纤维母细胞高表达 VEGF-C 在头颈部肿瘤侵袭转移中的作用

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摘要： 目的：研究纤维母细胞中血管内皮生长因子 C（vascular endothelial growth factor-C，VEGF-C）高表达与头颈部鳞癌肿瘤的生长与转移的关系。方法：将带 Luciferase 基因的质粒转染到头颈部肿瘤细胞；带 VEGF-C 的质粒转染到成纤维 3T3 细胞，通过体内外实验，探索 VEGF-C 在头颈部鳞癌肿瘤的生长与转移中发挥的作用及其可能机制。结果：Western blot 与 ELISA 实验共同证明 VEGF-C 基因已成功转染到上述头颈部肿瘤细胞中。细胞划痕实验验证了 VEGF-C 能够增强头颈部肿瘤细胞的侵袭、转移能力。体内成瘤实验观察到了两组大鼠体内肿瘤大小存在显著性差别，处死取出肿瘤后，肿瘤体积的差异性在统计学上具有显著性（P＜0.05）。结论：VEGF-C 是通过作用于肿瘤相关性纤维母细胞，在头颈部肿瘤的早期转移中起主导作用。

关键词：头颈部鳞状上皮细胞癌；纤维母细胞；血管内皮生长因子-C
中图分类号：R739.85

The rold of high expression VEGF-C of fibroblasts in head and neck squamous cell

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Abstract: Objective To study the relationship between the high expression of vascular endothelial growth factor C (VEGF-C) in fibroblasts and the growth and metastasis of head and neck squamous cell carcinoma. Methods We transfected plasmid containing Luciferase gene into head and neck cancer cells；We transfected plasmid containing VEGF-C into 3T3 fibroblasts，through experiments in vivo and in vitro to explore the role of VEGF-C in the growth and metastasis of head and neck squamous cell carcinoma and its possible mechanism. Results Western blot and ELISA experiments showed that VEGF-C gene was successfully transfected into the fibroblasts cells. There were significant differences in tumor size between the two groups of rats in vivo. The difference of tumor volume was statistically significant after the tumor was removed. (P < 0.05) Conclusions VEGF-C plays an leading role in the early metastasis of head and neck tumors by acting on cancer- associated fibroblasts.

Keywords: Head and neck squamous cell carcinoma； fibroblast；vascular endothelial growth factor

0 引言

VEGF-C 是 Joukov 以 Flt4 亲和层析法从前列腺癌细胞 PC-3 中分离一种的细胞因子，在后来的研究中被证实为主要的淋巴管形成生长因子[1]，它通过与血管内皮生长因子受体 3 结合，对肿瘤间质新生淋巴管内皮细胞的增殖和迁移进行调控，从而促进肿瘤的淋巴管形成。同时其也可以通过与血管内皮生长因子受体 2 结合，促进血管内皮细胞的增生，诱导新生血管的形成，因此其在血管形成的通路中也扮演了一定的角色。而头颈部肿瘤因其易通过分布广泛的头颈部淋巴结进行转移，故与 VEGF-C 的联系也就更加紧密。

近年来，肿瘤间叶在肿瘤发生发展中的作用越来越受到关注，肿瘤相关性纤维母细胞

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（cancer- associated fibroblasts，CAFs）和肌纤维母细胞被证实可促进肿瘤发生。肿瘤间叶包括：细胞外基质（ECM）和血液脉管系统、炎症细胞和纤维母细胞（fibroblasts，FB）等，其中纤维母细胞是间叶中最丰富的细胞。而所谓的肿瘤相关性纤维母细胞，实际上是一群不同来源的纤维母细胞的混合体，在正常的皮肤里不存在[2-3]，它们被激活后能和上皮细胞、免疫细胞等相互作用；正如 2004 年 Nature 杂志提到的：CAFs 负责许多肿瘤细胞外基质的合成、分解和重塑，产生许多影响肿瘤细胞增生和侵入间叶的细胞因子，对肿瘤发生发展的意义非常重大。 De Francesco 等人的实验则证明了在缺氧的肿瘤微环境下，CAFs 通过激活 HIF-1α/ GPER 转导通路的靶基因——VEGF-C，参与了肿瘤新生血管的形成，从而促进了肿瘤的发展[4]。提示 VEGF-C 可能在 CAFs 促进肿瘤转移的过程中扮演重要角色。因此，我们将带有荧光素酶的 VEGF-C 基因转染到成纤维细胞中，与头颈部肿瘤细胞混合进行体内成瘤实验，研究高表达 VEGF-C 的纤维母细胞在头颈部肿瘤侵袭转移中的作用。

1. 材料、主要试剂与仪器

1.1 材料

1.1.1 材料

培养头颈部肿瘤细胞 MDA1986、Tu159 以及成纤维细胞 3T3，均来自中山大学口腔医学院研究所，scid 大鼠

1.1.2 主要试剂

2.5%异氟醚：CD34 抗体和 VEGF 抗体(福州迈新公司)：SP 免疫组化试剂盒(福州迈新公司)。带荧光素酶、VEGF-C 的质粒（美国 MD ANDERSON CANCER CENTER 的 Fan Zhen 教授惠赠）。

1.2 方法

1.2.1 细胞转染

将带荧光素酶 Luciferase 基因的质粒转染到头颈部肿瘤细胞；将带 VEGF-C 的质粒转染到 3T3 细胞，另一组转染带 GFP 基因的质粒作为对照。质粒图如下：

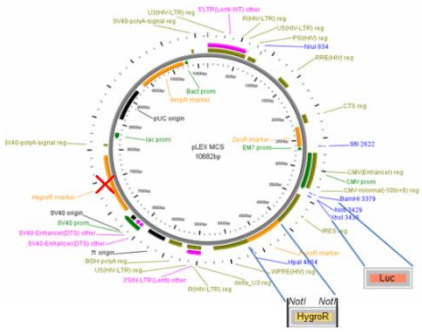


图 1　带 VEGF-C 的质粒结构图

Fig。 1　Plasmid structure diagram of with VEGF-C

转染步骤： 1）、转染前一天细胞换新的培养基 2）、制备 DNA-Lipofectamine 2000 复合物，如下：a、用 50μl 无血清培养基稀释 DNA（0.8g），轻轻混匀；b、Lipofectamine 2000 使用前，轻轻混匀，用 48μl 无血清培养基稀释 2μl Lipofectamine 2000，轻轻混匀，室温孵育 5 分钟；c、将 a+b 的液体加在一起，轻轻混匀，室温孵育 20 分钟，使 DNA-Lipofectamine 2000 复合物形成。3）、将 100μl DNA-Lipofectamine 2000 复合物加入培养板中，轻轻前后摇匀。4）、放入等量头颈部肿瘤细胞 MDA1986、Tu15 两组 37℃孵箱中培养 48h 后，1：10 传代，加入合适浓度的 G418 筛选。5）、等细胞在 G418 作用下，长满后冻存几支保种，再克隆化培养：将细胞消化后计数 50 个细胞，加入 22ml 完全培养基中，混匀，分装在 96 孔板中（200μl/孔）。第二天在显微镜下，寻找孔中有单个细胞的孔并做标记，等做标记的孔长满后，消化、转移到培养板中，最后转移到培养瓶中培养，冻存，鉴定。 6）、同时转染带 VEGF-C 的质粒到 3T3 细胞，步骤同上所述。

1.2.2 转染结果验证：Western blot 实验检测肿瘤细胞 Luciferase 蛋白

步骤：将转染 24h 后的细胞用冷的 PBS（转移缓冲液）40ml 洗一次，弃去残余的 PBS，加入 200μl 预冷的蛋白裂解液冰上裂解 2h，4℃、14000 r/min 离心 20 min，收集上清液。采用 BCA 法检测蛋白浓度，加入 5×SDS 上样缓冲液，100℃加热 5 min。10%SDS-PAGE 电泳后转膜，5%脱脂奶粉室温摇床封闭 2h，加入一抗 4℃缓慢摇床过夜孵育。TBST 漂洗 3 次，每次 15 min，加入辣根过氧化物酶标记二抗，室温低速摇床孵育 2 h。加入 ECL 显色液，室温避光孵育 1~3 min，曝光、显影。

