

Role of Tumor-Associated Macrophages on Cathepsin-B, Cathepsin-D, MMP-2, and MMP-9 in HSC-3 Oral Cancer Cells

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Abstract

This study was aimed to investigate the role of macrophages on an expression of cathepsin B and D (CTSB and CTSD) and the production of matrix metalloproteinase 2 and 9 (MMP-2 and 9) in lymph node metastatic human oral cancer, HSC-3 cell line. Human monocytic cell line (THP-1) was differentiated into 3 types of macrophages; M0, M1 and M2 macrophage using phorbol 12-myristate 13-acetate (PMA), Porphyromonas gingivalis lipopolysaccharide (Pg LPS) and interleukin-4 (IL-4), respectively. Total RNA was extracted from THP-1 and macrophages to detect the expression of CD14, CD68 and CD206 by real-time PCR. The non-contact co-culture of HSC-3 with conditioned media (CM) from different phenotype of macrophages was performed. After 24 hours, the expression of CTSB and CTSD in HSC-3 was investigated using real-time PCR. At 48 hours of co-culture, MMP-2 and MMP-9 activities were detected using gelatin zymography. We successfully converted THP-1 into M0, M1 and M2 macrophages. Co-culture of HSC-3 with CM from M2 macrophage significantly elevated the expressions of CTSB and CTSD ($p < 0.05$). Co-culture of HSC-3 with CM from M2 macrophages also increased MMP-9 activity ($p < 0.05$) but not MMP-2 activity was found no difference. Hence, M2 macrophages could elevate the gene expression of CTSB and CTSD and the production of MMP-9 in HSC-3, and may play role in invasion, and metastasis of oral cancers.

Experimental article (J Int Dent Med Res 2021; 14(1): 156-162)

Keywords: Macrophage, oral squamous cell carcinoma, cancer progression.

Received date: 20 December 2020

Accept date: 20 January 2021

Introduction

Oral squamous cell carcinoma (OSCC) is the most common (more than 90%) malignant epithelial neoplasm affecting the oral cavity. The percentage of 5-year survival for patients with OSCC varies from 40-50%. There is a relative higher frequency of comorbidities among the patients with OSCC when comparing with other cancers. The most common comorbidities associated with oral cancer are diabetes mellitus and hypertension.¹ Macrophages are innate immune cells that play a role in the host defense and the maintenance of tissue homeostasis. They originate from circulating bone marrow-derived monocytic precursors. These precursor

cells extravasate into target tissues where they differentiate into mature macrophages and polarize into diverse subsets that have different responses to microenvironmental challenges.²

In general, macrophages have been classified into two subsets: the M1 and the M2 macrophages. The M1 macrophages (inflammatory macrophages) serve as a critical cellular component involved in the inflammatory response and anti-tumor immunity. On the other hand, the M2 macrophages exert anti-inflammatory and pro-tumorigenic activities.³ Within the tumor, macrophages are a major stromal component, where they are commonly termed "tumor associated macrophages" (TAMs). TAMs are derived from circulating monocytes or tissue-resident macrophages. The mobilization of macrophage into tumor tissues is regulated by multiple microenvironmental such as cytokines, chemokines, extracellular matrix (ECM) components, and hypoxia.⁴

TAMs generally have M2-like phenotype. However, the specific phenotype of TAMs actually depends on the tumor progression stage.

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In the regression stages of tumors, TAMs adopt the M1-like phenotype for the anti-tumor immunity. In contrast, in progression stage of tumor, TAMs shift to a M2-like stage to enhance tumor angiogenesis, immunosuppression, and tumor progression.⁵ The polarization of macrophages is regulated by hypoxia and immunosuppressive factors derived from tumor and stromal cells.⁶ The previous study revealed that in primary oral squamous cell carcinomas with lymphogenic metastasis showed increasing macrophages infiltration and an increasing M2 macrophage.⁷

