The TGF-β1/COX-2-dependant pathway serves a key role in the generation of OKC-induced M2-polarized macrophage-like cells and angiogenesis

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Abstract. An odontogenic keratocyst (OKC) is a common oral cyst arising from the odontogenic epithelium, which has the characteristics of a tumor. Previous studies have demonstrated that M2-polarized macrophages and angiogenesis have important roles in the progression of OKCs. As transforming growth factor (TGF)-β1 is important in growth and developmental processes, and early studies have indicated that TGF-β1 is upregulated in OKCs, the present study aimed to investigate the expression levels of TGF-β1 as a first step. Flow cytometric analysis suggested that TGF-β1 induced M2-polarization of macrophages in a dose-dependent manner. Expression levels of cyclooxygenase (COX)-1 and -2 were measured after treatment of M2 macrophages with TGF-β1 and OKC homogenate supernatant. COX-2 expression was influenced by TGF-β1 in a concentration-dependent manner and in OKC induction. In addition, inhibition of COX-2 resulted in the induction of M2-polarization of macrophages via TGF-β1 and OKC disruption. Because the extracellular matrix (ECM) is altered in individuals with chronic diseases, the present study analyzed the expression of matrix metalloproteinase (MMP)-9, which is able to degrade the ECM. The present study observed a decrease in MMP-9 activity following treatment with TGF-β1 and OKC homogenate supernatant. Additionally, the present study analyzed tube formation caused by OKC with or without a COX-2 inhibitor. The results of the present study suggested that angiogenesis increased following treatment with OKC homogenate supernatant but decreased after treatment with a COX-2 inhibitor. These findings indicated that the TGF-β1/COX-2 pathway may have an important role in the progression of OKC.

Introduction

Odontogenic keratocyst (OKC) is a common oral cyst arising from the odontogenic epithelium, which has the characteristics of a tumor; however, the name OKC is controversial. Now in the World Health Organization classification, non-inflammatory odontogenic cyst head and neck tumors are termed OKCs (1); however, before 2017, they were termed keratinous cystic odontogenic tumors (2). An OKC grows aggressively with a high rate of recurrence, exhibiting the behavior of a malignant tumor; therefore, due to their locally destructive growth they may require radical surgery (3). Numerous pathways representing therapeutic targets may be useful for the treatment of an OKC. For example, mutations in protein patched homolog 1, a transmembrane protein in the hedgehog (HH) signaling pathway, have been demonstrated in OKC, and such mutations increase HH signaling activity that promotes the proliferation and neoplastic growth of the tumor (4). Other signaling pathways, such as epidermal growth factor receptor, Wnt and AKT signaling, have been reported to be abnormal in OKC (5).

An increasing number of studies have demonstrated that macrophages perform a central role in the development of tumors (6,7). The two subtypes of polarized macrophages are M1 (classical macrophage) and M2 (alternative macrophage); these macrophages promote tumor rejection or stimulate growth, respectively (8). Tumor-associated macrophages (TAMs) are present in abundance in the microenvironment of solid tumors, and are involved in tumor angiogenesis and metastasis (6). TAMs mostly have an M2-like phenotype (6,9). It has previously been demonstrated that TAMs are involved in the development of oral squamous cell carcinomas, as well as functions such as proliferation, angiogenesis and tumor cell invasion in the stroma of the lesion (10). The heterogeneity

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and plasticity of TAMs in the development of cancer suggests that the differentiation processes of TAMs may be targets for immunotherapy (11). Hence, knowledge of TAMs in tumors is important for their diagnosis and therapy.

Cyclooxygenase (COX)-2 is an inflammation-inducible enzyme that is upregulated in numerous types of carcinoma (12,13). COX-2 is able to promote carcinoma progression and metastasis, and reduce patient survival (14-16). In addition, COX-2 is generally absent or at very low levels in cells, but can be induced by pro-inflammatory and mitogenic stimuli (17,18). Transforming growth factor (TGF)-β is an important growth factor which performs various roles in biological growth and development (19). A previous study suggested that TGF-β1 may upregulate the expression of COX-2 in the early stages of human dental pulp inflammation (19); thus indicating that TGF-β1-induced COX-2 expression may serve an important role in human dental disease.

A previous study demonstrated that M2-polarized macrophages are present in OKCs where they promote tumor angiogenesis (20). The present study aimed to explore whether TGF-β1-induced COX-2 affects the development of OKC and angiogenesis, as well as the possible mechanisms of action.