1.2.3 转染效率验证

ELISA 实验验证 3T3 细胞 VEGF-C 表达情况。收集细胞上清，并裂解细胞作为检测样品。

1）包被：每孔加入适当稀释的鼠抗人 VEGF 单克隆抗体 100 μl，4℃过夜，PBST 洗 3 次，每次 3 min。 2）封闭：每孔加 2% BSA 200 μl，37℃，60 min，PBST 洗 3 次，每次 3 min。 3）样品：每孔加 100μl 待测样品，37℃放置 40~60 min。 4）一抗： 每孔加入适当稀释的兔抗人 VEGF 抗体 100μl，37℃反应 40 ~60 min。 5）二抗：每孔加入酶标二抗 100μl(山羊抗兔 VEGF-HRP) ，37℃反应 40~60 min。 6）显色：加 100μl OPD 底物。 7）终止：每孔加入 2mol/L 的 H_2SO_4 终止液 50 μl 终止反应，于 20min 内测定实验结果。 8）结果判断：在 ELISA 检测仪上，于 450nm 处，以空白对照孔调零后测各孔 OD 值。

1.2.4 CAF 标记物检测

3T3-VEGF-C 细胞培养，进行 α-平滑肌肌动蛋白（α-SMA）、FSP1 的免疫荧光实验。

步骤：用上皮细胞标志物 E-cadherin，pan keratin 和间叶细胞标志物 vimentin 以及 CAF 标志物 α-SMA，FSP1 的抗体分别进行免疫荧光单染（PE 标记），对照细胞是上皮来源的 Tca8113。

主要步骤如下：培养好的细胞用 PBS 洗去培养液，4%多聚甲醛固定 30min；PBS 洗 3 次，每次 5min；0.2%TritonX-100 处理 5min；PBS 洗 3 次，每次 5min；10%正常山羊血清封闭 30min；一抗 4℃孵育过夜；PBS 洗 3 次，每次 5min；荧光标记二抗（1：100）室温湿盒避光孵育 1h；PBS 洗 3 次，每次 5min；DAPI 室温湿盒避光孵育 5min；PBS 洗一次，5min；荧光抗淬灭剂封片；镜检，拍照。

1.2.5 体内成瘤实验

$6*10^6$ 头颈部肿瘤细胞混合 $6*10^5$ 的 3T3 细胞注射在裸鼠皮下，每周荧光素酶报告基因显像，观察并记录肿瘤大小、生长速度，观察转移情况。

步骤：分为对照组与实验组，其中每组又分为 MDA1986、Tu159 两种头颈部肿瘤细胞组，每组各有五只（5-6 周龄）scid 大鼠，共二十只。将上述培养的两种头颈部肿瘤细胞、经 VEGF-C 转染的 3T3 细胞与未经 VEGF-C 转染的 3T3 细胞分别用胰酶分散、洗涤、计数后，按照每只大鼠 $6*10^6$ 个头颈部肿瘤细胞与 $6*10^5$ 个 3T3 细胞的规格，分别在四组大鼠体内接种，用公式 $\pi/6 \times ab2$ 计算肿瘤体积（a：长度；b：宽度，a > b），处理开始的第十五天，当移植瘤体积达到约 120 mm^3 时，注射 3.3 毫克/100 μL 的 D-荧光素和 2.5%异氟醚后在体外成像系统观察生物发光图像。生物发光成像数据用 Living Image 4.3.1 软件进行分析。随后处死小鼠，取出肿瘤测量体积。

1.3 统计分析

采用 SPSS18.0 for windows 统计软件对数据进行统计学分析。采用未配对的双侧 t 检验来比较两组独立样本，以 P＜0.05 为具有统计学意义。

2 结果

2.1 Western blot 实验结果

可见作为VEGF-C标志的myc标签含量在有无VEGF-C转染的3T3细胞中有显著差别。

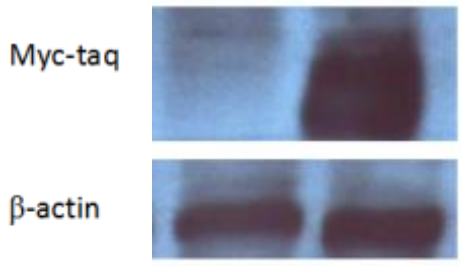


图 2Wester
n blot 检测 VEGF-C
Fig. 2Detection results of VEGF-C by Western blot

2.2 ELISA 实验

结果可见转染了 VEGF-C 的 3T3 细胞分泌 VEGF-C

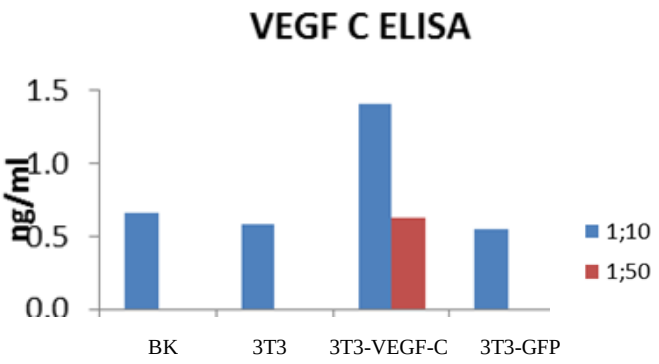
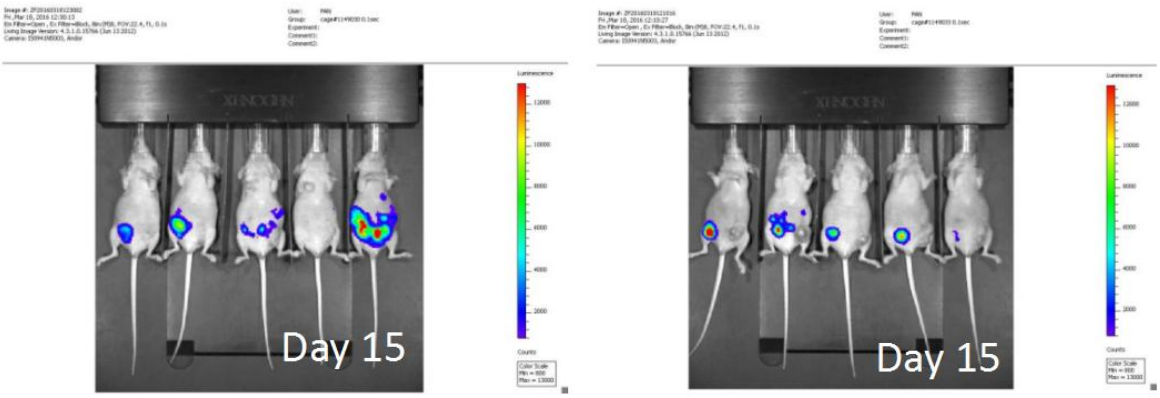


图 3ELISA 检测 VEGF-C

Fig. 2Detection of VEGF-C by ELISA

2.3 体内成瘤实验

可见 MDA1986 + 3T3-vc 组皮下成瘤体积较大，有两只裸鼠腹腔转移明显。



MDA1986 + 3T3-vc　　MDA1986 + 3T3-GFP

图 4 活体成像仪观察大鼠体内肿瘤生长状况

Fig. 4Observation of tumor growth in vivo by bioluminescence imaging

3 讨论

转移瘤是一种常见且致命的肿瘤并发症，血管与淋巴管的形成在其中扮演了重要角色，头颈部因淋巴结数量多、分布广使得头颈部肿瘤更容易发生转移。VEGF-C 是目前已知最主要的淋巴管形成生长因子，它通过与血管内皮生长因子受体 3 结合，对肿瘤间质新生淋巴管内皮细胞的增殖和迁移进行调控，促进肿瘤的淋巴管形成。细胞划痕实验中，我们在镜下对划痕处理后 0h、24h、48h、72h、96h 的两种头颈部肿瘤细胞划痕宽度进行观察，发现了明显的差异，测量得到数据运用统计软件进行处理后也得到差异具有统计学意义。大量研究表明，原发性肿瘤中 VEGF-C 的表达增加与肿瘤细胞向局部淋巴结的传播呈正相关，其表达强弱可作为评估肿瘤预后的独立因素。

