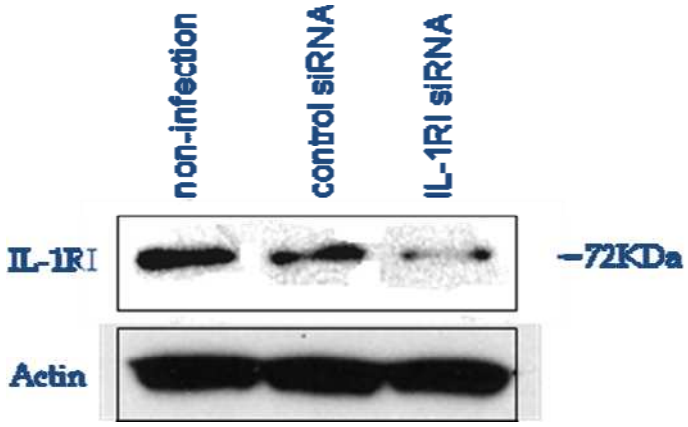


Fig. 6. Neutralization of IL-1α attenuated invasive growth of cancer cells. (Scale bar, 150 μm, ** designates a P value of <0.05 compared to neutralized IL-1α)

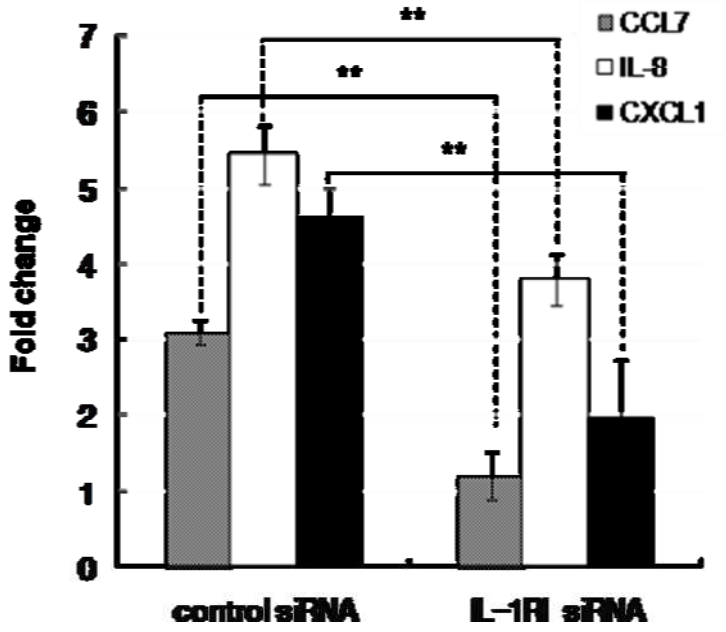
7. Effects of IL-1RI knockdown on the release of chemokines by CAF in response to IL-1α stimulation

This study investigated whether IL-1RI on CAF plays an important role in releasing chemokines, such as IL-8, CXCL1, and CCL7, in response to paracrine IL-1α stimulation. To do so, IL-1RI siRNA into CAF was introduced to knockdown the protein expression. The down-regulated IL-1RI protein expression on CAF was confirmed by western blot analysis (Figure 7a). The chemokine release from the transfected CAF stimulated by IL-1α was determined by ELISAs specific to IL-8, CXCL1, and CCL7. Inhibiting IL-1RI expression in CAF significantly reduced IL-8, CXCL1, and CCL7 secretion (Figure 7b), when compared to control siRNA-transfected CAF. These observations indicate that IL-1α acts to enhance the chemokine release from CAF through the IL-1RI on fibroblasts. Then in order to find out the changes in the invasion of OSCC cells co-cultured with siRNA transfected CAF, the invasion assay was performed and found that the invasion of OSCC cells co-cultured with IL-1RI siRNA transfected CAF was significantly reduced as compared to controls ($P < 0.05$ by Mann-Whitney test).

a.


non-infection
control siRNA
IL-1RI siRNA
IL-1RI
~72KDa
Actin

b.


