Oral cancer-associated fibroblasts inhibit heat-induced apoptosis in Tca8113 cells through upregulated expression of Bcl-2 through the Mig/CXCR3 axis

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Abstract. The aim of this study was to detect oral cancer-associated fibroblast-secreted cytokines and their regulation of heat-induced apoptosis in the human tongue squamous cell carcinoma cell line Tca8113. We isolated cancer-associated fibroblasts (CAFs) from human tongue squamous cell carcinoma and normal fibroblasts (NFs) from normal oral mucosa. The expression profiles of cytokines secreted by CAFs and those secreted by NFs were detected using the RayBio® human cytokine antibody microarray. The conditioned medium was prepared by mixing the CAF or NF supernatant cell culture medium with fresh complete medium. The expression levels of Bax, Bcl-2 and CXCR3 in Tca8113 cells were detected by western blot analysis. The heat-induced apoptosis ratio of the Tca8113 cells was detected by propidium iodide staining combined with flow cytometry. The quantity of the Mig factor, one of the chemokines secreted by CAFs, was clearly increased 19-fold when compared with the level in NFs. The conditioned medium of NFs had no obvious effect on the expression levels of Bax/Bcl-2 and the heat-induced apoptosis ratio in the Tca8113 cells. However, the expression levels of Bcl-2 were significantly upregulated and heat-induced Tca8113 cell apoptosis was inhibited in the CAF-conditioned medium. After adding neutralizing antibodies against Mig or its receptor CXCR3, the enhanced expression of the Bcl-2 protein and the inhibited heat-induced apoptosis of Tca8113 cells in the CAF-conditioned medium were significantly attenuated. CAFs may increase the expression levels of Bcl-2 through the paracrine secretion of Mig and reduce the thermosensitivity of Tca8113 cells.

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Introduction

Hyperthermia therapy is an important approach to tumor treatment. Acting as an external stimuli, high temperatures activate the mitochondrial apoptosis pathway to promote the apoptosis of tumor cells, which is regulated by the Bcl-2 gene family. Interference and disruption of the hyperthermia-induced apoptosis pathway stimulates tumor cell insensitivity to heat-induced cell apoptosis(1).

For many years, research on the effect and regulation of hyperthermia treatment has focused mainly on the tumor cell (2,3). However, the regulation of the thermal destruction of tumor cells in a stromal microenvironment is rarely investigated. Recent studies have shown that tumor development and treatment are not only determined by tumor cells but are also closely related to the balance of the tumor-stroma microenvironment (4,5).

The majority of tumor stromal cells are fibroblasts. Compared to normal fibroblasts (NFs), tumor fibroblasts are significantly altered. Tumor fibroblasts have certain biological characteristics of both fibroblasts and myofibroblasts and are also known as cancer-associated fibroblast cells (CAFs). CAFs produce a tumor extracellular matrix, which provides a suitable microenvironment to support the growth of tumors (6,7) and also to promote tumor cell proliferation, invasion and metastasis (8). Animal experiments have demonstrated that CAFs shorten the incubation period and increase the formation rate of tumor cells, reduce the dose of tumor cells required for tumor formation, promote the invasion and metastasis of tumor cells and reduce the effects of tumor treatment when tumor cells are injected into nude mice (7,9-11). Previous studies have shown that in *in vitro* models of pancreatic cancer, the efficacy of chemotherapy was significantly decreased and cell apoptosis was reduced when cancer cells were co-cultured with CAFs (12,13). It was also found, based on *in vitro* experiments, that the addition of CAFs in breast cancer enhanced the tolerance of the cancer to tamoxifen (chemotherapeutic drug) (14). In clinical studies, it was found that patients with rectal or pancreatic cancer had a poor prognosis if their pathological examination of surgical specimens showed high expression levels of α-SMA, which

Table I. Preparation of the conditioned medium.