VEGF-C 与头颈部肿瘤的联系非常密切：有相关文献报道，VEGF-C 的表达可以用于预测舌癌的转移，VEGF-C 可以增强食管癌的侵袭性和转移性[2]，VEGF-C 的高表达还可以作为预后不良的食管鳞状细胞癌的生物指标[3]。总而言之，VEGF-C 在肿瘤局部浸润、远处转移过程中起到了重要的作用，研究其调控因子对于肿瘤的病理学研究、临床肿瘤防治有重要意义。

我们在 MTT 实验中观察 VEGF-C 对头颈部肿瘤细胞生长速度的影响，发现二组细胞虽然在生长速度上有差异，但差异很小，不具有统计学意义。猜想 VEGF-C 对头颈部肿瘤生长不起明显促进作用的原因可能是 VEGF-C 的促血管生成能力较弱，微弱的促血管生成作用可以在一定程度上帮助肿瘤细胞获得获取营养物质的能力，但是 VEGF-C 强大的促淋巴管生成能力又提高了肿瘤细胞的代谢水平，二者在肿瘤生长方面的拮抗作用导致肿瘤细胞在宏观上表现出微弱的生长加快。

至于肿瘤实质细胞与间叶的关系。早在 1889 年就被提出的“seed and soil”假说就认为癌变的上皮细胞像一颗种子一样，必须有合适的间叶土壤才能繁殖生长。半个多世纪以来，虽然普遍接受的观点认为上皮细胞发生突变的累积是最终产生癌症的原因，但同时人们也发现，在许多肿瘤中，上皮细胞的异常和肿瘤的表型、临床行为不平行。在肿瘤形成的最早期，正常的微环境可抑制肿瘤细胞增生和肿瘤的形成。但是在肿瘤的发展过程中，CAFs 和肌纤维母细胞被证实可促进肿瘤发生[4]。肿瘤上皮细胞和间叶细胞之间存在不同形式的对话（crosstalk），其共同发展（co-evolution）有两种途径：间叶细胞先发生改变，引发上皮细胞的恶性转化；或者转化的上皮细胞以旁分泌的方式激活间叶细胞。最新的研究发现，间叶与癌症发生发展密切相关，并可能是致癌物的关键作用靶点，也被认为是肿瘤预防和治疗的一个可行的靶点。

最近的证据还提示纤维母细胞在某些肿瘤如乳腺癌中，已经不仅仅是“旁观者”，而是积极主动的“参与者”甚至“产生者”了。也就是说，纤维母细胞并非如人们以前所认为的那样，以一种比较被动的方式参与肿瘤，即只是接受上皮细胞的某些信号产生反应，而可能是主导者。目前有关 CAFs 的研究较多集中在乳腺癌、卵巢癌和前列腺癌，其中乳腺癌的研究最深入，已有学者建立了间叶来源的预测程序（stroma-derived prognostic predictor，SDPP）来预测乳腺癌患者的预后[6]。

CAFs 与头颈部肿瘤的关系也引起人们关注：已有研究发现正常纤维母细胞可抑制头颈部肿瘤的生长而 CAFs 可促进其侵袭和转移；CAFs 中的 Galectin-1 基因敲除后，通过降低 CCL-2 的表达，可抑制口腔鳞癌的转移[7]；Chatzistamo 等在口腔癌细胞中用 siRNA 下调 p21 后发现其参与了 α-平滑肌肌动蛋白（α-SMA）的激活，和 CAFs 的活化有关[8]；还有学者发现 TGF-β 受体 II、 III 在口腔鳞癌的上皮和 CAFs 中都表达下降[9]；CAFs 的异常引起部分 T 淋巴细胞的循环功能障碍，进而抑制了另一部分功能正常的 T 淋巴细胞的免疫功能[10]。

总之，头颈部肿瘤的进程是非常复杂的，其中间叶成分中的 CAFs 可以通过多种调节机制来引起头颈部肿瘤细胞的改变，而肿瘤细胞也可以通过细胞间接触、细胞外基质、可溶性因子等途径影响间叶。因而，肿瘤相关性纤维母细胞可能也是头颈部肿瘤早期干预、中期治疗、晚期预测与监控的有效的靶点。

De Francesco 等人的实验证明了在缺氧的肿瘤微环境下，CAFs 通过激活 HIF-1α/ GPER 转导通路的靶基因——VEGF-C，参与了肿瘤新生血管的形成，从而促进了肿瘤的发展。此外，VEGF-C 能激活 TGF-β/Smad 和 MAPK/ERK 通路[11]， 他们是控制细胞纤维化过程的主要信号通路；在各类纤维化疾病中都检测到了 TGF-β/Smad 通路和 MAPK/ERK 通路的激活[12] 。另有研究证明，VEGF-C 能促进 I 型和 III 型胶原的合成，同时具有促进肌成纤维细胞分泌基质金属蛋白酶 2（matrix metalloproteinases-2，MMP-2）和 MMP-9 的功能，而 MMPs 在胶原降解中起到核心作用。

4 结论

综合上述研究结果与实验结果，我们认为 VEGF-C 很可能通过作用于肿瘤相关性纤维母细胞，在头颈部肿瘤的早期转移中起到“主导者”的作用。

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Effect of matrix metalloproteinase 8 inhibitor and chlorhexidine on the cytotoxicity, oxidative stress and cytokine level of MDPC-23

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ABSTRACT

Objective. This study investigated the effect of matrix metalloproteinase-8 inhibitor I (MMP8-I) and chlorhexidine (CHX) on the viability, oxidative stress and cytokine secretion of MDPC-23 under short-term (30 min) and long-term (3 days) culture.

Methods. MDPC-23 were treated with MMP8-I or CHX for 30 min, 1 day, 2 days and 3 days to detect the proliferation by 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT) assay. In the following assays, MDPC-23 treated with 0.0003% CHX were referred to CHX group, treated with 8 μM MMP8-I were MMP8-I group. Cells without additional treatment were regarded as control group. The cell cycle, reactive oxygen species (ROS) level, and apoptosis were assessed by flow cytometry. The cytokine level was quantified by enzyme-linked immunosorbent assay (ELISA).

Results. In 30 min, CHX at concentrations higher than 0.0003% dilution inhibited cell proliferation when compared to the control group. MMP8-I (0.1–500 μM) showed no obvious cytotoxicity to MDPC-23, and MMP8-I (1000 μM) inhibited cell proliferation. In 3 days, CHX (0.0003%) significantly inhibited cell growth, while MMP8-I (8 μM) had no cytotoxicity. In the CHX group, the S phase population was decreased, and cellular ROS were increased in 3 days. In the MMP8-I group, the change of S phase population and cellular ROS was not significant compared with the control group. Cell apoptosis was not elevated in the MMP8-I group, while the apoptosis rate was increased in the CHX group both in 30 min and 3 days. In 30 min, CHX treatment significantly increased the secretion of interleukin (IL)-1β and IL-8, but slightly increased the secretion of IL-10, while MMP8-I caused no change in cytokines. In 3 days, CHX treatment significantly increased the secretion of IL-1β, IL-6, and IL-8, and inhibited the secretion of IL-10. MMP8-I treatment caused the increase of IL-6.

Significance. Compared with CHX, MMP8-I at low concentration did not result in cytotoxicity, oxidative stress, or the disorder of immune response.

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1. Introduction

The deterioration of resin–dentin bonds has become a focus of research in contemporary adhesive dentistry due to the proteolysis of collagen matrix of the hybrid layers [1]. Chlorhexidine (CHX), a nonspecific protease inhibitor of matrix metalloproteinase (MMP) and cysteine cathepsins, has been found to be beneficial to maintaining the integrity of the hybrid layer and preventing the loss of bond strength with time [2,3]. However, emerging evidence suggests that CHX exhibits both cytotoxicity and genotoxicity to oral cells, which greatly restricts its application in adhesive dentistry [4–6]. Recently, we reported that the synthetic MMP-8 inhibitor I (MMP8-I, 8 uM) protects the degradation of resin–dentin bonds over time [7]. Compared to CHX, MMP8-I is a selective inhibitor of MMP-8, which improved bond strength, preserved collagen integrity, and decreased nanoleakage after 1 year of in vitro storage [7]. In order to verify the security of MMP8-I application in adhesive dentistry, it is necessary to explore the effects of MMP8-I on odontoblast-like cells (MDPC-23).

