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ORIGINAL ARTICLE

The growth and osteoclastogenic effects of fibroblasts isolated from keratocystic odontogenic tumor

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OBJECTIVES: To investigate the growth characteristics and effects on osteoclastogenesis in fibroblasts isolated from keratocystic odontogenic tumor (KCOT) fibrous capsule.

MATERIALS AND METHODS: Fibroblasts isolated from KCOT fibrous capsule and normal gingival mucosa were cultured *in vitro*. Their colony-forming units and proliferative activity were investigated, and the osteoclastogenic effects were also observed by a co-culture system with osteoclast precursor cell line Raw264.7. The mRNA of several genes related to bone resorption (IL-6, VEGF, COX-2, and M-CSF) was analyzed by real-time PCR.

RESULTS: Keratocystic odontogenic tumor fibroblasts developed fewer CFU and had longer population doubling time than gingival fibroblasts ($P < 0.05$). In contrast to gingival fibroblasts, KCOT fibroblasts expressed less IL-6, COX-2, and M-CSF ($P < 0.05$); however, the Raw264.7 co-cultured with KCOT fibroblasts developed more osteoclast-like cells and expressed higher level of nfatc1 than that co-cultured with gingival fibroblasts. Increased COX-2 expression and VEGF expression were detected in KCOT fibroblasts and Raw264.7 co-culture system ($P < 0.05$).

CONCLUSION: Although KCOT fibroblasts showed lower level of cell proliferation than gingival fibroblasts, higher osteoclastogenic ability was detected when co-cultured with Raw264.7. These results suggest that the cell–cell interaction in the co-culture system, possibly by increasing COX-2 and VEGF expression, may be responsible for the increased osteoclastogenic effects of KCOT fibroblasts.

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Keywords: tumor stroma; fibroblast; keratocystic odontogenic tumor; osteoclastogenesis; COX-2; VEGF

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Introduction

In most tumors, epithelium and stroma interact with each other. Tumors attract mesenchymal stroma stem cells (MSCs) and make them tumor-associated fibroblasts (TAFs), which compose the main cellular component of tumor stroma and express different gene profile compared with fibroblasts near normal epithelium (Dakhova *et al*, 2009). TAFs influence tumor aggressiveness; fibroblasts in breast cancer destruct bone through enhanced Gli2 and PTHrP expression (Sterling *et al*, 2011). The osteoblasts, which derive from fibroblasts, secrete receptor activator of NF-κB ligand (RANKL) and stimulate osteoclast differentiation in breast cancer stroma (Thomas *et al*, 1999), but osteogenesis is inhibited by TGF-β released from bone resorption sites (Langley and Fidler, 2011). Similar phenomenons also occur in prostate cancer (Chung, 2003).

Keratocystic odontogenic tumor (KCOT) is the most frequent benign odontogenic tumor as reclassified by WHO in 2005, which mainly occurs in the jaw bone (Luo and Li, 2009), it appears as single or multiple cystic lesion, and the latter is one of the stigmata of nevoid basal cell carcinoma syndrome (NBCCS)(Mendes *et al*, 2010b). KCOTs are locally aggressive jaw lesions with a putative high growth potential and a propensity for recurrence (Morgan, 2011). Therefore, many studies were naturally concentrated on its intrinsic growth potential and related behavior.

Mural growth of the epithelial lining as well as the fibrous tissue walls of KCOT is believed to be the main contributor to the expansion and the aggressiveness of the tumor (Mendes *et al*, 2010a). Several studies have indicated that KCOT epithelial lining exhibited a significantly higher level of proliferative activity in comparison with dentigerous and radicular cyst linings, but similar to gingival mucosa (Li *et al*, 1994, 1995). These proliferating epithelial cells expressed various genes, such as IL-1α, IL-6, TNFα, and RANKL (Mendes *et al*, 2010a), related to osteoclastogenesis and bone resorption. In fact, the supernatant of KCOT fragments incubated *in vitro* promoted calcium released from rat calvaria, and the calcium content was

correlated with IL-6 in the supernatant (Gao and Li, 2005). The stroma of KCOTs may be another contributor to the aggressiveness of the lesion. Vered *et al* demonstrated that unequal amount of myofibroblasts may be responsible for different bone resorption capacity between KCOT and ameloblastoma (Vered *et al*, 2005). Fibroblasts within the fibrous capsule or isolated from KCOTs produce various molecules related to osteoclastogenesis or bone resorption, including IL-6, VEGF, PGE_2, and its synthase COX-2 (Ogata *et al*, 2007; Mitrou *et al*, 2009; Senguven and Oygur, 2011). Therefore, further studies into the growth characteristics of the KCOT stromal cells may provide new insights in understanding the behavior of the lesion and the development of new therapeutic approaches.