	NF-conditioned medium			CAF-conditioned medium	
No.	NF supernatants	Complete medium	No.	CAF supernatants	Complete medium
1	0/6	6/6	4	0/6	6/6
2	1/6	5/6	5	1/6	5/6
3	2/6	4/6	6	2/6	4/6

indirectly proved that CAFs protect and promote tumor development (15,16).

As mentioned above, considering the regulation of the tumor stroma in cancer therapy, this study is focused on the main component of tumor stroma, CAFs, to screen differentially expressed secreted proteins in oral cancer CAFs and NFs using cytokine chip technology. Additionally, we aimed to explore the characteristics of the secreted factor of oral CAFs and the regulation of the oral CAFs in cell apoptosis of heat-induced Tca8113 cells through *in vitro* indirect co-culture of oral cancer CAFs and Tca8113 cells as a simulation of *in vivo* tumor-stroma interaction.

Materials and methods

Materials. The following materials were used in this study: tongue squamous carcinoma cell line Tca8113 (Shanghai Bio-Cell Bank, China); RPMI-1640 medium (Hyclone Biochemical Products, Beijing Co., Ltd., China); mouse anti-human α-smooth muscle actin, cytokeratin, vimentin monoclonal antibodies and EnVision immunohistochemistry kit (Fuzhou Maixin Biotechnology Development Co., Ltd., China); mouse anti-human Bcl-2, Bax, CXCL9/MIG, CXCR3 antibodies (USA RD Cos.); and RayBio® human cytokine antibody chip kit (RayBiotech, Inc., USA).

Fibroblast culture. Oral NFs and oral CAFs were isolated from normal oral mucosa and oral squamous cell carcinoma tissues confirmed by pathological analysis, respectively, following standard tissue culture instructions. Cells were cultured in an RPMI-1640 medium containing 10% FBS and incubated at 37˚C, in 5% CO_2 in a humidified incubator.

Tca8113 cell culture and heat treatment. The Tca8113 cells were cultured in an RPMI-1640 medium containing 10% FBS (complete medium) and incubated at 37˚C, in 5% CO_2 in a humidified incubator. Heat treatment was performed in a 43˚C water bath for 80 min.

Immunocytochemistry. Logarithmic-growing fibroblasts and Tca8113 cells were seeded and then fixed by cold acetone. Immunocytochemistry was performed using the EnVision immunocytochemistry kit, visualized by DAB and restained with hematoxylin.

Cytokine antibody microarray hybridization. The cells were cultured for 24 h and the culture supernatants were collected. Cytokine antibody microarray hybridization was performed using a RayBio® human cytokine antibody chip kit according to the manufacturer's instructions and the differences between the 2 groups were analyzed with ScanAlyze software after X-ray film exposure.

Preparation of conditioned medium. NFs and CAFs were cultured in an RPMI-1640 medium containing 10% FBS (complete medium) and the supernatants were collected. Conditioned medium was prepared by mixing the collected supernatants with a complete medium at a different volume ratio (Table I).

Antibody neutralization. Tca8113 cells were cultured with a conditioned medium containing 20 μg/ml of an antibody against CXCL9/MIG or CXCR3 for 24 h, and then incubated in a 43˚C water bath for 80 min.

Apoptosis analysis by flow cytometry. Cells were harvested, fixed with 70% cold ethanol and stained with propidium iodide (PI) staining solution (100 μg/ml PI, 0.1 mg/ml RNase, 0.1% Triton X-100). The DNA content was analyzed using Beckman Coulter Epics XL flow cytometry, and the apoptosis rate was calculated by using the sub-diploid peak prior to the G0/G1 phase as the apoptosis signal.

Western blot analysis. Cellular proteins were transferred to a PVDF membrane after the SDS-PAGE electrophoresis. The membrane was incubated with a primary antibody solution (1:1000 diluted in TBST) at 4˚C overnight, washed with 1X TBST, incubated with a secondary antibody solution (1:5000 diluted in TBST), washed with 1X TBST for 1 h and then exposed to film.