CCL7
IL-8
CXCL1
**
**
**
Fold change
0
1
2
3
4
5
6
7
control siRNA
IL-1RI siRNA

c.

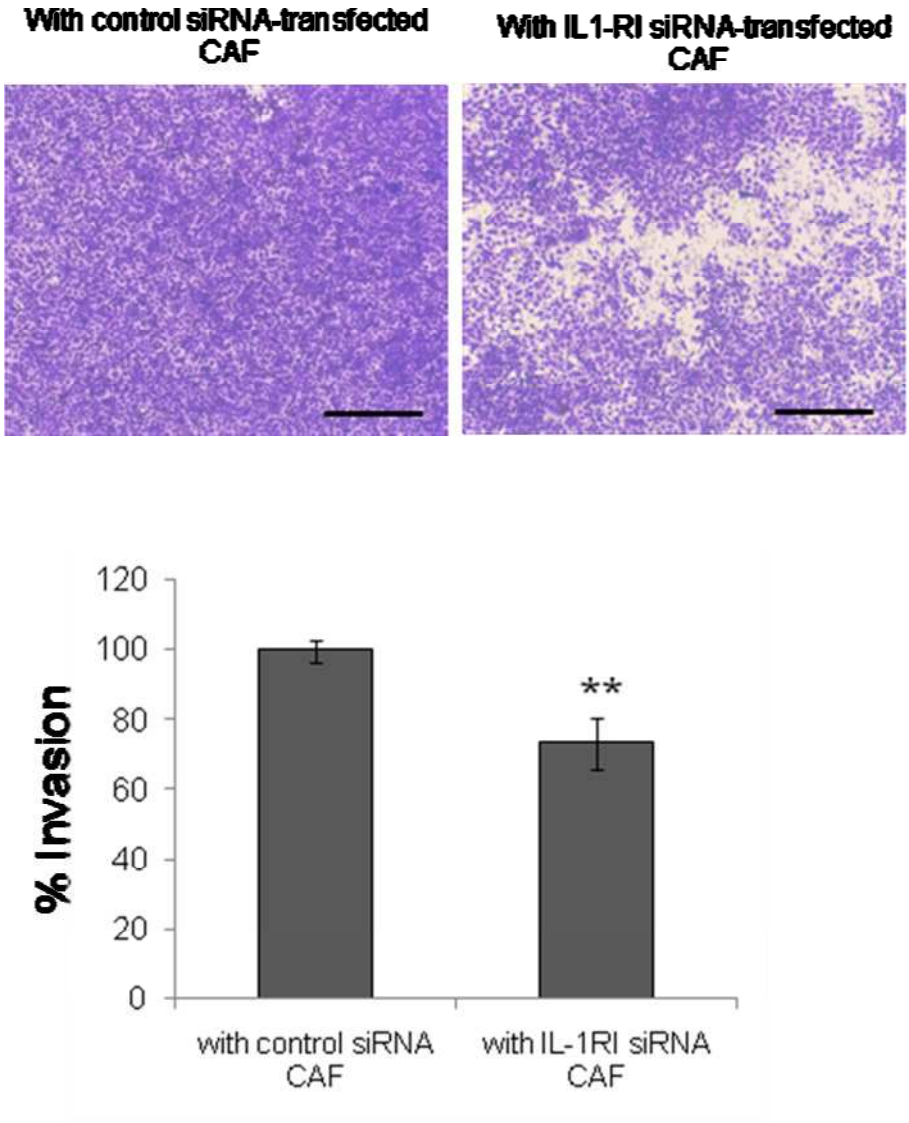


Fig. 7. IL-1RI siRNA decreases the level of chemokines released by CAF. a, Expression of IL-1RI protein on non-infected, control siRNA-treated, and IL-1RI siRNA-treated CAFs was analyzed by western blot analysis. β-Actin

was used for equal loading. b. The release of IL-8, CXCL1, and CCL7 from control siRNA-treated and IL-1RI siRNA-treated CAFs was analyzed by IL-8, CXCL1, and CCL7-specific ELISAs, respectively. Fold change was calculated from non-infected control. c. Type-1 IL-1 receptor siRNA reduced the invasion of OSCC cells induced by co-cultured CAF. (** designates a P value of <0.05 by Mann-Whitney test, Scale bar, 450 μm)