We compared the growth characteristics of fibroblasts isolated from KCOT fibrous capsule and that from gingival mucosa. The osteoclastogenic effects of these fibroblasts were also observed by a co-culture system with a murine osteoclast precursor cell line Raw264.7.

Materials and methods

Cell culture

Fresh tissue specimens of KCOT and gingival mucosa were obtained from patients admitted to the Peking University Hospital of Stomatology under institutionally approved protocols after informed consent was obtained. As described previously (Kubota *et al*, 2000), fibroblasts were isolated from KCOT surgical specimens, as well as from the excess normal gingival mucosa collected during surgical extractions of the third molars.

Fibroblasts were routinely cultured in Delbucco's modified Eagle's medium (DMEM/F12) (GIBCO, Grand Island, NY, USA) containing 1% antibiotics (100 U ml^{-1} penicillin, 100 g ml^{-1} streptomycin), 2 mM L-glutamine, and 10% fetal bovine serum (FBS). The murine osteoclast precursor cell line Raw264.7 was routinely cultured in Dulbecco's modified Eagle's medium (DMEM) (GIBCO) containing 1% antibiotics (100 U ml^{-1} penicillin, 100 g ml^{-1} streptomycin), 2 mM L-glutamine, and 10% fetal bovine serum (FBS). All the cells were routinely cultured under 95% humidity and 5% CO_2 atmosphere.

Colony-forming unit assay

The fibroblasts were seeded onto 90-mm dishes at the density of 1000 per dish cultured with DMEM/F12 for 14 days. After stained with methylene blue, cell aggregates containing more than 50 cells were counted as colony-forming unit (CFU).

Population doubling time assay

The fibroblasts were seeded into 96-well plates at the density of 3000 per well cultured with DMEM/F12 mentioned above. After 24 h, the cell number was counted in six wells every 2 days with Cell Counting kit-8 (Dojindo, Kumamoto, Japan) according to the manufacturer's protocol. Briefly, 10 μl of the kit reagent was added to the cells and incubated for 2.5 h. Cell number was assessed using the ELISA plate reader at 450 nm. Population doubling time (PDT) was calculated with the formula: PDT = $(t-t0)$*lg2/lg$(N/N0)$ ($N0$ and N represent the cell numbers at time $t0$ and t, respectively).

Co-culture fibroblasts with Raw264.7

According to methods described previously (Hashizume *et al*, 2008), fibroblasts were plated in 24-well plates. Raw264.7 cells were inoculated at the density of 1×10^4 per well onto fibroblasts layer when the latter grown to 60% confluency. The co-culture systems were routinely cultured in α-MEM supplemented with 1% antibiotics (100 U ml^{-1} penicillin, 100 g ml^{-1} streptomycin), 2 mM L-glutamine, and 10% fetal bovine serum (FBS).

The medium was replaced every 2 days for 10 days, the cells were stained for tartrate-resistant acid phosphatase (TRAP) (TRAP kit, Sigma, St. Louis, MO, USA) according to protocol, the TRAP-positive cells containing no < 3 nuclei were counting as osteoclast-like cells, as TRAP-positive multinucleated ($TRAP^+$ MNCs). The mRNA of the co-culture system was collected with TRIZOL (Invitrogen) at the 3nd or 5th day, to investigate osteoclastogenic marker nfatc1 and other genes related to osteoclastogenesis.

RNA extraction, reverse transcription, and PCR

Total RNA was isolated from fibroblasts and co-culture system using TRIZOL Reagent (Invitrogen). 2 μg total RNA was reverse-transcribed into cDNA using the Superscript first-strand synthesis system (Invitrogen) according to protocol. Reactions were conducted in a 20-μl reaction mixture with ABI 7500 real-time PCR system (ABI), incubated for 10 min at 95°C, followed by 40 cycles of a two-step amplification procedure, composed of annealing/extension at 60°C for 1 min and denaturation for 15 s at 95°C. The expression of genes related to osteoclastogenesis was normalized to human β-actin expression, and osteoclastogenic marker nfatc1 was normalized to mus musculus actin beta (Actb), as described previously (Kang *et al*, 2011). The expression of the different genes is expressed as $2^{-(\Delta Ct)}$, and the used primers are listed in Table 1.