Statistical analysis. Statistical analysis was performed with SPSS17.0 statistical software using a one-way ANOVA and a comparison of the 2 groups was performed using an LSD test; $P<0.05$ was considered to indicate a statistically significant difference.

Results

Culture of fibroblasts. Oral mucosa NFs have an elongated spindle shape and have no cell-overlapping growth. However, oral tongue squamous CAFs have a short spindle shape with clear cell protuberances, they are irregular, multinucleated and have local cell-overlapping growth (Fig. 1). Immunocytochemical staining shows NFs are α-SMA negative, while CAFs are α-SMA positive (Fig. 2).

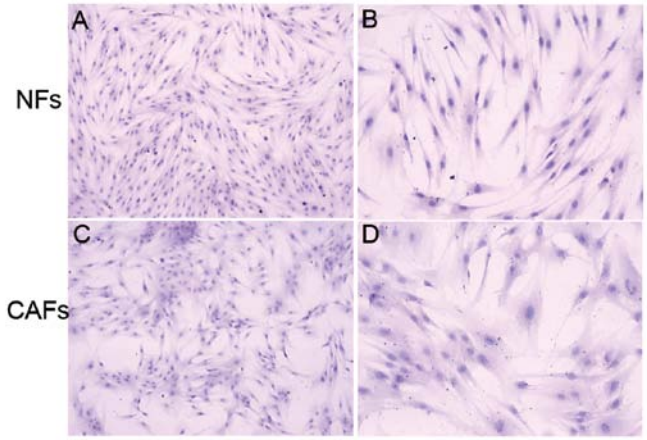

Figure 1. Hematoxylin and eosin (H&E) staining of fibroblasts. NFs have an elongated-spindle shape, in radial or spiral arrangement when they densely grow and have no cell-overlapping growth [(A) H&E x40, (B) H&E x100]. CAFs have a short-spindle shape, with clear cell protuberances. The CAFs are irregular, multinucleated and have local cell-overlapping growth [(C) H&E x40, (D) H&E x100].

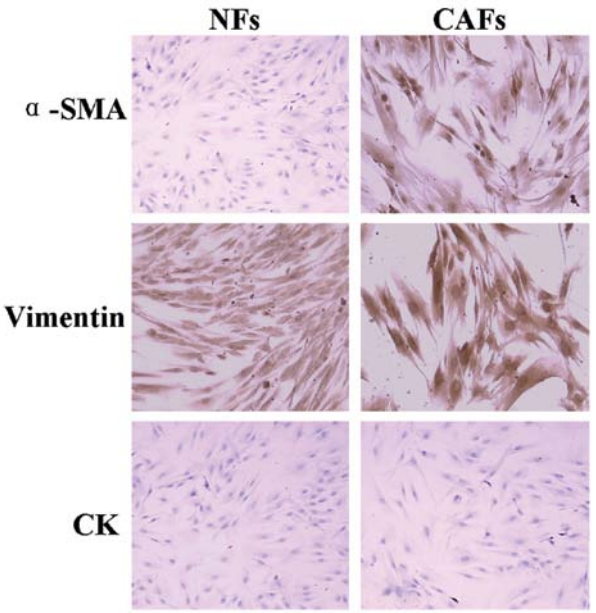

Figure 2. Immunohistochemical EnVision staining of fibroblasts. Oral mucosal NFs are α-SMA negative, vimentin positive and CK negative (EnVision x100). CAFs are α-SMA positive, vimentin positive and CK negative (EnVision x100).