During aerobic metabolism, cells regularly generate reactive oxygen species (ROS), including the superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radicals (OH^-) [8,9]. Moderate amounts of ROS are considered to be signaling molecules in biological and physiological processes [10–12]. However, oxidative stress occurs when the generation of ROS disrupts the cellular redox homeostasis [11]. Oxidative stress plays an important role in delaying the cell cycle, increasing cell apoptosis, and modulating cytokines levels [13,14].

Cytokines, produced by a broad range of cells, are signaling proteins playing vital roles in biological phenomena, such as proliferation, inflammation, and immunomodulation [15,16]. Inflammation always accompanies the dysregulation of pro- and anti-inflammatory cytokines [16,17]. Therefore, cytokines reflect immune and cellular defensive responses when cells are destroyed by external factors. To our knowledge, the relationship between MMP8-I and cytokines remains unknown.

The aim of this study was to compare the effects of CHX and MMP8-I on the viability, oxidative stress, and cytokine secretion of MDPC-23. Because the MMP8-I was used as pretreatment, which is long-lasting in dental-resin interface, we explored the short-term (30 min) and long-term (3 days) effect of MMP8-I on MDPC-23.

2. Materials & methods

2.1. Culture of odontoblast-like cells

Immortalized odontoblast-like cells (MDPC-23) were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Gibco-BRL, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (Gibco-BRL), 100 μg/mL streptomycin (HyClone, Logan, UT, USA), 100 U/mL penicillin (HyClone), and 5 mmol/L glutamine (Gibco-BRL) at 37 °C in 5% CO_2.

2.2. Cell proliferation assay

Cell proliferation of MDPC-23 was determined by MTT assay as described previously [18]. MDPC-23 were seeded at a density of 3000 cells per well and a volume of 100 μL of fresh medium in 96-well plates. CHX (Sigma, St. Louis, MO, USA) or MMP8-I (Merck KGaA, Darmstadt, Germany) was added in the culture medium at the various concentrations for different observation times (30 min, 1 day, 2 days and 3 days). Cells cultured in fresh medium without CHX or MMP8-I served as the negative control group. Briefly, 10 μL of MTT (5 mg/mL) solution was added to each well and incubated for 4 h at 37 °C. The supernatants from each well were discarded. Dimethyl sulfoxide (100 μL) was added and incubated for 10 min to dissolve the crystals. Cell number and viability were determined by measuring the absorbance at a wavelength of 490 nm with a microplate reader (Bio-Tek, Winooski, VT, USA).

2.3. Cell cycle analysis

For cell cycle analysis, each group was washed with cold PBS and fixed in 75% ethanol overnight at 4 °C. After being incubated with ribonuclease A (100 μg/mL in PBS), cells were stained with 50 μg/mL of propidium iodide (PI) at 4 °C for 30 min and analyzed using cytometry (Beckman Coulter, Krefeld, Germany). Data were analyzed using CytExpert software.

2.4. ROS staining

ROS staining was performed using an ROS staining kit (Beyotime, China) following the manufacturer's instructions [19]. MDPC-23 from each group were stimulated with serum-free medium containing 10 μM dichloro-dihydro-fluorescein diacetate (DCFH-DA) for 20 min at 37 °C, shaking slightly every 5 min. After removing the medium and washing the cells with serum-free culture medium, the cells were collected for examining the fluorescence intensity of each sample using flow cytometry (Beckman Coulter).

2.5. Apoptosis evaluation

The cytotoxic responses of MDPC-23 in the presence of CHX or MMP8-I were evaluated using cell apoptosis analyses. The MDPC-23 cultured in fresh medium were exposed to 0.0003% CHX or MMP8-I (8 μM) for 30 min and 3 days, while MDPC-23 cultured in fresh medium without CHX or MMP8-I were used as the negative control group. Cell apoptosis was analyzed using an PE Annexin V Apoptosis Detection Kit (BD Biosciences, Franklin Lakes, NJ, USA) following the manufacturer's protocol [20]. Briefly, 1×10^6 MDPC-23 were suspended in 1 × binding buffer, stained using PE-annexin V and 7-AAD staining solutions and analyzed by cytometry (Beckman Coulter).

2.6. Enzyme-linked immunosorbent assay (ELISA)

To quantify the secretory cytokines produced by MDPC-23, ELISA was performed according to the manufacturer's instructions. Briefly, after cells being cultured in each group for 30 min or 3 days, the supernatant was collected and analyzed with separate ELISA kits for mouse interleukin (IL)-1β, IL-6, IL-8, and IL-10 (NeoBioscience Technology, Guangdong, China). The

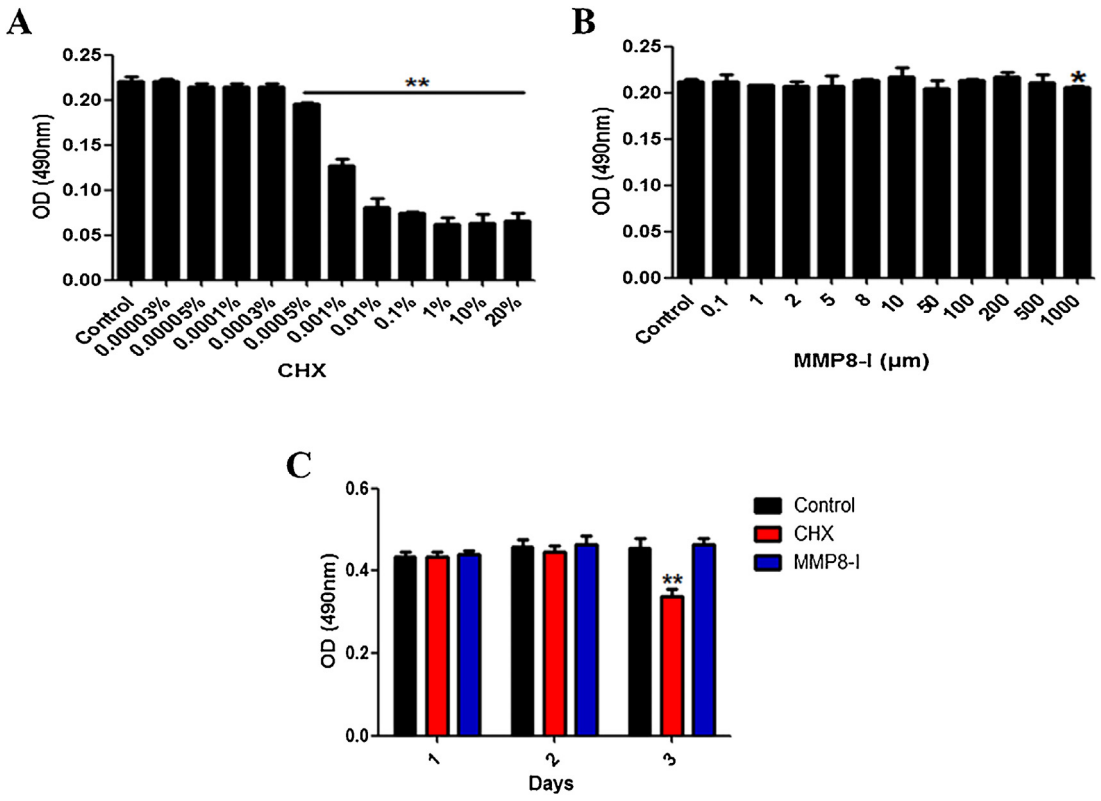

Fig. 1 – Assessment of effects on cell proliferation with CHX and MMP8-I.
MTT assay was performed after cells were treated with CHX (A) or MMP8-I (B) for short-term (30 min) and long-term (3 days) culture (C). All data are expressed as mean ± SD. *$p < 0.05$, **$p < 0.01$ compared with control group.