Table 1 Primer sequences

Genes	*Forward primers (5'-3')*	*Reverse primers (5'-3')*	*Gene ID*
Human β-actin	catgtacggttgctatccaggc	ctccttaatgtcacgcacgat	NM_001101.3
IL-6	aacctgaaccttccaaagatgg	tctggcttgttcctcactact	NM_000600.3
VEGF	ttatgcggatcaaacctcacc	gaagctcatctctcctatgtgc	NM_001171630.1
COX-2	ccagtataagtgcgattgtaccc	tcaaaaattccggtgttgagca	NM_000963.2
M-CSF	agacctcgtgccaaattacatt	aggtgtctcatagaaagttcgga	NM_172210.2
Actb	tctggcaccacaccttcta	aggcatacagggacagcac	NM_007393.3
nfatc1	cagtgtgaccgaagatacctgg	tcgagacttgatagggacccc	NM_016791.4

Statistics

The counting of CFUs and TRAP$^+$ MNCs was performed, respectively, in two independent sets containing three samples, altogether six samples in each experiment. The experiment of cell PDT was performed in two independent sets containing seven samples, altogether 14 samples. The comparison of genes expression between fibroblasts was made in two independent sets containing nine samples; altogether, 18 samples were performed. The comparison of gene expression between co-culture systems was made in two independent sets containing three samples, altogether six samples; each sample was done at least three technical repeats in the two experiments.

The data of gene expression were compared by means of Mann–Whitney *U*-test using SPSS 18.0 (IBM, Armonk, NY, USA); the data of TRAP$^+$ MNCs, CFUs, and PDT were compared by means of Student's *t*-test using EXCEL. Data are displayed as mean ± s.d. The statistical significance risk rate was set at $P < 0.05$.

Results

Fibroblasts isolated from KCOT and gingival mucosa

After passage 1, cells were tightly adhered, displayed well-spread, spindle-shaped, and elongated fibroblast-like appearance as described in previous reports (Motaln *et al*, 2010). Non-adherent cells or polygonal epithelium-like cells were not visible in cultures. These fibroblast-like cells became 70–80% confluence between 10 and 15 days, and no obvious morphological differences between the two types of fibroblasts were observed (Figure 1a).

Colony formation and cell proliferation

By classical CFU assay, we confirmed that the fibroblasts derived from KCOT and gingival mucosa were clonogenic, and these fibroblasts began to establish multiple but small colonies around the day 7 of culture (Figure 1b). The cell population in primary culture was homogenous and proliferated with attached well-spread morphology at each passage. At the 14th day, we counted the number of CFUs developed from the two types of fibroblasts with methylene blue staining (Figure 1c), and CFUs developed from KCOT fibroblasts were significantly fewer than that from gingival fibroblasts (CFUs, 34 ± 10.54/1000 cells *vs* 114.33 ± 21.94/1000 cells, respectively) ($P = 0.005$, Figure 2a).

We further compared the PDT of the two types of fibroblasts. Firstly, we depicted the cell proliferating curves according to cell counting by CCK-8 at the interval of 1 day and then selected index growth periods according to curve fitting using SPSS 18.0 (Figure 2b). The PDT of KCOT fibroblasts was 3.82 ± 0.88 days on average, significantly longer than that of gingival fibroblasts, which was 2.54 ± 0.85 days on average. There was statistically significant difference of PDT between the two types of fibroblasts ($P = 0.017$, Figure 2c).

Co-culture of fibroblasts with Raw264.7-induced osteoclastogenic differentiation

The murine osteoclast precursor cell line Raw264.7 was co-cultured with fibroblasts isolated from either KCOT fibrous capsule or gingival mucosa for 10 days. In the co-culture system, only the TRAP-positive cells containing no <3 nuclei were counted as osteoclast-like TRAP$^+$ MNCs. TRAP$^+$ MNCs appeared in both the KCOT and gingival co-culture systems, without obvious morphological differences (Figure 3a). But the KCOT co-culture systems developed 40.33 ± 10.21 TRAP$^+$ MNCs/plate, significantly more than that developed in gingival co-culture system (20.67 ± 7.37 TRAP$^+$ MNCs/plate, $P = 0.05$, Figure 3b).