Cathepsins are lysosomal proteases and can be divided into different families. Each cathepsin family performs different function in tumorigenic processes.⁸ Cathepsin B (CTSB) is a lysosomal cysteine protease which functions as an endopeptidase and an exopeptidase. CTSB can degrade the basement membrane and ECM components, allowing invasion of tumor cells.⁹ The evidence showed that a decrease in CTSB levels in vitro leads to reduction in migration and invasion of tumor cells.¹⁰ Cathepsin D (CTSD) is an aspartic protease which can be found in eccrine sweat, extracellular matrix and synovial fluid cartilage.⁸ Elevation of CTSD level leads to an increase in fibroblast motility and invasion as well as an increase in micro-vessel density.¹¹ Also, CTSD is suggested to stimulate early phases of tumor progression.¹² Most importantly, CTSD is associated with the local recurrence and metastasis formation of cancer.¹³ There is a correlation between the expression of CTSB and CTSD.¹⁴

Matrix metalloproteinases (MMPs) are a large group of secretory proteases that are involved in normal physiological and pathologic processes such as embryogenesis, wound healing, angiogenesis, tissue remodeling, tumor invasion, and metastasis. Matrix metalloproteinase 2 and 9 (MMP-2 and 9), are believed to play an important role in head and neck cancer progression.¹⁵

During inflammation and injury, high level of MMP expression leads to tumor growth, invasion, migration and angiogenesis.¹⁶ The purpose of this study was to investigate the role of macrophages in expression of CTSB and CTSD and production of MMP-2 and MMP-9 in lymph node metastatic HSC-3 human oral cancer cell lines.

Materials and methods

Cell cultures

The human monocytic cell line, THP-1 (ATCC[®] TIB-202[™], Manassas, VA, USA) were cultured in Roswell Park Memorial Institute 1640 (RPMI 1640) medium (Gibco, Life Technologies, Grand island, NY, USA) supplemented with 1% penicillin 100 IU/ml (Capricorn Scientific, Ebsdorfergrund, Germany), streptomycin 100 µg/ml (Capricorn Scientific), 1% amphotericin B (Capricorn Scientific) and 10% fetal bovine serum (Biowest, Nuaille, France) in a 37°C humidified 5% CO₂ atmosphere.

The human OSCC HSC-3 cells were obtained from the Japanese cancer research resource bank (Tokyo, Japan). They were established from a metastatic tumor found in a cervical lymph node of a 64-year-old man with poorly differentiated carcinoma of the tongue. Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Gibco) containing 1% penicillin 100 IU/ml, streptomycin 100 µg/ml, 1% amphotericin B and 10% fetal bovine serum in a 37°C humidified and 5% CO₂ incubator.

Conversion of monocyte to M0, M1 and M2 macrophages

THP-1 cells (10⁶ cells) were cultured in 6-well plates and differentiated toward M0 macrophage by treating with 12.5 ng/ml phorbol myristate acetate (PMA, Sigma, Saint Louis, MO, USA) for 48 hours, in supplemented RPMI 1640, 37°C humidified and 5% CO₂ incubator.

To drive M0 into M1 phenotype, M0 were starved with 1% Fetal bovine serum supplemented RPMI 1640, 37°C humidified and 5% CO₂ incubator. After 16 hours starvation, M0 cells were treated with 1 µg/mL *Pg* LPS, 37°C for additional 24 hours.

To drive THP-1 into M2 phenotype, THP-1 was first driven into M0 phenotype. Then 20 ng/ml recombinant human interleukin 4 (IL-4) (Peprotech Asia, Rehovot, Israel) was add to M0 macrophage for another 36 hour. M0, M1, M2 macrophages conditioned media were used to co-culture with HSC-3 cancer cells.

Confirmation of M0, M1 and M2 macrophage differentiation

CD14, CD68 and CD208 surface markers mRNA expression levels were measured to confirm M0, M1 and M2 macrophages

differentiation using real-time PCR. Total RNA was isolated from THP-1, M0, M1, and M2 macrophages using Total RNA Mini Kit (Blood/cultured cell, Geneaid Biotech Ltd., New Taipei city, Taiwan). The quantity and purity of the RNA was measured by NanoDrop (ND-2000 spectrophotometer, Thermo Fisher Scientific, Wilmington, DE, USA). A PrimeScript 1st strand cDNA Synthesis Kit (Takara Bio, Inc., Otsu, Japan) and a KAPA SYBR® FAST qPCR Kit Master Mix (Kapa Biosystems, Wilmington, MA, USA) were used for reverse transcription and real-time PCR detection, respectively, according to the manufacturer's instructions. The forward and reverse primer sequences were listed in table 1.