Materials and methods

Preparation of tissue homogenate. A total of 14 OKC samples were collected by The Department of Stomatology, Zhejiang Provincial People's Hospital (Zhejiang, China) between March 2017 to July 2017. Patients with a history of tumors other than OKC were excluded. The patients comprised of 8 males and 6 females, with an average age of 32 years and age range, 21-48 years. This study was approved by the Ethics Committee of Zhejiang Provincial People's Hospital. All patients were informed of the purpose of this study and provided written informed consent.

Tissue homogenate was prepared as previously described (20). Fresh OKC tissues were collected and washed thoroughly. The samples were immediately placed in serum-free RPMI-1640 medium (Gibco; Thermo Fisher Scientific, Inc.). Samples were homogenized in a glass homogenizer, and were then transferred into tubes and centrifuged at 10,000 x g for 1 h at room temperature. Each supernatant was collected and stored at -80°C.

Cell culture. THP-1 cells were purchased from The Kunming Cell Bank, Chinese Academy of Sciences and human umbilical vein endothelial cells (HUVECs) were from Shanghai Fuxiang Biotechnology Co., Ltd. THP-1 cells and HUVECs were cultured in RPMI-1640 and high-glucose DMEM (Gibco; Thermo Fisher Scientific, Inc.), respectively. Both were supplemented with 10% FBS (Hyclone; GE Healthcare life Sciences) and 1% penicillin/streptomycin (Gibco; Thermo Fisher Scientific, Inc.). Cells were cultured at 37°C in an atmosphere containing 5% CO2.

Treatment of cells. THP-1 cells were cultured in 6-well plates then treated with 25 ng/ml phorbol-12-myristate-13-acetate (PMA, M2 macrophages inducer; Sigma-Aldrich; Merck KGaA) at 37°C. After 12 h, the culture medium was removed and OKC tissue supernatant was added, with or without niflumic acid (NA, COX inhibitor; Selleck Chemicals). Following THP-1 cell induction to M2 macrophages with PMA treatment, M2 macrophages were cultured with 1, 5 and 10 ng/ml concentrations of TGF-β1 with or without NA, 20 ng/ml interleukin (IL)-4 and 2 ng/ml IL-13, then polarized for 36 h. The cells were collected for flow cytometric analysis, reverse transcription-quantitative PCR (RT-qPCR) and western blot analysis. Collected medium was centrifuged at 500 x g for 20 min at room temperature and stored at -80°C.

Flow cytometry. After cells were treated, they were collected and washed in PBS three times. The cells were suspended in loading buffer (Sangon Biotech Co., Ltd.) to a density of 2.5x105 cells/ml, then incubated with a phycoerythrin (PE)-conjugated mouse anti-CD163 monoclonal antibody (1:100; cat. no. sc-20066; Santa Cruz Biotechnology) in the dark for 30 min at 37°C. After removal of the antibody solution, the cells were washed with PBS twice and then resuspended in loading buffer. A BD FACSCanto™ II flow cytometer (BD Biosciences) was used to detect surface markers of the cells. The results were analyzed by FlowJo software v.10.0.7 (FlowJo LLC).

RT-qPCR. Total RNA was extracted from cells using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.) in accordance with the manufacturer's protocol. First-strand cDNA was synthesized according to the manufacturer's instructions at 42°C for 60 min using a RevertAid First strand cDNA synthesis kit (Thermo Fisher Scientific, Inc.). cDNA and primers were mixed with SYBR® Green Master Mix (Starbiolab) and RT-qPCR was conducted on a Roche LightCycler® 480 system (Roche Diagnostics) by using the following thermocycling procedure: 10 min at 95°C, followed by 40 cycles of 20 sec at 95°C, 30 sec at 60°C and 30 sec at 72°C. The following primers were used: TGF-β, forward 5'-GGGACTATCCACCCCTGAAAGA-3', reverse 5'-CCTCTCTGCTGTAGTG-3'; COX-2, forward 5'-TGGGACTATCCACCCCTGAAAGA-3', reverse 5'-CTCTCTGCTGTAGTG-3'; GAPDH was used as the endogenous reference gene and gene expression was comparatively quantified using the 2-ΔΔCq method (21).