Cytokine microarray hybridization. Cytokines secreted by CAFs and NFs were measured by the Human-L Series of protein factors (RayBio), which simultaneously measures 507 types of protein factors, including growth factors, interleukins, tumor necrosis factor and chemokines. The results showed that the following CAF-secreted cytokines significantly increased: CD40/TNFRSF5 (4.39-fold of NFs), IL-17C (6.55-fold of NFs), MFRP (16.70-fold of NFs), MIG (19.22-fold of NFs), NCAM-1/CD56 (4.60-fold of NFs), NeuroD1 (4.42-fold of NFs) and Smad4 (5.95-fold of NFs). The cytokines which were significantly reduced were CCL28/VIC (0.09-fold of NFs), GFR α-1 (0.08-fold of NFs), Granzyme A (0.03-fold of NFs), Neuregulin

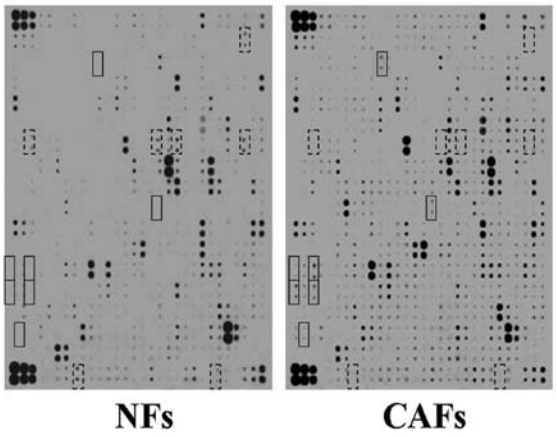

Figure 3. Chemiluminescence of the cytokine microarray. Hybridization results of NF or CAF supernatant using RayBio human cytokine antibody microarray. The cytokines which were significantly increased as shown in the solid line boxes were: CD40/TNFRSF5 (rows 5 and 6, column 11), IL-17C (rows 19 and 20, column 17), MFRP (rows 25 and 26, column 1), MIG (rows 25 and 26, column 3), NCAM-1/CD56 (rows 27 and 28, column 1), NeuroD1 (rows 27 and 28, column 3) and Smad4 (rows 31 and 32, column 2). The cytokines which were significantly reduced as shown in the dotted line boxes were: CCL28/VIC (rows 3 and 4, column 27), GFR α-1 (rows 13 and 14, column 3), Granzyme A (rows 13 and 14, column 18), GRO (row 13 and 14, column 20), Heregulin/NDF/GGF/Neuregulin (rows 13 and 14, column 28), TSLP (rows 35 and 36, column 8) and WISP-1/CCN4 (rows 35 and 36, column 25).