The expression of osteoclast differentiation marker nfatc1 was investigated at the 3rd day of co-culturing in the two co-culture systems. In the KCOT co-culture system, the relative expression of nfatc1 was significantly higher than that in gingival co-culture system (0.01309 ± 0.00392 *vs* 5.68748E-4 ± 5.17503E-4, $P = 0.029$, Figure 3c),

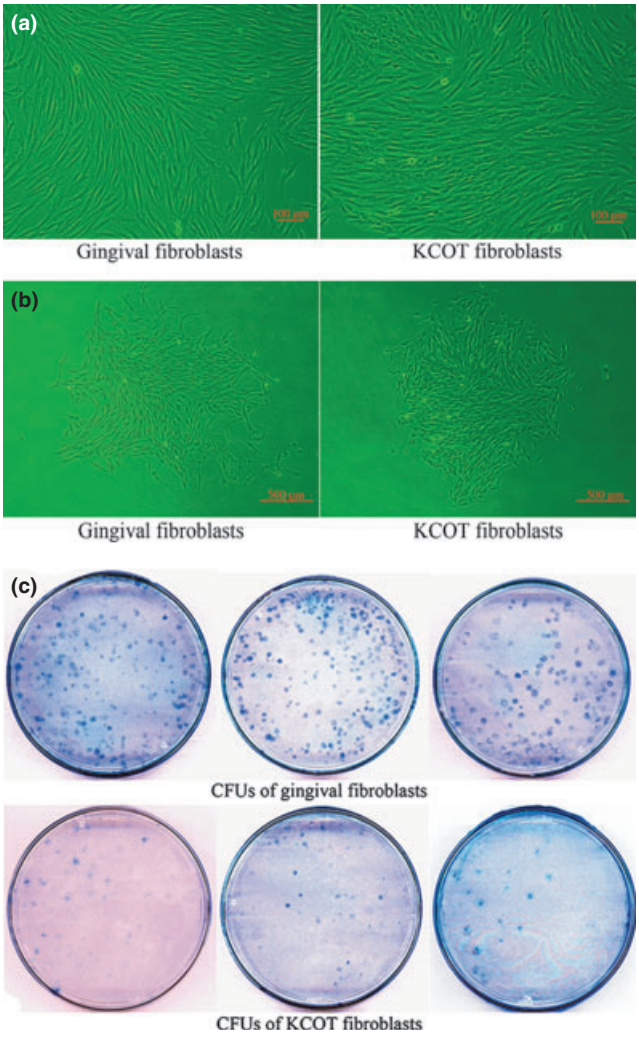

Figure 1 Images of cultured fibroblasts, cell colonies, and CFU-F assays. All the cells isolated from keratocystic odontogenic tumor fibrous capsule and gingival mucosa showed fibroblastic morphology and adherence to plastic culture dishes (with original magnification ×100, scale bar: 100 μm) (**a**). The cell aggregates containing more than 50 cells were counted as colony-forming unit (CFU) (with original magnification ×40, scale bar: 500 μm) (**b**). The cell colonies were stained with 1% methylene blue at day 14 of culture (**c**)