Western blotting. Cells were lysed in RIPA buffer (Beyotime Institute of Biotechnology) containing protease and phosphatase inhibitor cocktails (Thermo Fisher Scientific, Inc.). Protein concentrations in samples were quantified using a bicinchoninic acid assay kit (Beyotime Institute of Biotechnology). Equal quantities of protein (25 µg) were separated by SDS-PAGE on 10% gels. Proteins were then transferred onto polyvinylidene difluoride membranes (EMD Millipore). Membranes were blocked with 5% non-fat milk for 1 h at room temperature, then incubated with the appropriate primary antibodies overnight at 4°C. The following primary
antibodies were used to detect proteins: Rabbit polyclonal anti-COX-2 (1:500; cat. no. 12375-1-AP; Wuhan Sanying Biotechnology), rabbit polyclonal anti-TGF-β1 (1:500; cat. no. 21898-1-AP; Wuhan Sanying Biotechnology) and rabbit polyclonal anti-MMP-9 (1:500; cat. no. 27306-1-AP; Wuhan Sanying Biotechnology). Membranes were then washed with TBST and incubated with a horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG antibody or a HRP-conjugated goat anti-mouse IgG antibody (1:1,000, Beyotime Institute of Biotechnology), as appropriate, for 1 h at room temperature. Incubation with a mouse anti-GAPDH monoclonal antibody (1:1,000; cat. no. ab8245; Abcam) was used as a loading control. Blots were analyzed and proteins comparatively semi-quantified using a Clarity MAX Western ECL Substrate (Bio-Rad Laboratories, Inc.) in a ChemiDoc XRS+ system (Bio-Rad Laboratories, Inc.).

In vitro tube formation assay and immunofluorescence staining. A tube formation assay was performed to measure the effects of OKC supernatant on angiogenesis in vitro. A total of 100 µl Matrigel (BD Biosciences) was placed into the wells of a 48-well plate (Corning, Inc.) and then chilled at 4˚C overnight. The Matrigel was then polymerized at 37˚C for 30 min. M2 macrophages (20,000 cells/well) in combination were added to the Matrigel-coated wells. After 24 h at 37˚C, images of the tubular structures were captured using a light microscope (magnification, x100; Leica DM18 microscope, Leica Microsystems, Inc.) equipped with a Leica MC170 HD digital camera (Leica Microsystems, Inc.) and were analyzed using ImageJ software v.1.8.0 (National Institutes of Health).

Tubular structures were fixed in 4% paraformaldehyde (Sangon Biotech Co., Ltd.) for 30 min at room temperature, then washed in PBS (Sangon Biotech Co., Ltd.) three times. Slides were blocked with 10% goat serum (Beyotime Institute of Biotechnology) for 1 h at room temperature and then stained using a mouse anti-CD31 monoclonal antibody conjugated with FITC (1:100; cat. no. sc-376764; Santa Cruz Biotechnology, Inc.) overnight at 4˚C in the dark. The antibodies were removed and the slides were washed in PBS three times prior to staining the cell nuclei with DAPI for 15 min at room temperature in the dark. After washing three times in PBS, the images were captured using a fluorescent microscope and (magnification, x100; Leica Microsystems, Inc.) equipped with a Leica DFC450C digital camera (Leica Microsystems, Inc.).

MTT cell viability assay. A total of 5,000 cells/well were seeded in 96-well plates and various treatments (PMA, PMA+OKC, PMA+OKC+NA or PMA+IL4/13) were added, as appropriate. After 48 h at room temperature of incubation, 100 µl MTT solution (1 mg/ml) was added to the cells and incubated at 37˚C for 4 h. The MTT solution was aspirated and 150 µl isopropanol was added to solubilize the formazan crystals formed by cell metabolism, followed by gentle agitation for 15 min. Cell viability was calculated from relative absorbance of 595 nm light using a Cytation 3 Multi-Mode plate reader (BioTek Instruments, Inc.).

Statistical analysis. Each experiment was repeated three times. Data are presented as the mean ± standard deviation. Data were compared using a two-tailed Student's t-test (unpaired) or one-way analysis of variance with a Tukey’s multiple comparison test. Data were presented graphically using GraphPad Prism 6 (GraphPad Software). P<0.05 was considered to indicate a statistically significant difference.