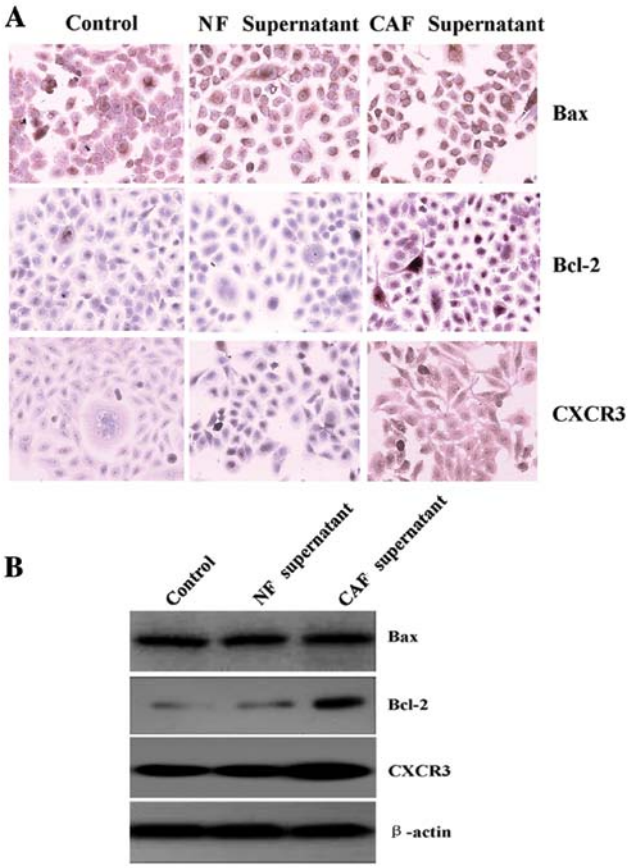

Figure 4. CAFs increased the expression of Bcl-2 and CXCR3, but not Bax in the Tca8113 cells. (A) Immunohistochemical staining of the Tca8113 cells (EnVision x200). (B) Western blot analysis, with β-actin as the loading control, detected the total proteins of the Tca8113 cells and the expression levels of Bax, Bcl-2 and CXCR3. Cells that were treated with a 2/6 volume of NF or CAF supernatant were extracted using western blot analysis.

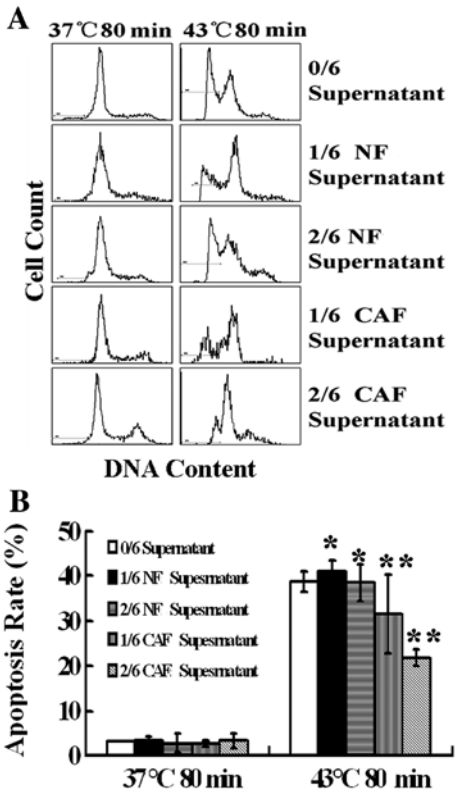

Figure 5. CAF supernatant inhibits heat-induced Tca8113 cell apoptosis. (A) The apoptosis rate was determined by flow cytometry using propidium iodide staining. The sub-G1 population was used to represent the apoptotic rate. (B) The results were obtained from 3 independent experiments; bars, ±SD. *Group compared with 0/6 supernatants and heated at 43˚C for 80 min, P>0.05. **Group compared with 0/6 supernatants and heated at 43˚C for 80 min, P<0.05.

(0.04-fold of NFs), GRO (0.08-fold of NFs), TSLP (0.09-fold of NFs) and WISP-1/CCN4 (0.06-fold of NFs) (Fig. 3).

CAF supernatant promotes Tca8113 cell expression of Bcl-2 and CXCR3. The expression level of Bax in the Tca8113 cells had no obvious change, after treatment with a 2/6 volume NF- or a CAF-conditioned medium supernatant. After treatment with a 2/6 volume NF-conditioned medium supernatant, expression levels of Bcl-2 and CXCR3 in the Tca8113 cells had no obvious changes. However, after treatment with a 2/6 volume CAF-conditioned medium supernatant, the expression levels significantly increased (Fig. 4).

CAF supernatant inhibits heat-induced Tca8113 cell apoptosis. Heat treatment at 43˚C for 80 min induced Tca8113 cell apoptosis, and the apoptosis rate was 38.8% according to propidium iodide staining combined with flow cytometry. After treatment with a 1/6 and 2/6 volume of the NF supernatant, the apoptosis rates were 41.06 and 38.43% (P>0.05), respectively; thus, there was no significant effect on the heat-induced cell apoptosis rate. After treatment with a 1/6 volume of CAF supernatant, the apoptosis rate was 31.6%. After a pretreatment with a 2/6 volume of CAF supernatant, the rate of apoptosis was only 21.8% (P<0.05), significantly inhibiting the heat-induced Tca8113 cell apoptosis (Fig. 5).