OD values were measured at 450 nm using a microplate reader (Bio-Tek).

2.7. *Statistical analysis*

All experiments were performed in triplicate. The SPSS 20.0 software package (SPSS, Chicago, IL, USA) was used for the statistical tests. Values are expressed as the mean ± SD. A one-way analysis of variance (ANOVA) with Tukey's test was performed to determine the statistical differences. In this study, $p < 0$ 05 was considered to indicate statistical significance.

3. Results

3.1. *Assessment of effects on cell viability with CHX and MMP8-I treatment*

For the MTT assay, cells were treated with CHX or MMP8-I at different concentrations for 30 min in order to evaluate cell growth. As the results showed, cells with CHX at concentration higher than 0.0003% had decreased cell proliferation when compared to the control group (Fig. 1A). No statistical differences were shown for MMP8-I treatment at concentrations ranging from 0.1 to 500 μM. However, 1000 μM MMP8-I may led to the decrease of cell proliferation (Fig. 1B). Then, we evaluated the cytotoxicity of long-term culture by treating cells with CHX (0.0003%) or MMP8-I (8 μM) and testing cell proliferation at 1, 2, and 3 days. CHX significantly inhibited cell growth at 3 days and MMP8-I treatment did not induce cytotoxicity (Fig. 1C). This indicated that CHX inhibited cell viability in dose- and time-dependent manners, while MMP8-I showed no cytotoxicity at concentration up to 500 μM.

3.2. *Assessment of cell cycle after CHX and MMP8-I treatment*

To further examine the cytotoxicity of CHX (0.0003%) and MMP8-I (8 μM) on MDPC-23, the cell cycle of different groups was detected. The results revealed that there was no significant difference in the S phase population among three groups for 30 min (Fig. 2A–C). In 3 days, the S phase population significantly decreased in the CHX group, while MMP8-I treatment did not decrease the S phase population of MDPC-23 (Fig. 2B–D).

3.3. *Measurement of ROS*

We further detected the effects of CHX (0.0003%) and MMP8-I (8 μM) treatment on the formation of intracellular ROS using a DCFH-DA probe. Neither CHX nor MMP8-I treatment elevated intracellular ROS in 30 min (Fig. 3A). However, in 3 days, CHX treatment caused the obvious increase of intracellular ROS, and MMP8-I treatment maintained the same level of ROS as the control group (Fig. 3B), which indicated that MMP8-I treatment did not induce cell oxidative stress.

3.4. *Assessment of cell apoptosis with CHX and MMP8-I treatment*

To test the effect of CHX (0.0003%) and MMP8-I (8 μM) treatment on cell apoptosis, PE-Annexin V/7AAD staining assay was performed. Our results showed that the percentage of apoptotic cells was significantly increased with CHX treatment for 30 min and 3 days, while MMP8-I treatment did not affect cell apoptosis in 30 min or 3 days (Fig. 4).

3.5. *Detection of chemokine secretion of cells treated with CHX or MMP8-I*

Finally, we tested the effect of CHX (0.0003%) and MMP8-I (8 μM) treatment on chemokine secretion of MDPC-23 by ELISA assay. Cells cultured with CHX treatment for 30 min significantly increased the secretion of IL-1β and IL-8, and did not influence the secretion of IL-6 and IL-10 (Fig. 5A). Treatment with MMP8-I for 30 min did not influence chemokine secretion of MDPC-23 compared to the control group (Fig. 5A). In 3 days, CHX increased the pro-inflammatory factors (IL-1β, IL-6 and IL-8) and decreased secretion of IL-10 (Fig. 5B). In the MMP8-I group, the secretion of IL-6 was increased (Fig. 5B).

4. Discussion

CHX has broad-spectrum antibacterial activity and can inhibit the catalytic activity of MMPs, so it's use is recommended as a cavity disinfectant to improve resin–dentin bond durability. However, CHX showed cytotoxic effects in MDPC-23, human osteoblastic, and fibroblastic cells [21–23]. Similarly, we found that CHX (0.005%–20%) is cytotoxic to MDPC-23 in short-term treatment (Fig. 1A). Therefore, cells are greatly damaged during the application of CHX in adhesive density.

MMP8-I, a selective inhibitor of MMP-8, based on the hydroxamate structure which specifically interferes with the action of the zinc catalytic domain in the MMP-8 molecule [24,25], protects against the degradation of resin–dentin bonds over time [7]. In our study, the cytotoxicity of CHX to MDPC-23 was dose- and time-dependent in short- and long-term treatment (Fig. 1A–C). MMP8-I at concentration up to 500 μM showed no cytotoxicity to MDPC-23 (Fig. 1B). The decrease of cell vitality is often connected with cell cycle arrest, and the S phase of the cell cycle is the phase in which DNA is replicated and the cells prepare for mitotic division [26,27]. CHX inhibits the viability of dermal fibroblasts by impeding

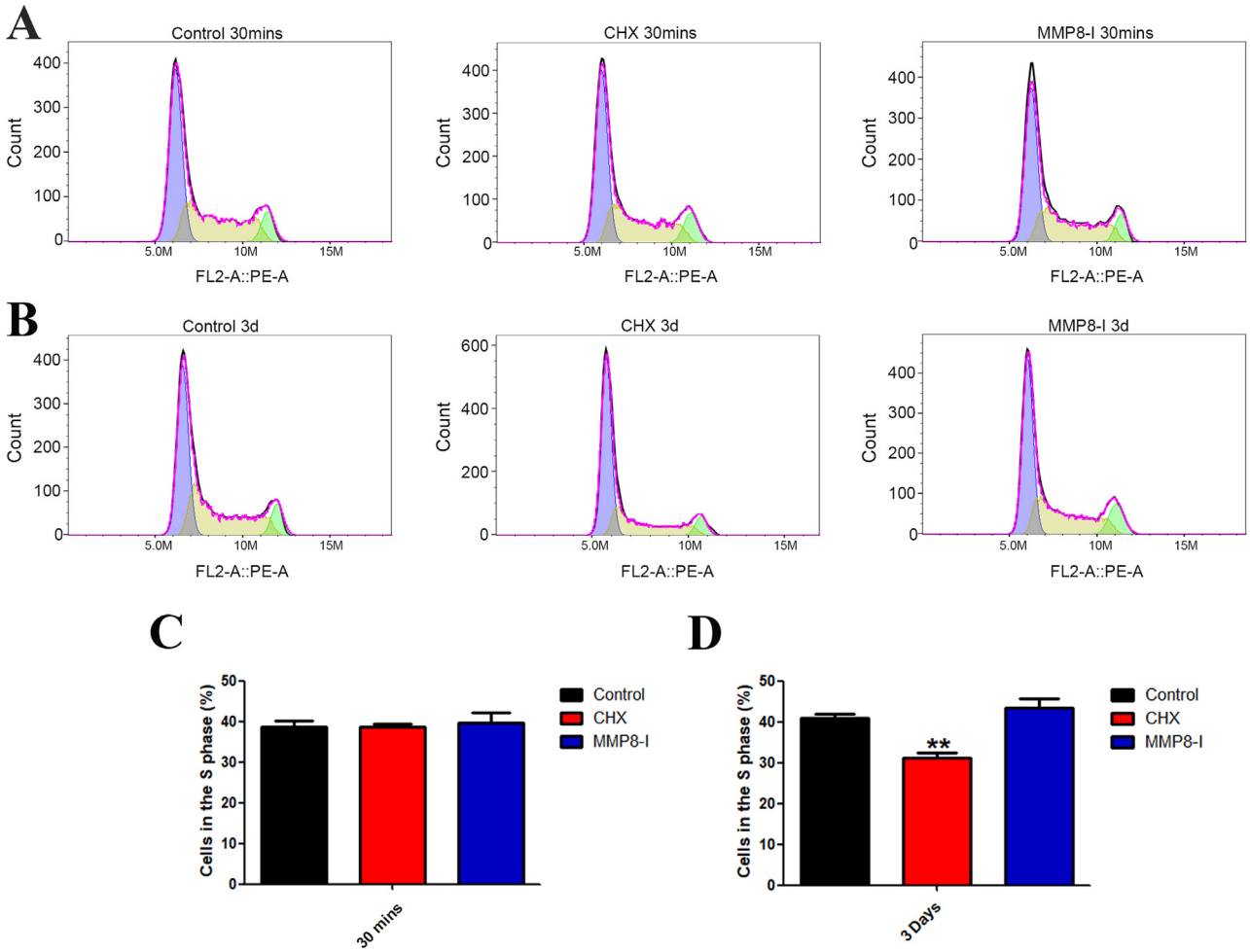

Fig. 2 – Cell cycle of MDPC-23.
Cell cycle of MDPC-23 in different groups in 30 min (A). Cell cycle of MDPC-23 in different groups in 3 days (B). Quantitative analysis of cell cycle (C and D). All data are expressed as mean ± SD. $^{**}p < 0.01$ compared with the control group.