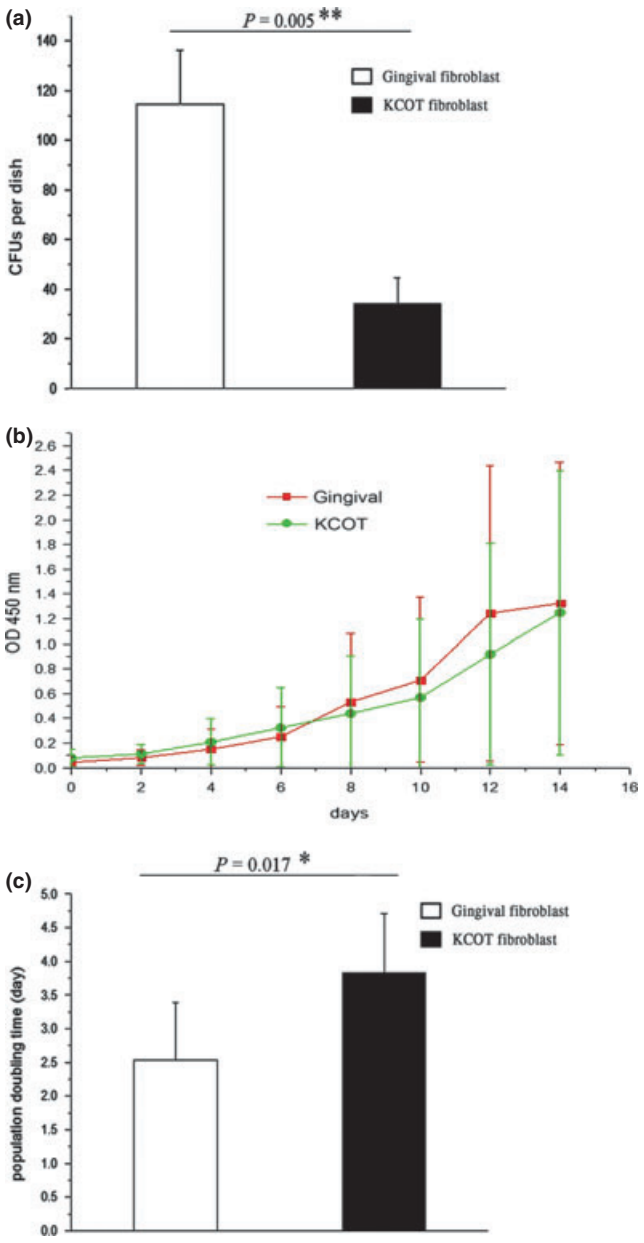

Figure 2 The investigation of colony-forming units (CFUs) and cell population doubling time (PDT). The CFUs were counted in keratocystic odontogenic tumor (KCOT) *vs* gingival fibroblasts. Significantly fewer CFUs developed from KCOT fibroblasts were detected (**a**). Growth curves of the KCOT and gingival fibroblasts were depicted (**b**). The PDT of KCOT fibroblasts was significantly longer than that of gingival fibroblasts (**c**). (*$P < 0.05$; **$P < 0.01$)

which was consistent with the results of $TRAP^+$ MNCs counting.

Co-culture of KCOT fibroblasts with Raw264.7 and the expression changes in genes related to osteoclastogenesis
Before the fibroblasts co-cultured with Raw264.7, the levels of IL-6, COX-2, and M-CSF mRNA in KCOT fibroblasts were significantly lower than that in gingival fibroblasts ($P = 0.001$, $P = 0.019$ and $P = 0.003$), but the difference of the VEGF mRNA level between the