CAFs enhance Bcl-2 expression and inhibit heat-induced Tca8113 cell apoptosis through the Mig/CXCR3 axis. As mentioned above, the protein level of the Mig factor in the CAFs was significantly higher than that in the NFs, and the expression level of CXCR3 in the Tca8113 cells significantly increased

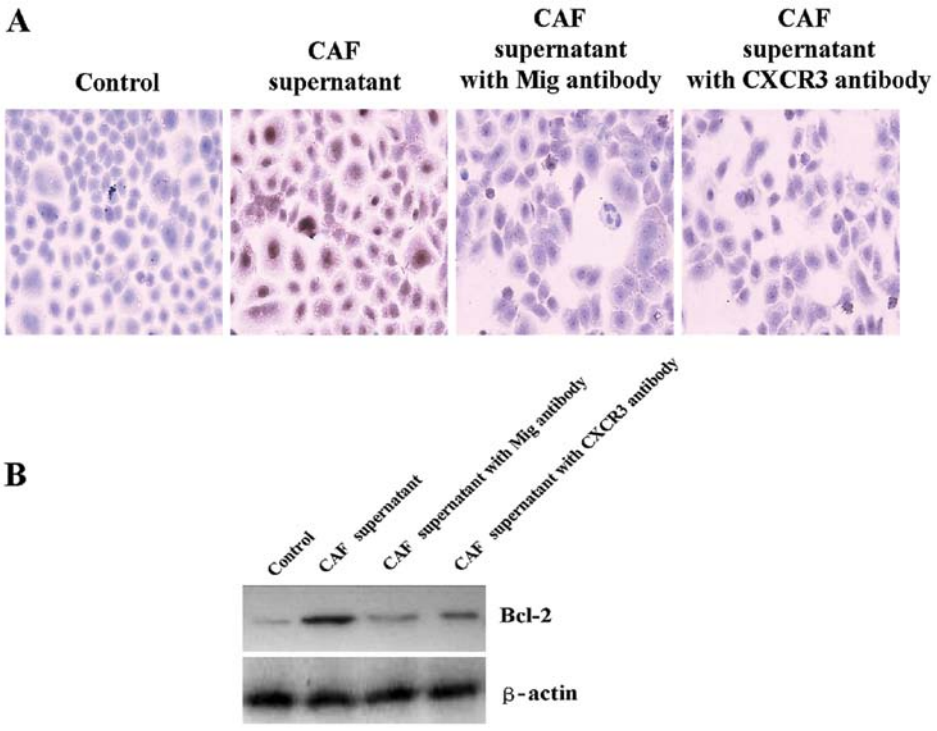

Figure 6. Neutralizing antibodies against Mig factor and CXCR3 reduced enhanced expression of Bcl-2 protein, which was originally induced by the CAFs. (A) Immunohistochemical staining of the Tca8113 cells (EnVision x200). (B) Western blot analysis: total proteins of the Tca8113 cells were extracted and the expression level of Bcl-2 was detected by western blot analysis, with β-actin as a loading control.