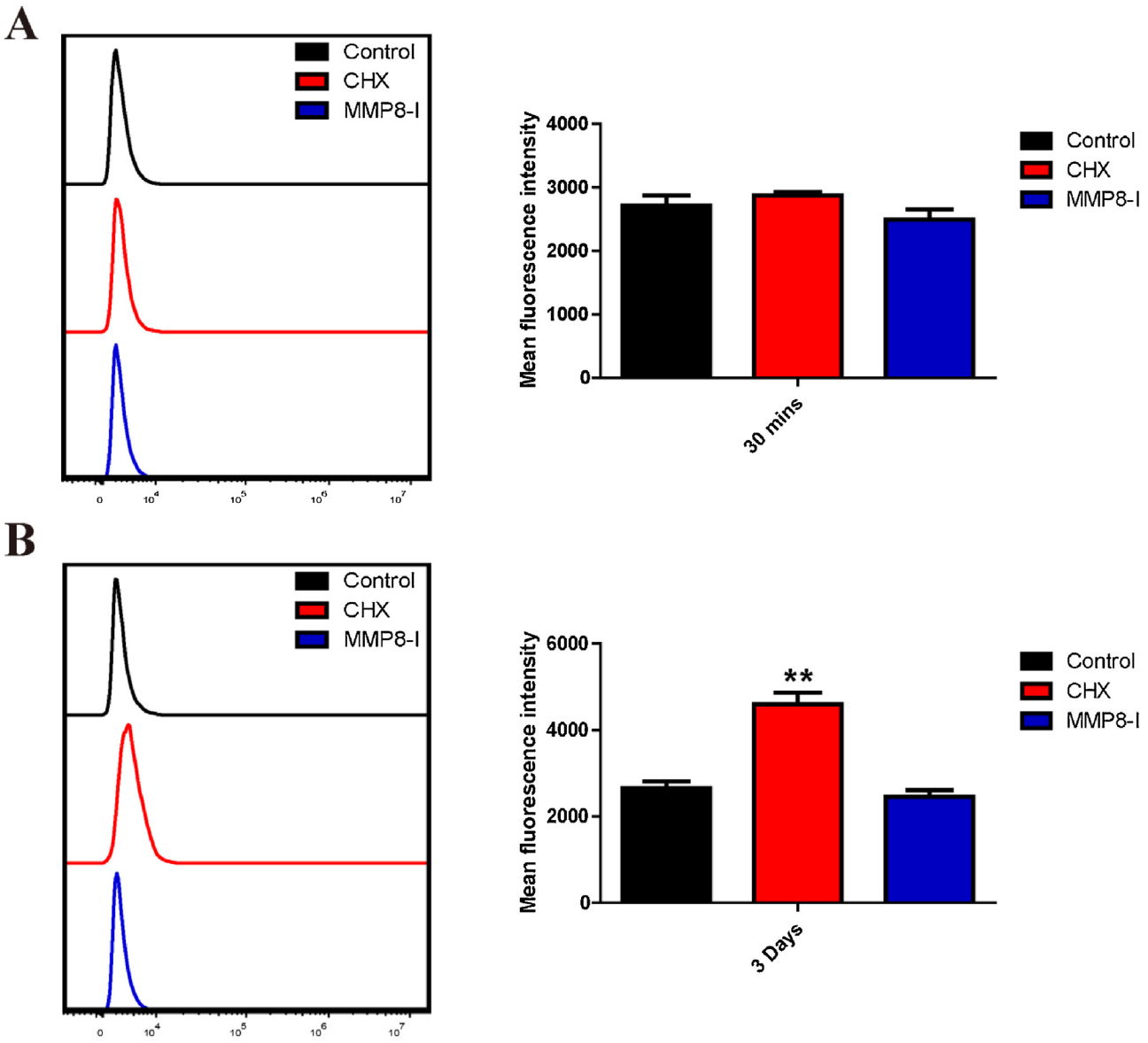

Fig. 3 – Measurement of intracellular ROS.
ROS production and quantitative analysis induced by CHX or MMP8-I treatment for 30 min (A) or 3 days (B). $^{**}p < 0.01$ compared with control group.

cells from advancing to the S phase [28]. In long-term treatment, the S phase population decreased in the CHX group, which inhibits cell proliferation through cell cycle arrest, while MMP8-I does not interrupt the cell cycle of MDPC-23 (Fig. 2). This demonstrated that MMP8-I is safer than CHX during the clinical application. The mechanism of CHX-induced cytotoxicity is associated with the increase of ROS and the induction of cell apoptosis [23,26].

ROS have been identified as chemical mediators in the processes of cell growth, cell apoptosis, and cytokine secretion. In osteoblasts, CHX induces cytotoxicity by promoting total ROS, which may cause further osteoblast impairment, such as cellular structure and DNA damage [29]. In MDPC-23, we found that CHX significantly increased the cellular ROS and apoptosis rate in 3 days (Figs. 3 and 4), while there was no difference between the MMP8-I group and the control group (Figs. 3 and 4), which further indicated that MMP-I had no obvious toxic effect on MDPC-23, possibly due to the concentration of MMP8-I and specificity to MMP-8 [30,31].

IL-1β is produced by many immune cells, including monocytes, macrophages, and dendritic cells, which is regarded as an early inflammatory biomarker [32,33]. In the CHX group, the levels of IL-1β were significantly increased both in 30 min and 3 days, while there were no significant changes between the MMP8-I group and the control group. This demonstrated that MMP8-I treatment did not cause early inflammation in MDPC-23. IL-6 is a pleiotropic cytokine produced by a variety of immune cells, which plays a role of both pro- and anti-inflammatory function in local immune response [34,35]. In CHX and MMP8-I groups, the levels of IL-6 were increased in 3 days, which showed that both CHX and MMP8-I stimulated the IL-6-related immune response in MDPC-23. Our