Gene	Primer sequence (5'/3')	Amplicon size (base pairs)
GAPDH	(F) ATCACCATCTTCCAGGAG	318
	(R) ATCGACTGTGGTCATGAG	
CD14	(F) GACCTAAAGATAACCGGCACC	161
	(R) GCAATGCTCAGTACCTTGAGG	
CD68	(F) CTTCTCTCATTCCCTATGGACA	105
	(R) GAAGGACACATTGTACTIONACC	
CD206	(F) GGGTTGCTATCACTCTCTATGC	126
	(R) TTTCTTGCTCTGTTGCCGTAGTT	
CSTB	(F) AGAGTTATGTTTACCGAGGACCT	159
	(R) GATGCAGATCCGGTCAGAGA	
CSTD	(F) TGCTCAAGAACTACATGGACGC	81
	(R) CGAAGACGACTGTGAAGCACT	

Table 1. Target and housekeeping genes. Forward (F) and reverse (R) primer sequences used in the real-time polymerase chain reaction (PCR) GAPDH = glyceraldehyde-3-phosphate dehydrogenase; CSTB = cathepsin B; CSTD = cathepsin D.

Non-contact co-culture M0, M1, and M2 macrophages with HSC-3

HSC-3 cells (10⁶ cells) were cultured in 6-well plates and incubated at 37°C humidified and 5% CO₂ incubator for 24 hours. After incubation, the wells were washed with PBS and then M0, M1, M2 macrophages conditioned media were used to co-culture with HSC-3 cancer cells in a proportion 1:1 (macrophage conditioned media:fresh DMEM without fetal bovine serum). After 24 hours, the HSC-3 cells were collected for the detection of CSTB and CSTD mRNA expression levels. After 48 hours, the supernatant was collected and centrifuged at 1,000 g and 4°C for 10 min and stored at -80°C until used for gelatin zymography.

Detection of cathepsin B and cathepsin D

After 24 hours of non-contact co-culture M0, M1, and M2 macrophages with HSC-3, CTSB and CSTD mRNA expression levels were detected using real-time PCR. Total RNA was isolated from each HSC-3 group using Total RNA Mini Kit. The quantity and purity of the RNA was measured by NanoDrop. A PrimeScript 1st strand cDNA Synthesis Kit and a KAPA SYBR® FAST qPCR Kit Master Mix were used for reverse transcription and real-time PCR detection, respectively, according to the manufacturer's instructions. The forward and reverse primer sequences were listed in table 1.

Gelatin zymography

Total protein in the CM was estimated using the Pierce™ BCA protein assay kit (Thermo Fisher Scientific, Waltham, MA, USA). After adjustment protein concentration of all supernatant collected from cancer cell culture, gelatin zymography was used for detection of MMP-2, MMP-9 activities as previously described.¹⁷ Briefly, gelatin (bloom 300, Sigma) was added to a 10% acrylamide separating gel at a final concentration of 0.2%. Samples containing equal amounts of total protein were mixed with non-reducing sample buffer and added to the gel. Following electrophoresis, the gels were washed in 2.5% Triton X-100 for 30 min at 37°C. The gels were incubated at 37°C overnight in developing buffer. The gels were stained with 0.5% Coomassie blue G250 in a 30% methanol and 10% glacial acetic acid solution for 30 min and destained in the same solution without Coomassie blue. The gelatin-degrading enzymes were identified as clear bands against the blue background of the stained gel. Images of the stained gels were captured under illumination using a G:BOX gel documentation system (Syngene, Frederick, MD, USA). The gelatinolytic bands were quantified using GeneTools software (Syngene). Conditioned media collected from HT1080 fibrosarcoma cell line was used as a positive control.