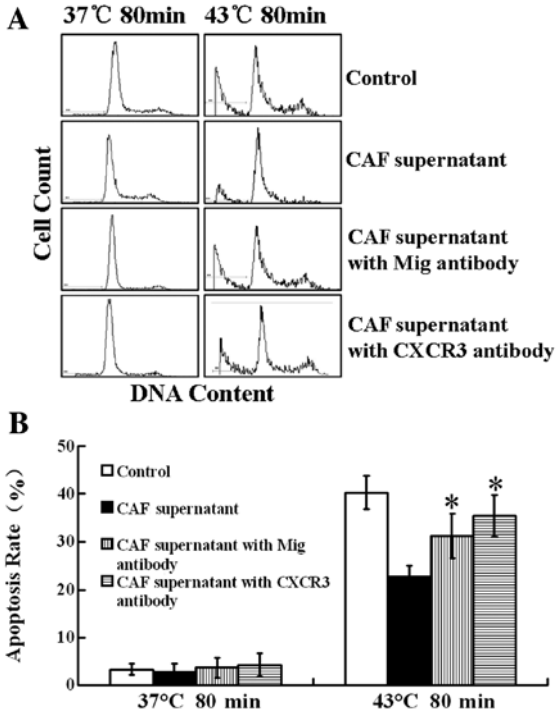

Figure 7. Blocking Mig or CXCR3 with neutralizing antibodies rescues heat-induced Tca8113 cell apoptosis. (A) The apoptosis population was determined with flow cytometry by propidium iodide staining. The sub-G1 population was used to represent the apoptotic population. (B) The results were obtained from 3 independent experiments; bars, ±SD. *Group compared with CAF supernatants and heated at 43˚C for 80 min, P<0.05.

after treatment with the CAF supernatant. Therefore, we used neutralizing antibodies against the Mig factor or its receptor CXCR3, treated the Tca8113 cells with a 2/6 volume of CAF supernatant and tested the expression of the Bcl-2 protein in the Tca8113 cells using western blot analysis. We tested the rate of the heat-induced Tca8113 cell apoptosis using flow cytometry. The results showed that after neutralizing antibodies against the Mig factor or its receptor CXCR3, enhanced expression of Bcl-2 protein in Tca8113 and inhibition of heat-induced Tca8113 cell apoptosis in CAF supernatant were significantly reduced. After a heat treatment at 43˚C for 80 min, the heat-induced Tca8113 cell apoptosis rate was 40.3%. After pretreatment with a 2/6 volume CAF supernatant and heat treatment at 43˚C for 80 min, the apoptosis rate dropped to 22.6%. After adding neutralizing antibodies against the Mig factor, the apoptosis rate increased to 31.2%. After adding a neutralizing antibody against CXCR3, the apoptosis rate increased to 35.4% (Figs. 6 and 7). These results suggest that CAFs may enhance Bcl-2 expression and inhibit heat-induced cell apoptosis through the Mig factor and its receptor CXCR3.

Discussion

A tumor is composed of tumor cells, extracellular matrix and a vascular system. The majority of tumor stromal cells are fibroblasts. CAFs produce a tumor extracellular matrix that provides a suitable microenvironment to support the growth of the tumor (6,7). Through their interaction with tumor cells, CAFs alter their morphological and physiological characteristics, express various cytokines, proteases, adhesion molecules and increase the malignant phenotype of tumor cells to different degrees. These events play an important role in cancer proliferation, development, invasion and metastasis (8).

In this study, we derived CAFs from oral tongue squamous carcinoma tissue. Compared with NFs, the biological characteristics of the CAFs were altered. The secreted factors in the CAFs and NFs were significantly different as determined by the cytokine microarray test. Compared with the NFs, the levels of the CAF-secreted cytokine MIG was significantly higher.