IV. DISCUSSION

The results of the present study demonstrate that CAF are different from NF morphologically and functionally, and these differences make CAF have potential to the invasiveness of cancer cells. In order to characterize CAF, this study attempted to develop an experimental model akin to in vivo by creating a collagen matrix intermixed with fibroblasts as a dermal equivalent.

Firstly, CAF showed stronger expression of vimentin and α-smooth muscle actin (SMA) than NF. This result is congruent with previous studies (Sappino et al., 1988; Mueller and Fusening, 2004; Bissell and Radisky, 2001; Orimo et al, 2001 and 2005) that fibroblasts extracted from within and around cancer cells exhibit increased α-SMA expression as well as increased contractility, both indicative of myofibroblasts. Myofibroblasts are stromal fibroblasts with features of both myoblasts (e.g., expression of smooth muscle actin) and fibroblasts that have been implicated in cancer invasion, extracellular matrix remodeling, wound healing, and chronic inflammation (De Wever and Mareel, 2003). But the present experiments did not address how CAF acquire these unique phenotypes and how these cells maintain the myofibroblastic phenotypes. Further research is needed to examine this phenomenon.

Then, this study showed that, in transwell invasion assay employing

collagen type 1 –coated filters, OSCC cell invasion was significantly increased by co-culturing with fibroblasts as compared to that without co-cultured fibroblasts. And CAF enhanced OSCC cell invasion at significantly higher levels than did NF. Che et al. (2006) showed that CAF play an essential role in cancer invasion and suggested that soluble factors released from CAF may be related to this process. To examine soluble factors released from CAF for cancer cell invasion, Che (2007) preformed microarray and real-time PCR, comparing cytokine expression between monolayer cultured CAF and co-cultured CAF with OSCC. From the results, he found that several cytokines such as CCL7, CXCL1, CXCL2, CXCL3, and IL-8 highly expressed in co-cultured CAF. In that experiment, CAF is transformed NF by carcinogen directly or through transformed epithelial cells or cancer cells. Therefore, this study attempted to identify the role of fibroblasts in cancer invasion by investigating the difference of cytokine expression between CAF and NF. This study found that CAF secreted CCL7, IL-8 and CXCL1 more prominently than NF did, and this difference was more pronounced when fibroblasts were co-cultured with cancer cells. From these results, it is conceivable that increased secretion of cytokines from CAF may cause cancer cell invasion. The reason is that CXCL1 and IL-8 (CXCL-8) are two of the CXC chemokines family members that are ELR positive and promote angiogenesis (Gomperts and Strieter, 2006). Tumor growth, invasion and

metastasis are dependent on a pro-angiogenic environment, while several factors have been found to promote angiogenesis, specific CXC chemokines have increasingly been demonstrated to significantly contribute to net angiogenesis in a variety of tumors. CXC chemokines are heparin binding proteins that contain four highly conserved cysteine amino acid residues with the first two cysteines separated by a non-conserved amino acid residue (Strieter et al., 2004). Several CXC chemokines also possess three amino acid residues – Glu-Leu-Arg; the so-called 'ELR' motif – at the NH2 terminus preceding the first cysteine amino acid residue. Chemokines that contain the ELR motif (ELR-positive) are pro-angiogenic, whereas members that lack the ELR motif (ELR-negative) and are in general interferon-inducible inhibit angiogenesis (Strieter et al., 2004). The ELR-positive CXC chemokines have the ability to act as both autocrine growth factors and as potent paracrine mediators in the promotion of tumor associated angiogenesis. On the other hand, CCL7 is one of the NH2-terminally cleaved monocyte chemotactic proteins and have impaired capacity to attract cancer-associated macrophages and function as receptor antagonists for intact CC chemokines (Damme et al., 2004).