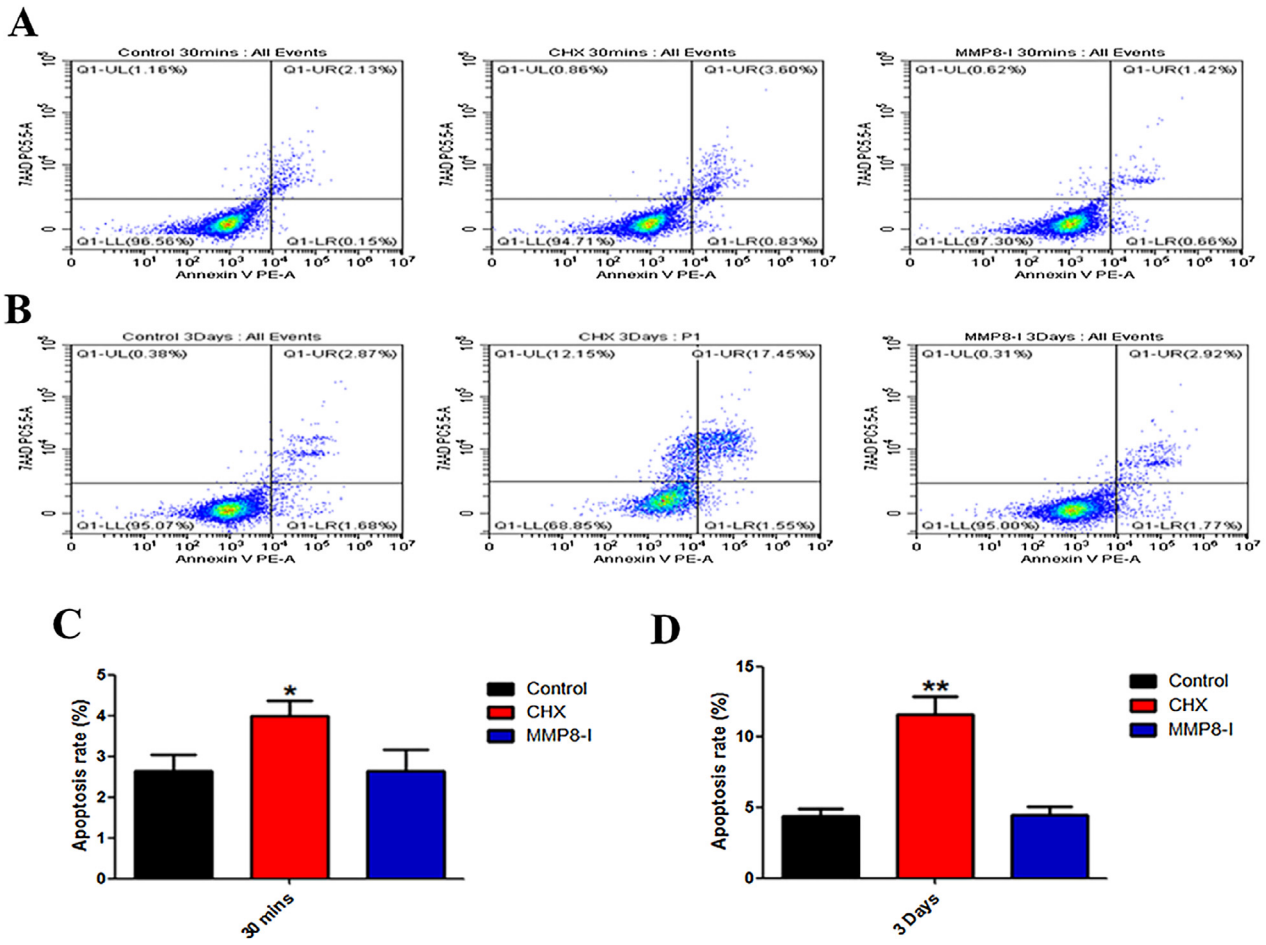

Fig. 4 – Assessment of cell apoptosis with CHX or MMP8-I treatment.
The cell apoptosis of MDPC-23 were treated with CHX or MMP8-I for 30 min (A) or 3 days (B). Quantitative analysis of apoptotic cells (C and D). $^{*}p < 0.05$, $^{**}p < 0.01$ compared with control group.

results correspond to the previous study showing that both CHX and epigallocatechin-gallate increased the secretion of IL-6 [34]. IL-8 is another pro-inflammatory cytokine involving the chemotaxis of neutrophils [36,37]. Similar to the results of IL-1β, the production of IL-8 was enhanced in the CHX group, while MMP8-I caused no obvious change in IL-8 secretion, which further confirmed that MMP8-I caused more mild inflammatory response than CHX in MDPC-23. IL-10 is an anti-inflammatory cytokine, which plays a crucial role in immune defense [38]. CHX significantly inhibited the IL-10 secretion in 3 days, while the level of IL-10 in the MMP8-I group did not decrease, which showed that MMP8-I did not cause the disor-

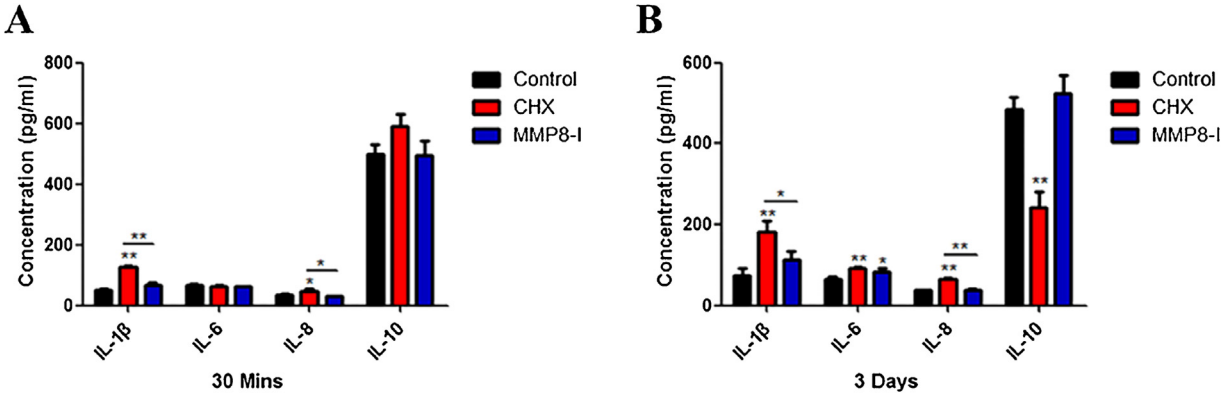

Fig. 5 – Chemokine secretion of cells treated with CHX or MMP8-I.
Cells were treated for 30 min (A). Cells were treated for 3 days (B). All data are expressed as mean ± SD. $^{*}p < 0.05$, $^{**}p < 0.01$ compared with control group.

der of the anti-inflammatory immune response. After treated for 30 min, MDPC-23 in CHX group secreted higher level of pro-inflammatory cytokine including IL-1β and IL-8. Cytokines did not present significant change in secretion levels in MMP8-I group. After treated for 3 days, cells in CHX group increased the level of pro-inflammatory cytokine (IL-1β and IL-8) and cytokine owning both pro- and anti-inflammatory function (IL-6). But the production of anti-inflammatory cytokine (IL-10) was decreased in the CHX group. In 3 days, IL-1β, IL-8 and IL-10 did not present significant change in secretion levels in MMP8-I group, the level of IL-6 was increased in MMP8-I group. The different patterns of cytokine secretion indicated CHX may be cytotoxic to MDPC-23 due to pro-inflammatory reaction, while MMP8-I is less toxic for MDPC-23.

Studies found that MMP-8 plays a pivotal role in arthritis, inflammatory and neurological disorders [39–41]. MMP-8 is upregulated in LPS-stimulated microglia, and the treatment of MMP8-I suppresses proinflammatory molecules, such as TNF-α [40]. In neuroinflammatory disorders, the treatment of MMP8-I decreases the secretion of ROS and proinflammatory molecules, particularly TNF-α, IL- 1β, and IL-6 [25].

Therefore, combined with the previous results [7], this study showed that MMP8-I is a promising adhesive in adhesive bonding systems due to its effectiveness of improving bond strength and low cytotoxicity in MDPC-23. In future research, because the caries-infected dentin is common in clinical practice, we will concentrate on the function of MMP8-I to dental pulp cells in order to explore the role of MMP8-I in dental bonding of caries-infected dentin. We will also focus on the improvement of drug-delivery strategy in order to extend the efficacy of MMP8-I, such as nanoparticle delivery carrier.

5. Conclusion

Compared with CHX, the application of MMP8-I caused lower cytotoxicity, oxidative stress, and the disorder of immune response, indicating that MMP8-I is less toxic than CHX for clinical application in adhesive dentistry.