Hyperthermia not only induces cell apoptosis but may also have a direct lethal effect on a tumor. Commonly used temperatures in clinical hyperthermia are between 40 and 45˚C, and the death of tumor cells is mainly caused by cell apoptosis during this treatment (17). For many years, research on the effect and regulation of hyperthermia treatment has been mainly focused on the tumor cell itself; for example, examining the relationship between the expression levels of apoptosis-related proteins and heat shock proteins in tumor cells and the efficacy of hyperthermia (2,3). However, the regulation of the thermal destruction of tumor cells in the stromal microenvironment is rarely investigated. Recent studies have shown that the incidence of tumor development and treatment is not simply determined by tumor cells or stroma. It is closely related to the balance of the tumor-stroma microenvironment formed through the interaction between both the tumor and the stroma (4,5). CAFs are the most important host cells in this microenvironment, and they play an important role in regulating the balance of the system, which is dependent on direct cell-to-cell contact, secretion of soluble factors and extracellular matrix modification (18,19). Studies have shown that CAFs promote tumor development and enhance tumor capacity to fight against radiation therapy and chemotherapy (12-14). In this study, we derived CAFs from oral tongue squamous carcinoma, established an *in vitro* co-culture model for CAFs and Tca8113 cells and found that the CAF supernatant significantly inhibited heat-induced Tca8113 cell apoptosis. Heat treatment at 43˚C for 80 min induced Tca8113 cell apoptosis, and the apoptosis rate was 38.8%. After treatment with a 1/6 or 2/6 volume NF supernatant, there was no significant effect on the heat-induced cell apoptosis rate of Tca8113. However, after treatment with a 1/6 volume CAF supernatant, the rate was impacted and lowered to 31.6%. A pretreatment with a 2/6 volume CAF supernatant significantly inhibited the heat-induced Tca8113 cell apoptosis and the rate of apoptosis was only 21.8%. These results suggest that CAFs significantly enhance the ability of cancer cells to fight against thermal destruction. Furthermore, the tumor microenvironment is another important factor in determining the efficacy of hyperthermia.

A high temperature also inhibits the expression of the anti-apoptotic genes, such as Bcl-2, activates apoptosis-related pathways and induces cell apoptosis (1,17,20,21). Interference and disruption of a hyperthermia-induced apoptosis pathway may induce tumor cells which are no longer sensitive to the stimulation of cell apoptosis. Our previous studies found that the intracellular expression of Bax and Bcl-2 is one of the intrinsic factors determining the efficacy of hyperthermia. In this study, we tested the expression levels of Bax and Bcl-2 in Tca8113 cells after treatment with CAF and NF supernatants, and we found that the expression level of Bax was not affected. We also found

that the expression level of Bcl-2 after the NF treatment was not affected whereas the level was significantly enhanced after the CAF treatment. These results suggest that CAFs promote the expression of an anti-apoptotic gene, Bcl-2, enhance tumor resistance to heat-induced apoptosis and mediate thermal tolerance.

As mentioned above, the secreted cytokines of CAFs are significantly different from the secreted cytokines of NFs. CAFs can mediate the heat tolerance of cancer cells dependent on these factors. Comparing secreted cytokines of oral cancer CAFs with those of NFs shows that the quality of the Mig factor, one of the chemokines, is clearly increased by 19-folds compared with that in NFs. Recent studies have shown that the chemokine receptor, CXCR3, is highly expressed in a variety of tumor cells. Furthermore, specific binding of CXCR3 with its ligand chemokine Mig (CXCL9), IP-10 (CXCL10) or I-TAC (CXCL11) regulates tumor invasion, metastasis, survival and proliferation (22-24). The results from tissue-specific analysis and research among various types of tumors are varied, which may be related to different shearing variants of CXCR3 and the culture conditions of *in vitro* studies, such as the presence or absence of serum (25). In this study, we found that Tca8113 cells expressed CXCR3, and the expression level of CXCR3 in the Tca8113 cells increased after treatment with the CAF supernatant. Furthermore, we treated the Tca8113 cells with the CAF supernatants while using neutralizing antibodies against the Mig factor or its receptor CXCR3, and we found that this enhanced the expression of the Bcl-2 protein in Tca8113 cells and that the inhibition of heat-induced Tca8113 cell apoptosis in the CAF supernatant was significantly reduced.

In summary, secreted cytokines of oral tongue squamous cell carcinoma CAFs are significantly different from NFs. CAFs increase the expression level of the Bcl-2 protein in Tca8113 cells, enhance resistance to heat-induced cancer cell apoptosis and mediate thermal tolerance. Further studies should investigate whether or not CAFs initiate cancer-related downstream signaling pathways and regulate the expression and activation of target proteins to regulate heat-induced tumor cell apoptosis by secreting the Mig chemokine factor to directly bind the CXCR3 receptor of cancer cells, the Mig/CXCR3 paracrine axis.

Acknowledgements

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