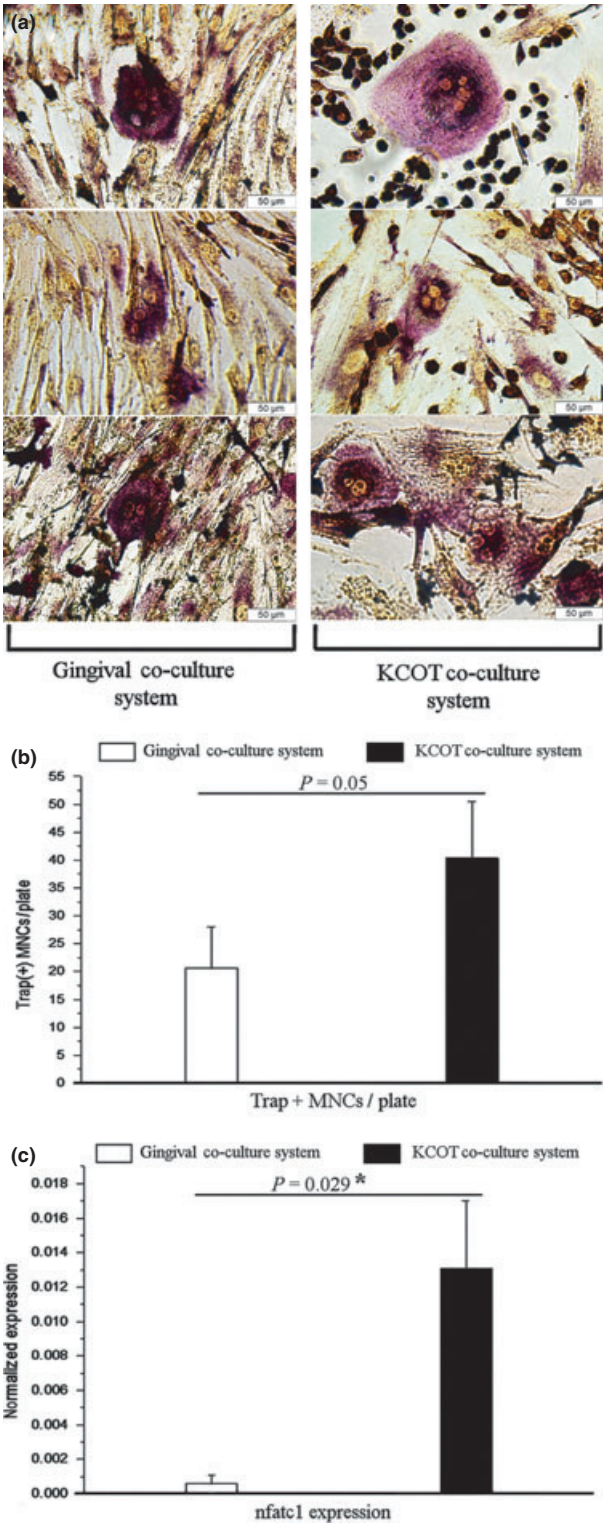

Figure 3 Effect of fibroblasts on osteoclastic differentiation of Raw264.7 cells. keratocystic odontogenic tumor (KCOT) or gingival fibroblasts were co-cultured with Raw264.7 cells for 10 days. Cells were stained with tartrate-resistant acid phosphatase (TRAP) (with original magnification ×200, scale bar: 50 μm) (**a**). At the 10th day of co-culturing, TRAP + MNCs were investigated in KCOT *vs* gingival co-culture systems. More TRAP + MNCs were detected in KCOT co-culture system (**b**). At the 3rd day of co-culturing, a significant higher expression of nfatc1 mRNA was detected in KCOT co-culture system (**c**). (*$P < 0.05$; **$P < 0.01$)