Results

TGF-β1 is upregulated in M2-polarized macrophages treated with OKC homogenate supernatant. In this study, RT-qPCR and western blotting were used to find that TGF-1 in M2-polarized macrophages was upregulated when treated with OKC homogenate supernatant. IL-4/13 promotes M2-polarization of macrophages; therefore, these two factors were used to induce M2 macrophages as a positive control. The mRNA and protein expression levels of TGF-β1 were clearly upregulated in M2-polarized macrophages induced by treatment with a homogenate supernatant of OKC tissues compared with the control group (PMA only) (Fig. 1). These findings suggested that OKC increased the expression of TGF-β1.
TGF-β1 induces M2-polarization of macrophages. Multiple markers are differentially expressed on the surface of different macrophages. For example, CD206, CD163 and CD301 are M2-specific markers (22). In the present study, CD163+ was used to characterize M2-polarization of macrophages using flow cytometry.
The results demonstrated that M2-polarization of macrophages was induced by treatment with OKC homogenate supernatant, IL-4/13 and TGF-β1 (Fig. 2). In addition, the proportion of M2-polarization increased with TGF-β1 in a concentration-dependent manner. These results indicated that TGF-β1 induced M2-polarization of macrophage-like cells in vitro.
COX-2 is upregulated in TGF-β1- and OKC homogenate supernatant-induced M2-polarized macrophages. COX exists as two isoforms: COX-1 and COX-2. COX-1 is universally expressed in high concentrations, whereas COX-2 is expressed in low concentrations but can be induced by multiple stimuli (19,23). In the present study, the expression levels of the two genes were quantified in response to different treatments. TGF-β1 induced a significant increase in COX-1 mRNA expression levels, except for the highest concentration (Fig. 3A), compared with the levels in the PMA-only group. Conversely, the mRNA expression levels of COX-2 were significantly upregulated in a concentration-dependent manner by TGF-β1 (Fig. 3B). In addition, following treatment with OKC homogenate supernatant, the mRNA and protein expression levels of COX-1 and COX-2 were significantly upregulated compared with those in the PMA-only group (Fig. 3C and D). In subsequent experiments, 10 ng/ml TGF-β1 was selected and western blot analysis was conducted to evaluate the protein expression levels of COX-2. Treatment with TGF-β1 or OKC homogenate supernatant significantly increased the protein expression levels of COX-2 compared with those in the PMA-only group (Fig. 3E). These results suggested that COX-2 can be induced by both OKC and TGF-β1, in particular in high doses.

Inhibition of COX-2 influences M2-polarization of macrophages induced by OKC. NA is an inhibitor of COX-2 (24), and can inhibit cancer cell proliferation and migration (25). After treatment with TGF-β1 combined with NA, or OKC homogenate supernatant combined with NA, the protein expression levels of COX-2 were significantly downregulated compared with those in the PMA-only group (Fig. 4C), indicating that NA effectively inhibited COX-2 in cells. The mRNA expression levels of TGF-β1 were significantly upregulated following treatment with TGF-β1, but the protein expression levels were not significantly different compared with those in the PMA-only group (Fig. 4B and D). Following treatment with NA, TGF-β1 was downregulated compared with the PMA-only group (Fig. 4D), suggesting that TGF-β1 was affected when COX-2 was inhibited. Flow cytometry was then used to analyze whether M2-polarized macrophages were affected by NA. These results demonstrated that NA reduced the M2-polarization of macrophages by OKC homogenate supernatant and TGF-β1 (from 51.6 to 25.2 and 62.4 to 34.8%, respectively) (Fig. 4E).

MMP-9 levels in cells treated with TGF-β1 and OKC homogenate supernatant with or without NA. Regulation of the ECM by TGF-β1 is altered in a number of chronic diseases (26) and MMP serves a significant role in ECM degradation; therefore, MMP-9 expression levels were measured to determine whether the ECM was affected as a result of different treatments. The results demonstrated that compared with in the PMA-only group, the expression levels of MMP-9 were significantly downregulated in all groups except for the mRNA levels after OKC homogenate supernatant treatment (Fig. 5). In addition, MMP-9 expression levels were markedly downregulated following treatment with NA. These results indicated that MMP-9 expression may be altered following treatment with TGF-β1 and OKC, especially when COX-2 is inhibited.

OKC promotes angiogenesis, as determined by measuring microvessel density (MVD). A previous study has indicated that angiogenesis is observed in OKC tissue (20); therefore, the ability of OKC to induce angiogenesis was examined. The present study performed a tube formation assay using HUVECs in vitro. The results demonstrated that M2-polarization of macrophage-like cells induced by OKC enhanced angiogenesis compared with in the PMA-only group (Fig. 6A and B). After NA was added to the PMA + OKC group to observe the effects of NA on angiogenesis caused by M2-polarized macrophage-like cells, the effect was inhibited (Fig. 6A and B). In addition, analysis of HUVEC viability, as measured by the MTT assay, demonstrated that M2-polarized macrophage-like cells induced by OKC homogenate supernatant promoted HUVEC viability, whereas this was inhibited by NA treatment (Fig. S1). These data indicated that inhibition of COX-2 prevents the angiogenesis caused by OKC-induced M2-polarized macrophage-like cells.
MVD can be used as a useful marker for evaluation of angiogenesis in tumors. In this assay, CD31 represents the MVD marker (27). Immunofluorescence staining was performed to measure MVD in capillary-like structures. The results demonstrated that CD31 was highly expressed in the PMA + OKC and PMA + IL-4/13 groups, but not expressed in the PMA + OKC + NA group (Fig. 6C). These results suggested that OKC may increase angiogenesis and enhance tumor cell invasion. Additionally, the effects of OKC on tumor development may be dependent on the regulation of COX-2.