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REVIEW
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Progresses and Perspectives of Anti-PD-1/PD-L1 Antibody Therapy in Head and Neck Cancers

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Head and neck cancer is the 6th most common malignancy worldwide and urgently requires novel therapy methods to change the situation of low 5-years survival rate and poor prognosis. Targeted therapy provides more precision, higher efficiency while lower adverse effects than traditional treatments like surgery, radiotherapy, and chemotherapy. Blockade of PD-1 pathway with antibodies against PD-1 or PD-L1 is such a typical targeted therapy which reconstitutes anti-tumor activity of T cell in treatments of cancers, especially those highly expressing PD-L1, including head and neck cancers. There are many clinical trials all over the world and FDA has approved anti-PD-1/PD-L1 drugs for head and neck cancers. However, with the time going, the dark side of this therapy has emerged, including some serious side effects and drug resistance. Novel materials like nanoparticles and combination therapy have been developed to improve the efficacy. At the same time, standards for evaluation of activity and safety are to be established for this new therapy. Here we provide a systematic review with comprehensive depth on the application of anti-PD1/PD-L1 antibodies in head and neck cancer treatment: mechanism, drugs, clinical studies, influencing factors, adverse effects and managements, and the potential future developments.

Keywords: PD-1, PD-L1, immune checkpoint inhibitor, head and neck cancer, immunotherapy, adverse effects

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INTRODUCTION OF HEAD AND NECK CANCERS

Head and neck cancers are composed of various kinds of epithelial malignant tumors, including oral cancers, maxillofacial cancers, larynx cancers, and many others, almost all of which are head and neck squamous cell carcinoma (HNSCC). Although, there are other pathological types such as verrucous carcinoma, basaloid squamous cell carcinoma, papillary squamous cell carcinoma, they only make up a small percentage (1). HNSCC is the 6th most common malignancy worldwide, with number of 650,000 new cases a year and 350,000 deaths (2). Around 2/3 of patients present with advanced disease, often with regional lymph node involvement, while 10% present with distant metastases (3). According to epidemiological survey, the 5-years survival rate of HNSCC in all stages was about 60%, and the survival rate was even worse for specific primary sites such as hypopharynx. The main causes of head and neck cancers are tobacco and alcohol consumption (1, 4–8). Chewing betel quid is also well-recognized as a risk factor for the cancer of oral cavity (9). And human papillomavirus (HPV) and p53 mutation are related to certain subsets of head

and neck cancers (10–12). About 25% of HNSCC contain HPV genomic DNA (13). However, HPV positivity is a favorable prognostic factor in HNSCC (14). Patients with HPV^+ HNSCC show better responsiveness to radiation, chemotherapy, or both, and might be more susceptive to immunosurveillance of tumor-specific antigens (14).

COMMON TREATMENT STRATEGIES FOR HEAD AND NECK CANCERS

The location of the cancers makes it necessary to take the spiritual and plastic factors into consideration. Primary tumor site, stage, and resectability are also treatment concerns as well as the patient factors such as swallowing, airway, organ preservation, and comorbid illnesses. For plan making, doctors are needed and organized from different departments which include head and neck surgeons, plastic surgeons, medical oncologists, radiation oncologists, radiologists, and dentists (2).

Common treatment strategies for head and neck cancers include surgery, radiotherapy, and chemotherapy. At present, surgery is still the standard therapy for HNSCC. However, surgical operations are limited, owing to the complexity of structures and the need for organ preservation. Most surgeons agree that the carotid artery, the base of the skull, and the invasion of the pre-vertebral muscle tissue are unresectable (2). Moreover, when the tumor is too extensive or there are multiple distant metastases, patients are generally not suitable for surgical treatment. Radiotherapy alone can improve the cure rate of early glottis, tongue, and tonsil cancers (15). However, prolonged interruption of radiotherapy or delayed post-operative radiotherapy may impair the patient's prognosis, which may be due to the proliferation of cancer cells (16). Delivery of radiation remains to be improved with continuous technological progress, and customization of radiation dose and volume (17). Chemotherapy is the core component of local advanced HNSCC treatment (18). Platinum compounds Cisplatin is a standard reagent for combination with radiotherapy or other drugs. Huperzine compounds are active and have been tested in locally advanced HNSCC chemotherapy (19, 20). Concurrent chemotherapy with normo-fractionated radiotherapy (2 Gy/day, 5 days/week, for 5–7 weeks) is used most in current practice (21).

Traditional therapy can result in serious complications, from pain to malnutrition, risk of infection, and psychological distress (21). In order to ameliorate these drawbacks, comprehensive treatments are currently preferred for the advanced tumors. Comprehensive treatments must be well-designed and planned according to the patient's general condition and the stage of tumor development. At present, the treatment of oral and maxillofacial malignant tumors emphasizes the comprehensive treatment based on surgery, especially the triple therapy, which combines surgery with radiotherapy and chemotherapy.

Modern research has been keen on identifying specific molecular targets involved in the occurrence and progression of head and neck cancers. EGFR and VEGF are two main targets which are overexpressed in majority of both precancerous oral lesions and HNSCC (22–24). EGFR can bind to and be activated by different ligands, including the epidermal growth factor (EGF) and transforming growth factor-α (TGF-α) (25). EGFR activation initiates subsequent signaling pathways, eventually resulting in tumor cell resistance to apoptosis and promoting angiogenesis, tumor cell migration, and tumor cell proliferation (**Figure 1**) (25, 26). Current EGFR-targeted therapies include monoclonal antibodies (mAbs) and tyrosine kinase inhibitors (TKIs). Antibodies target the extracellular domain of EGFR while TKIs hinder downstream signaling pathways by binding to the cytoplasmic region of EGFR (27). To date, Cetuximab remains the only FDA-approved EGFR-targeted mAb for the treatment of recurrent/metastatic (R/M) HNSCC. Cetuximab in combination with radiotherapy is a standard treatment option for locally or regionally advanced HNSCC (28). VEGF, is a key regulator of physiological angiogenesis during embryogenesis, skeletal growth, and reproductive functions (29). The biological effects of VEGF, mediated by two receptor tyrosine kinases (RTKs), VEGFR-1 and VEGFR-2, cause receptor TK activation and downstream signaling to stimulate endothelial cell proliferation, vessel permeability, and migration (27). Bevacizumab, a humanized monoclonal antibody targeting VEGF-A, was approved by the FDA for treatment of advanced cancer types. Bevacizumab could increase the sensitivity of HNSCC to radiotherapy in preclinical trials. Bevacizumab was evaluated in phase I and II clinical trials in combination with Erlotinib, an EGFR inhibitor, in patients with R/M HNSCC (30, 31) and the combined treatments increased the complete response rate by ∼15% and median survival by 7.1 months (30). The phase II trial on the combination of Bevacizumab with chemotherapy, radiotherapy or EGFR inhibitors are ongoing.

IMMUNOLOGICAL TARGETED THERAPY

Immunotherapies stimulate host antitumor immune system and can elicit endurable responses in subsets of patients across different types of tumors (**Figure 1**) (32). Immune checkpoints, like cytotoxic T-lymphocyte-associated antigen-4 (CTLA-4) and programmed cell death-1 (PD-1), work as inhibitory pathways, playing an important role in self-tolerance under healthy conditions. Checkpoint inhibitors are part of immunotherapies that enhance antitumor T cell activity by hindering initiation of suppressive signaling pathways of activated T cells. The 2018 Nobel Prize in Physiology or Medicine was recently given to James P. Allison and Tasuku Honjo for their discovery and contribution in cancer immunotherapy correlated with CTLA-4

Abbreviations: APC, antigen presenting cell; ATF, activating transcription factor; CRC, colorectal cancer; GEM, chemotherapy drug gemcitabine; GOx, glucose oxidase; HNSCC, head and neck squamous cell carcinoma; IGF, insulin-like growth factor; NFAT, nuclear factor of activated T cells; NSCLC, non-small cell lung cancer; ORR, objective response rate; OS, overall survival; PIP, phosphatidylinositol; PLGF, placental growth factor; RCC, renal cell carcinoma; ROS, reactive oxygen species; RTK, receptor tyrosine kinases; SAEs, severe adverse events; sPD-1/sPD-L1, soluble PD-1/ soluble PD-L1; TCR, T cell receptors; TGF, transforming growth factor; TILs, tumor-infiltrating lymphocytes; TKIs, tyrosine kinase inhibitors; TNF, tumor necrosis factor; T-NHL, T-cell non-Hodgkin's lymphoma; trAEs, treatment-related adverse events.