Statistical analysis

Data were analyzed using GraphPad Prism 6.0 (GraphPad software, La Jolla, CA, USA). The results are expressed as means and standard error of the mean (mean±SEM) of three separate experiments (n=3). Statistical analysis was performed by one-way ANOVA followed by

Tukey's multiple comparisons test. The level of significance was set at 0.05.

Results

Expression of surface marker in M0, M1 and M2 macrophages

In order to differentiate THP-1 to M0 macrophages, firstly, THP-1 was treated with PMA for 48 hours. This resulted in significantly increased expression of CD14 which is a surface marker of M0 macrophages when compared to that of THP-1 ($p < 0.05$) (Figure 1A). Then, *Pg* LPS, and IL-4 were added to differentiate M0 macrophages into M1, and M2 macrophages, respectively. The gene expression of CD68 which is a surface marker of M1 and M2 macrophages were significantly increased in THP-1+PMA+LPS and THP-1+PMA+IL-4 groups as shown in figure 1B ($p < 0.05$). While, the expression of CD206, which is a surface marker for M2 macrophages, were significantly increased in THP-1+PMA+IL-4 group, as shown in figure 1C ($p < 0.05$).

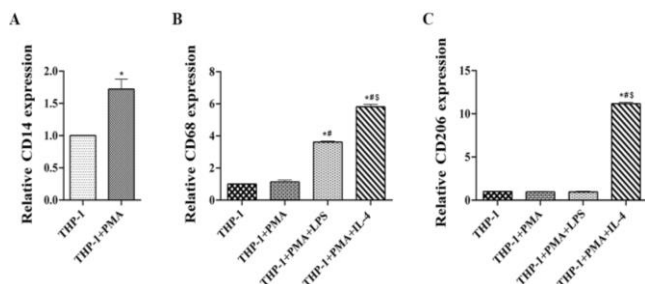


Figure 1. Gene expression of (A) CD 14, (B) CD 68, and (C) CD 206. Gene expression was determined by real-time polymerase chain reaction (PCR). Ratios of target genes were normalized to the housekeeping gene (GAPDH). Relative expression levels represent fold changes. Bars represent means±SEM (n=3). * indicates a significant difference compared with THP-1, # indicates a significant difference compared with THP-1 treated with PMA, and \$ indicates a significant difference compared with THP-1 treated with PMA and LPS ($p < 0.05$).

Expression of cathepsin B and cathepsin D in HSC-3 cancer cell co-cultured with macrophage conditioned media

Co-cultured of HSC-3 with conditioned media from M2 macrophages significantly increased the expression of CTSD compared with HSC-3, HSC-3+M0, and HSC-3+M1, respectively ($p < 0.05$) (Figure 2A). Furthermore,

co-cultured of HSC-3 with conditioned media from M2 macrophage also significantly increased expression of CTSD compared with HSC-3, HSC-3+M0, and HSC-3+M1, respectively ($p < 0.05$) (Figure 2B).

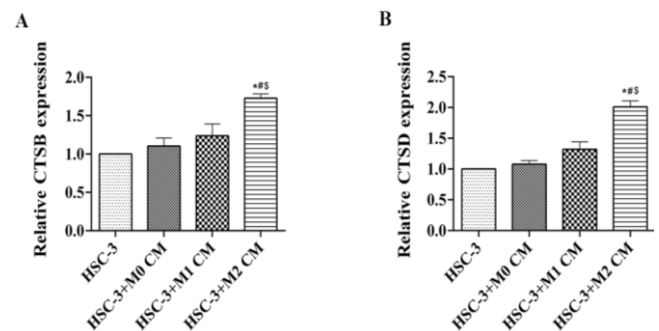


Figure 2. Gene expression of (A) CTSD, and (B) CTSD of HSC-3 when co-cultured with different phenotype of macrophages at 24 hours. Gene expression was determined by real-time polymerase chain reaction (PCR). Ratios of target genes were normalized to the housekeeping gene (GAPDH). Relative expression levels represent fold changes. Bars represent means±SEM (n=3). * indicates a significant difference compared with HSC-3 (control), and # indicates a significant difference compared with HSC-3 + M0 conditioned media, and \$ indicates a significant difference compared with HSC-3 + M1 condition media ($p < 0.05$).