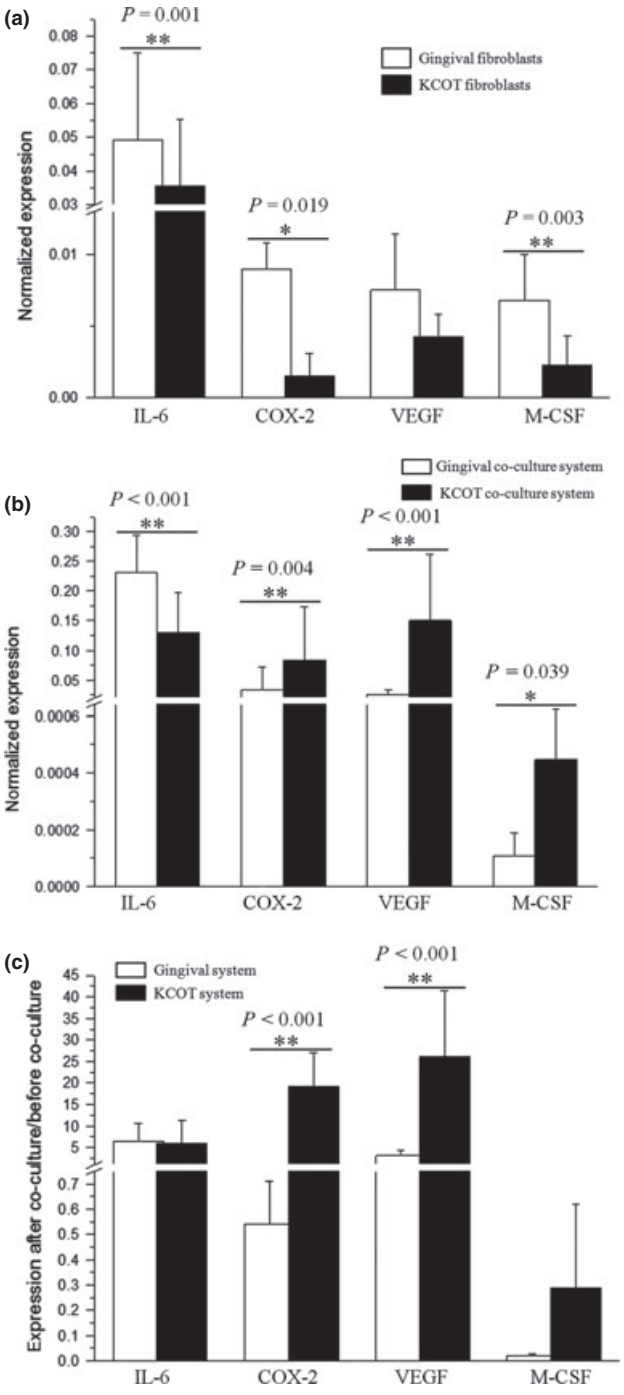

Figure 4 The expression changes in genes related to osteoclastogenesis in the co-culture systems. Before co-culturing with Raw264.7, the genes related to osteoclastogenesis were investigated in keratocystic odontogenic tumor (KCOT) *vs* gingival fibroblasts. The significantly lower IL6, COX-2, and M-CSF mRNA expression in KCOT fibroblasts were detected, while the difference of VEGF mRNA expression was not statistically significant (**a**). After co-culturing with Raw264.7 for 5 days, the same genes were investigated in KCOT *vs* gingival co-culture system. The significantly higher COX-2, VEGF, and M-CSF expressions, as well as the lower IL6 mRNA expression in KCOT co-culture system, were detected (**b**). The folds of the expression changes in these genes were calculated in KCOT *vs* gingival systems. The significantly greater expression increase in COX-2 and VEGF mRNA in KCOT system was detected, while the difference of the folds of decreased M-CSF and increased IL-6 mRNA was not statistically significant (**c**). (*:$P < 0.05$; **$P < 0.01$)

Table 2 The normalized expression of genes in fibroblasts before co-culturing

n = 9	*KCOT fibroblasts*	*Gingival fibroblasts*	P
IL-6	0.02784 ± 0.02187	0.07566 ± 0.02235	**0.001
VEGF	0.00342 ± 0.00296	0.00457 ± 0.00242	0.2
COX-2	0.000951769 ± 0.00194	0.00819 ± 0.01283	*0.019
M-CSF	0.00226 ± 0.00207	0.0068 ± 0.0032	**0.003

KCOT, keratocystic odontogenic tumor.
Including the fibroblasts used in co-culture systems later.
*$P < 0.05$; **$P < 0.01$.

Table 3 The normalized expression of genes in co-culture systems

n = 3	*KCOT co-culture system*	*Gingival co-culture system*	P
IL6	0.13048 ± 0.06691	0.23152 ± 0.06149	**<0.001
COX2	0.08384 ± 0.08978	0.03394 ± 0.03773	**0.004
VEGF	0.14988 ± 0.11146	0.02534 ± 0.00877	**<0.001
M-CSF	0.00045 ± 0.00018	0.00011 ± 0.00008	*0.039

KCOT, keratocystic odontogenic tumor.
*$P < 0.05$; **$P < 0.01$.

two types of fibroblasts was not statistically significant ($P = 0.2$). (Figure 4a, Table 2).

After being co-cultured with Raw264.7 for 5 days, the expression of IL6 mRNA in KCOT co-culture system was still significantly lower than that in gingival co-culture system ($P < 0.001$), but the expression of COX-2, VEGF, and M-CSF mRNA in KCOT co-culture system was significantly higher than that in gingival co-culture system ($P = 0.004$, $P < 0.001$ and $P = 0.039$) (Figure 4b, Table 3).

Overall, the expression of IL-6, COX-2, and VEGF mRNA increased in the co-culture systems, but the expression of M-CSF mRNA decreased after co-culture. The ratio of gene expression in co-culture system to that in fibroblasts before co-culturing was calculated, representing the folds or extent of the changes in genes expression in the co-culture system. In the KCOT co-culture system, the folds of the increase in COX-2 and VEGF mRNA expression were 19.25561 ± 7.72742 and 26.07758 ± 15.38089, respectively, and they were significantly higher than that in gingival co-culture (0.5417 ± 0.16973 and 3.07398 ± 1.32369) ($P < 0.001$). The folds of the changes in IL6 mRNA and M-CSF mRNA expression between the KCOT and gingival co-culture systems were not significantly different ($P = 0.256$ and $P = 0.237$) (Figure 4c).

Discussion

In this study, we cultured fibroblasts from KCOT fibrous capsule and gingival mucosa; both types of cells displayed spindle-shaped fibroblast-like appearance as described in previous studies (Motaln *et al*, 2010) (Figure 1a). By contrary to the consistent higher level of proliferative activity in the KCOT epithelia, KCOT fibroblasts showed lower colony-forming rate and longer PDT in comparison with gingival fibroblasts ($P < 0.05$, Figure 2). The counting of CFUs represents

the amount of MSCs, which have the capacity of self-renewal and multiple differentiation potential (Motaln *et al*, 2010). The relatively fewer MSCs within KCOT fibroblasts could explain the longer PDT and contribute to the fact that the fibrous capsules of KCOT are usually thin (Li, 2011). The lower level of proliferative capacity of KCOT fibroblasts is in sharp contrast to the consistent higher level of cell proliferation detected in its overlying epithelial cells (Li *et al*, 1994, 1995). The unique role of fibroblasts within the KCOT fibrous capsule as a special type of TAFs is yet to be explored by experiment involving co-culture of fibroblasts and epithelial cells, which is currently undertaken in our laboratory.