Next, this study investigated what soluble factors released from OSCC cells could induce cytokine secretion by CAF. For this study, cytokine antibody array was conducted to compare the difference of cytokine

expression released from CA 9.22 and YD-38 cells when co-cultured with CAF, based on the report of Che (2007) that CAF co-cultured with Ca9.22 cells showed highest CCL7 secretion and CAF co-cultured with YD-38 cells showed the lowest CCL7 secretion. Cytokine antibody array showed that VEGF and IL-1α showed remarkable intensity difference between CA 9.22 and YD-38 cells. Furthermore, when IL-1α was neutralized by their antibody, the invasiveness potential of OSCC cells by stimulation of CAF was markedly decreased. To confirm these results, this study examined CCL7, CXCL1, IL-8 levels in CAF by treatment of IL-1 α, and found that the cytokine levels were dose-dependently increased and were dramatically decreased by neutralizing antibody of IL-1α. From this result, IL-1α seems to be the most prime candidate of cytokines that can induce CAF to secrete chemokines to promote invasion for cancer cells. Human head and neck squamous cell carcinomas (HNSCC) constitutively express IL-1α (Wolf et al., 2001). IL-1α is an important regulatory cytokine, the release of which after an injury can induce activation of transcription factors nuclear factor (NF) κB and activator protein (AP-1), which promote expression of IL-8 involved in cell survival, proliferation and angiogenesis.

This study found that IL-1α secreted from cancer cells stimulated fibroblasts to release several cytokines. That was confirmed by neutralizing antibody of IL-1α. Then, the present study carried out knockdown of

expression of IL-1α receptor on fibroblasts and found that cytokine secretion from CAF was remarkably attenuated by down regulation of IL-1 receptor I in CAF, eventuating in decreased invasive growth of cancer cells.

In summary, the results of this study clarified the role of CAF by characterizing them morphologically and functionally and addressed cytokines that interact between CAF and cancer cells. Future studies will be required to ascertain why and how CAF obtain these characteristics in molecular level. Although this study may be an early step to understand the interaction process between cancer cells and fibroblasts, it could be a new branch point for research of cancer signaling and cancer development.

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ABSTRACT (IN KOREAN)

구강편평세포암종의 침윤성 성장을 유도하는 암종 주위 섬유모세포의 수용성 인자

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암 발생 과정은 단순히 암 유발유전자의 활성과 암 억제 유전자의 비활성에 의한 돌연변이 세포의 증식이라기 보다는, 암 실질세포와 그 주위 기질간의 상호작용의 결과로 볼 수 있다. 기질 세포는 암세포의 영향으로 인해 형태적 기능적으로 변질되며, 그 중에서도 섬유모세포가 암종의 개시와 진행, 침윤과정에 핵심적인 역할을 담

당한다. 또한 이러한 암세포와 섬유모세포의 상호작용은 다양한 수용성 인자들에 의해 매개된다. 따라서 본 연구에서는 구강편평세포암종의 침윤과정을 좀더 명확히 이해하기 위한 일환으로, 암종에서 유래한 섬유모세포(Carcinoma Associated Fibroblasts: CAF)의 생물학적 특성과 암세포와 섬유모세포의 상호작용을 매개하여 암세포의 침윤에 영향을 미치는 수용성 인자(soluble factors)들을 규명하고자 하였다.

먼저 CAF 와 정상섬유모세포 (Normal Fibroblasts: NF)의 조직학적인 차이를 비교하기 위하여 면역형광염색을 시행한 결과, CAF와 NF에서 vimentin과 α- smooth muscle actin (SMA)의 발현을 확인하였으며, CAF의 경우 NF보다 더 높은 발현 율을 보였다.