Discussion

OKC is a common oral disease arising from the odontogenic epithelium, which has characteristics similar to those of malignant tumors such as aggressive growth and high recurrence (28). Tumor development always requires angiogenesis and the expression of numerous cytokines; OKC also has this characteristic (29-31). The aim of the present study was to elucidate the regulation of OKC, its mechanism of action and disease progression.

In this study, THP-1 cells were used as a model of macrophages (32-34). However, THP-1 cells are derived from the peripheral blood of a patient with acute monocytic leukemia. Therefore, the results of the present study will be further verified using normal tissue-derived monocytes collected from patients in future studies. The existence of M2-polarized macrophages in OKC was first reported by Zhong et al (20), who also reported that TGF-β1 was highly expressed in OKC homogenate supernatant; therefore, to investigate the effects of TGF-β1 on OKC, RT-qPCR and western blotting were conducted to confirm its upregulation in the present study. At present, studies have reported that the rise of TGF-β1 is often associated with tumor development (35,36), thus the OKC cancerous characteristics may be related to TGF-β1. Accordingly, the mechanism by which TGF-β1 increases and how to inhibit this pathway will be explored in future studies. The present study also explored the relationships between TGF-β1 and M2-polarized macrophages. Flow cytometry measured the percentage of M2-polarized macrophages induced by different concentrations of TGF-β1. The results demonstrated that TGF-β1 induced M2-polarization of macrophages in a concentration-dependent manner. Since a number of studies have demonstrated that COX-2 can be induced by TGF-β1 (19,37), the present study aimed to determine whether the TGF-β1/COX-2 pathway also had a role in OKC. The results

Figure 6. M2-polarized macrophage-like cells enhance angiogenesis by promoting MVD. (A and B) Tube formation assay imaged after 4 h treatment and analyzed using ImageJ. (C) Immunofluorescence staining was performed to analyze MVD and CD31 location after treatment. The location of CD31 is demonstrated by the white arrows. PMA only was used as the control group. Scale bar, 500 μm. Data are presented as the mean ± SEM. **P<0.01, ***P<0.001. MVD, microvessel density; PMA, phorbol-12-myristate-13-acetate; OKC, odontogenic keratocyst; NA, niflumic acid; IL, interleukin; No., number.
of the present study indicated that COX-2 was upregulated by TGF-β1 in a concentration-dependent manner and that a high expression was observed in OKC-induced M2-polarization of macrophages. Unfortunately, the specific molecular mechanism was not determined in this experiment, and this will be studied further in future experiments. Taken together, these results indicated that the TGF-β1/COX-2 pathway may have a role in OKC.

The present study also inhibited COX-2 expression through the addition of NA and observed that the number of M2-polarized macrophages decreased when treated with NA. In addition, the expression of MMP-9, a protease with an active role in ECM remodeling, was downregulated in OKC-induced M2-polarized macrophages. These results suggested that the TGF-β1/COX-2 pathway not only affects macrophage polarization, but also the ECM of cells in OKC progression.

The present study also performed a tube formation assay with immunofluorescence staining. The results of the present study indicated that OKC induced capillary-like structures and markers of MVD were demonstrated in these structures; however, when COX-2 was inhibited by NA, these structures were destroyed. These results suggested that OKC enhanced angiogenesis and tumor development, and that this effect may be dependent on COX-2.

In summary, the present study demonstrated that TGF-β1/COX-2 may serve an important role in OKC-induced M2-polarization of macrophages and angiogenesis, suggesting that the TGF-β1/COX-2 pathway may regulate OKC progression.

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Availability of data and materials
The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions
GC and SW designed the research study. JG and LW performed the experiments. YD, QW and QBW analyzed the data. JX performed the flow cytometry experiments. GC wrote the manuscript. All authors have read and approved the manuscript.

Ethics approval and consent to participate
Not applicable.

Competing interests
The authors declare that they have no competing interests.

References


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