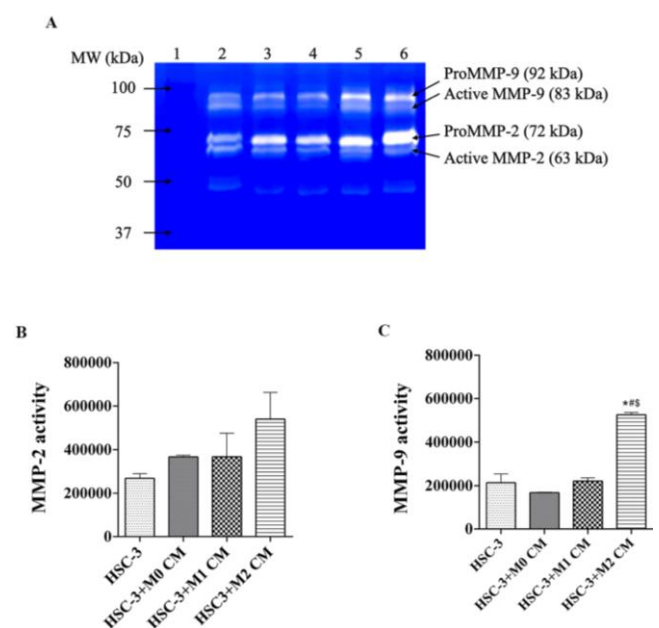


Figure 3. MMP-2 and MMP-9 activity. The HSC-3 cell lines were co-cultured with different phenotype of macrophages for 48 hours. The MMP activity in the different groups of co-culture

(A) were detected using zymography. Lane 1 = marker; lane 2 = HSC-3; lane 3 = HSC-3 + M0 conditioned media; lane 4 = HSC-3 + M1 conditioned media; lane 5 = HSC-3 + M2 conditioned media and lane 6 = HT1080 conditioned media (positive control). GeneTools software was used to quantify the gelatinolytic bands of (B) MMP-2 and (C) MMP-9 activity. Bars represent means \pm SEM (n=3). * indicates a significant difference compared with HSC-3 (control), # indicates a significant difference compared with HSC-3 + M0 conditioned media, and \$ indicates a significant difference compared with HSC-3 + M1 conditioned media ($p < 0.05$).

MMPs activities in HSC-3 cancer cell co-cultured with macrophage conditioned media

HSC-3 cells were co-cultured with conditioned media from M0, M1 and M2 for 48 hours. MMP-2 and MMP-9 activities of both cancer cell lines were detected by gelatin zymography. Gelatin zymography demonstrated 4 bands of MMPs activities with different molecular weights, including 92 (proMMP-9), 83 (active MMP-9), 72 (proMMP-2) and 63 kDa (active MMP-2), respectively, as shown in figure 3A. The MMP-2 activity in co-cultured of HSC-3 with conditioned media from M0, M1, M2 macrophages at 48 hours showed no statistically significant difference when compared with control ($p > 0.05$) as shown in figure 3A. Interestingly, co-cultured of HSC-3 with conditioned media from M2 macrophages showed a significant increase in MMP-9 activity at 48 hours when compared with control ($p < 0.05$), as shown in figure 3B.

Discussion

Cancer is one of the leading causes of death. Early detection of the oral mucosal changes plays a crucial role for the suspicion of the malignancy. Advanced stages of the disease have ultimately associated with poor prognosis.¹⁸ Currently, many studies have revealed that not only tumor cell itself, but also the tumor microenvironment plays a role in growth and survival of cancer cells. Immune cells which can be found in tumor microenvironment, can secrete several cytokines, chemokines, growth factors and enzymes. This microenvironment can promote the progression of cancer. The

prominent leukocyte immune cells that migrate to tumor microenvironment is macrophages.¹⁹ Macrophages have functional plasticity and can change their functional profiles repeatedly in response to environmental changes. When macrophages are exposed to lipopolysaccharides (LPS), they are polarized to M1 macrophages and have anti-tumor activities. When they are exposed to Th2 cytokines, such as IL-4 and IL-13, they are polarized to M2 macrophages and support tumor growth.²⁰ M2 macrophages can promote tumor progression, whereas M1-polarized macrophages have tumor-killing effect.²¹