Recent studies have demonstrated that fibroblasts from various tissues participate in osteoclastogenesis. In rheumarthritis, cell–cell contact between fibroblasts and monocyte precursor was required for osteoclast formation (Sabokbar *et al*, 2005); conditioned medium from normal gingival and skin fibroblasts also contribute to osteoclasts formation (Costa-Rodrigues and Fernandes, 2011). In this study, we co-cultured osteoclast precursor Raw264.7 with fibroblasts from KCOT fibrous capsule and gingival mucosa. A significantly higher number of osteoclast-like cells were observed in KCOT co-culture system than in gingival co-culture system. This was also confirmed by the higher expression of osteoclast differentiation marker nfatc1 in the KCOT co-culture system. It is thus interesting to suggest that KCOT fibroblasts may be involved in the bone resorption process through interacting with precursor cells of osteoclasts and enhance the osteoclastogenesis.

To explore the mechanism of the effects of KCOT fibroblasts on osteoclastogenesis, we compare the mRNA levels of IL-6, VEGF, COX-2, and M-CSF of the two types of fibroblasts in two different culture systems. We found that the levels of IL6, COX-2, and VEGF mRNA were increased when co-cultured with Raw264.7 cells, but that of M-CSF was decreased. In the KCOT co-culture systems, the increase in COX-2 and VEGF expression was significantly greater than that in gingival co-culture system; therefore, although the COX-2 and VEGF mRNA were lower in KCOT fibroblasts than in gingival fibroblasts before co-culturing, both became higher in KCOT co-culture systems. It has been reported that the upregulation of COX-2 in KCOT fibroblasts could enhance PGE_2 secretion (Ogata *et al*, 2007), which promotes osteoclast formation and increases its activity (Liu *et al*, 2005). VEGF functions as M-CSF, and recombinant human VEGF could induce osteoclastogenesis of both Raw264.7 and human monocyte precursor (Aldridge *et al*, 2005). It is therefore interesting to speculate that increased COX-2 and VEGF in KCOT fibroblasts may play a part in the increased osteoclastogenesis of Raw264.7 in the co-culture system.

The higher expression of COX-2 and VEGF in the KCOT co-culture system perhaps could be attributed to cell–cell interaction. Previous studies demonstrated that cell–cell contact between fibroblasts and osteoclast precursor is important in osteoclastogenesis (Sabokbar *et al*, 2005); when periodontal ligament fibroblasts contacted with osteoclast precursor directly, M-CSF and TNF-α mRNA are enhanced in the co-culture system, while the indirect co-culture system did not alter the expression of these genes related to osteoclastogenesis and developed fewer osteoclast-like cells (Bloemen *et al*, 2010); in fact, conditioned media from periodontal ligament fibroblasts inhibited the differentiation of osteoclast precursor into TRAP-positive cells (Kanzaki *et al*, 2001). It is likely that there were similar cell–cell interactions between KCOT fibroblasts and Raw264.7 in the present study, and such interaction may be responsible for the increased osteoclastogenic effects of KCOT fibroblasts, possibly by increasing COX-2 and VEGF expression. Unlike the previous report on periodontal ligament fibroblasts, however, M-CSF expression was decreased when KCOT fibroblasts were co-cultured with Raw264.7, suggesting that different mechanisms underlying cell–cell interactions may exist in the fibrous capsule of KCOT.

In this study, we compared the *in vitro* growth characteristics of KCOT and gingival fibroblasts. KCOT fibroblasts showed a lower proliferative activity than gingival fibroblasts, which is in keeping with the fact that the fibrous capsule of KCOT is usually thin and acellular. Higher osteoclastogenic ability was detected in KCOT fibroblasts co-cultured with Raw264.7, suggesting that the cell–cell interaction in the co-culture system, possibly by increasing COX-2 and VEGF expression, could enhance osteoclastogenic effects of KCOT fibroblasts.

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Conflict of interest statement

None declared.

Author contribution

The first author Hai-cheng Wang has mainly contribute to study design, literature studies, experimental studies, data acquisition and analysis, manuscript preparation and editing etc. The corresponding author Tie-jun Li has mainly contribute to guarantee the integrity of the study, study design, define the intellectual content, manuscript editing and review etc.

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