In vitro 에서 섬유모세포가 구강편평세포암종 (Oral Squamous Cell Carcinoma: OSCC)의 침윤을 촉진하는 지의 여부를 확인하기 위하여 Transwell invasion assay를 실시하였다. 그 결과 암세포의 침윤성 성장은 섬유모세포와 공동 배양했을 때 유의적으로 증가하였으며, 특히 CAF가 NF에 비해 암세포의 침윤성 성장을 더 촉진하였다.

OSCC 와 공동 배양한 섬유모세포가 단독 배양한 섬유모세포에 비해 chemokine의 분비가 더 증가하는지의 여부를 확인하기 위해 효소결합면역흡수 분석법 (Enzyme-Linked Immunosorbent Assay: ELISA)을 시행한 결과 OSCC 와 공동배양한 섬유모세포에서 chemokine(IL-8,

CXCL1, CCL7)의 분비가 증가하였으며, OSCC와 공동 배양한 CAF가 NF에 비해 chemokine의 분비가 유의적으로 증가하였다. 이로 인해 OSCC의 자극에 대해 CAF의 반응성이 NF보다 높음을 알 수 있었다.

CAF의 cytokine 분비를 유도하는 OSCC세포의 soluble factor를 확인하기 위해 cytokine detection kit을 사용하여 antibody array를 실시하였다. VEGF와 IL-1α는 Ca 9.22 와 YD-38에서 발현량의 차이가 크게 나타났다.

VEGF와 IL-1α가 CAF의 cytokine 분비를 조절하는 지의 여부를 확인하기 위해 CAF 에 VEGF 와/또는 IL-1α를 첨가하여 분비되는 CCL7, IL-8, CXCL1을 측정한 결과, IL-1α는 CAF에서 CCL7, IL-8, CXCL1의 발현을 농도 의존적으로 유도하였으나 VEGF 는 CCL7, IL-8, CXCL1의 발현을 유도하지 못했다. 또한 VEGF는 IL-1α 에 의해 발현이 유도된 CCL7, IL-8, CXCL1에 상승효과를 보였다. 이러한 현상은 IL-1α 에 대한 중화항체에 의해 완전히 감소하였다. 또한 IL-1α를 중화함으로써 CAF의 자극에 의한 OSCC의 침윤성도 현저하게 감소되었다.

paracrine IL-1α자극에 반응하여 CAF가 CCL7, IL-8, CXCL1등의 chemokine을 분비하는데 IL-1α에 대한 수용체(IL-1RI)가 중요한 역할을 담당하는지의 여부를 분석하기 위해 IL-1RI siRNA를 CAF에 주

입하였다. 그 결과 IL-1α 가 섬유모세포의 IL-1RI를 통해 CAF의 chemokine 분비를 촉진함을 알 수 있었다.

이상의 결과를 통해 암세포에서 유래한 섬유모세포가 정상섬유모세포에 비해 CCL7, IL-8, CXCL1 의 분비가 증가하였으며, 이러한 차이는 암세포와 공동 배양한 섬유모세포에서 더 현저하게 나타남을 알 수 있었다. 또한 암세포의 침윤성은 CAF를 암세포와 공동배양 하였을 때가 NF를 공동배양 하였을 때보다 현저하게 증가함도 확인되었다. cytokine 분비는 섬유모세포를 암세포와 공동배양 하였을 때 증가했기 때문에, 암세포에서 섬유모세포의 cytokine 분비를 자극하는 soluble factor를 확인한 결과 IL-1α가 가장 유력한 cytokine candidate임을 알게 되었다. 본 연구는 암종 주위 섬유모세포의 생물학적 특성을 확인하고, 구강편평세포암종에서 암세포와 섬유모세포의 상호작용을 매개하는 수용성인자들을 규명하였다. 이는 암의 진행과정을 이해하기 위한 기초 연구로, 후속 연구를 위한 초석이 되고자 한다.

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핵심어: 암종주위 섬유모세포, 정상섬유모세포, 구강편평세포암종, 침윤성, 수용성인자