In the previous study, M1 macrophages were differentiated using PMA and *Pg* LPS.²² Whereas M2 macrophages were differentiated using PMA and IL-4.²³ Therefore, the present study confirmed that the system of M1 and M2 macrophage differentiation in both studies were valid. Furthermore, this study demonstrated that co-cultured of oral cancer cells with conditioned media from macrophages could alter the behaviors of human HSC-3 oral cancer cell lines. Those behaviors may lead to an increase in invasive, and metastatic abilities of oral cancers.

The previous study has reported that expression of CTSD was correlated with CTSD in oral cancer tissue¹⁴ which was consistent to our study. This study demonstrated that CTSD and CTSD expressions were both increased in HSC-3, when exposed to M2 macrophage conditioned media. CTSD is shown to have both direct and indirect effects on tumor metastasis by regulating motility and invasion, dissemination, degradation of extracellular matrix (ECM), and angiogenesis of cancer cells.⁹ Besides, CTSD can activate other proteases including MMP and urokinase plasminogen activators which further facilitate formation of tumor microenvironment.¹⁰ CTSD is involved in mediating of motility and invasion, dissemination, and angiogenesis.¹¹ Intense expression of CTSD maybe a marker for invasive potential and aggressive behavior in high-grade carcinomas.²⁴ Among patients with positive lymph nodes, those with CTSD immunopositive tumor cells were at higher risk of relapsing.²⁵ Elevated expression of CTSD and CTSD may increase the aggressiveness and recurrence of oral cancer cells.

MMP-2 and MMP-9 activities are crucial in tumor invasion. Several studies have reported the expression and role of MMP-2 and MMP-9 in

head and neck cancer cell aggressiveness.^{26,27}

The M2 macrophage conditioned media also increased the MMP-9 activity, but not MMP-2 of HSC-3. This result was consistent with previous study that co-cultured of M2 macrophages with basal cell carcinoma induced COX-2-dependent production of MMP-9 but not MMP-2.²⁸ MMP-9 production in head and neck cancer cell is induced through various signaling pathways, including epidermal growth factor receptor (EGFR), mitogen-activated kinase (MAPK), and PI3K/Akt.^{27, 29} According to a previous study, MMPs, mainly MMP-9, played a role in the releasing of vascular endothelial growth factor (VEGF) in ovarian cancer.³⁰ It might be able to imply that high level of MMP-9 activity found in our study might induce VEGF secretion in oral cancer cells, which is essential for the growth and metastasis of solid tumor.³¹ However, the mechanism of M2 macrophage in promoting oral cancer cell aggressiveness require further investigation.

Conclusions

In the present study, THP-1 human monocytic cell line was successfully converted into M0, M1 and M2 macrophages. Co-cultured of lymph node metastatic, HSC-3 human oral cancer cell lines with conditioned media from M2 macrophages could elevate the expression of CTSB and CTSD when co-cultured with HSC-3. M2 macrophages could also induce MMP-9 activity in HSC-3 oral cancer cell lines. Taken together, it was concluded that M2 macrophages played crucial roles in the invasiveness, metastasis, and aggressive behaviors of oral cancer cells.

Acknowledgements

This work was supported by young scientist research funding, Thammasat University (2017). The authors would like to thank Assoc. Prof. Dr. Kusumawadee Utispan for the valuable suggestions, Dr. Paopanga Kokilakanit, Miss Pawinee Sirisawat, Miss Pitchaporn Khamphaeng, Miss Porntip Tansanthon, Miss Suchanya Wongnokkaewtong, Miss Virada Kanchanapun, and Mr. Worapong Teanchai for technical support. The English editing of this manuscript was kindly performed by Dr. Ahmed A. Abbas, Faculty of Dentistry, Thammasat University.

Declaration of Interest

The authors declare no conflict of interest relevant to this article.

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