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ORIGINAL ARTICLE

Separation, cultivation and biological characteristics of oral carcinoma-associated fibroblasts

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OBJECTIVES: Carcinoma-associated fibroblasts (CAFs) have been suggested to regulate the initiation and progression of many types of solid tumors. The aim of the study was to separate, cultivate, identify oral CAFs, and to investigate their biological characteristics.

MATERIALS AND METHODS: The primary CAFs and normal fibroblasts (NFs) of the tongue were obtained by tissue culture. Then cells were dissociated by 0.25% trypsin and purified by curettage method combining with trypsinization. The cells were verified according to morphological observation and immunohistochemical staining of certain proteins. Multiple proliferation indexes and karyotype of the cells were assayed.

RESULTS: Third passage purified oral CAFs and NFs were attained successfully. The morphological characteristics of the CAFs changed significantly comparing to the NFs. The CAFs showed positive staining for vimentin, α-smooth muscle actin and matrix metalloproteinases-2. The proliferation and mitosis ability of the CAFs were significantly increased compared with the NFs ($P < 0.05$). No karyotypic abnormalities were found in the CAFs.

CONCLUSIONS: There were obvious differences in the biological characteristics between oral CAFs and NFs. The results may provide us an experimental foundation for further studies on the roles of CAFs in the initiation and progression of oral cancer.

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Keywords: oral cancer; carcinoma-associated fibroblast; cell culture; biological characteristics

Introduction

The precise mechanisms of formation and development of oral squamous carcinoma are not known with certainty. Past research mainly focused on the oral epithelial or mesenchymal components alone, while ignoring the important role of mesenchyme–epithelium interactions in carcinogenesis in the oral mucosa. In recent years, it has been realized that the microenvironment of the tumor–host interface composed of aberrant epithelium and adjacent stroma provides a nutrient soil for survival and development of tumor cells (Liotta and Kohn, 2001; Radisky and Bissell, 2004). The primary host cells – carcinoma-associated fibroblasts (CAFs) – exert an important influence on both invasion and metastasis of the tumor (Tuxhorn *et al*, 2002; Wang and Tetu, 2002). Because we found few reports of oral CAFs, we separated, cultured and identified oral CAFs and investigated their biological characteristics. The results may lay the foundation for further studies on the roles of CAFs in the oral tumor–host microenvironment.

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Materials and methods

Specimens

Fresh, sterile tissue specimens, including oral squamous carcinoma and normal oral mucosal tissue, were attained after obtaining informed consent from patients in our clinic. The position of the resected tissue and the sex and age of subjects were required to be consistent. All samples contained epithelium and adjacent connective tissues. The pathological or normal state of the samples was confirmed by histopathological and clinical examination.

Primary culture reagents

Basic DMEM medium (Gibco, Grand Island, NY, USA); synthetic DMEM medium (pH 7.2) containing calf serum (10%), glutamine (20 μg ml^{-1}), penicillin (100 U ml^{-1}) and streptomycin (100 μg ml^{-1}); 0.25% trypsinase (Gibco); 100% dimethylsulfoxide (DMSO; Sigma, St Louis, MO, USA); 0.05% colchicine (Sigma); 0.5% 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma).

Separation, cultivation and purification of oral CAFs

The specimens were washed with phosphate-buffered solution (PBS) and antibiotics, and then the epithelial and

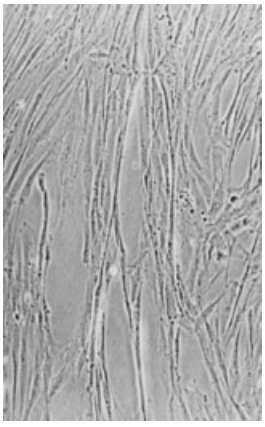

Figure 1 Oral carcinoma-associated fibroblasts at passage 3 (phase-contrast microscope, ×200)

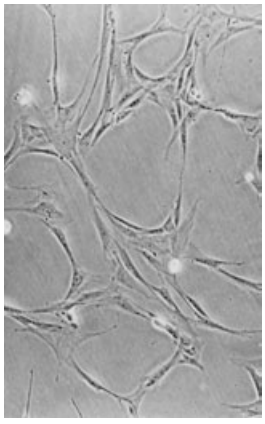

Figure 2 Oral normal fibroblasts at passage 3 (phase-contrast microscope, ×200)

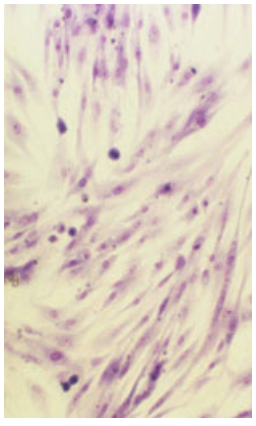

Figure 3 Oral carcinoma-associated fibroblasts at passage 3 (Giemsa stained, light microscope, ×100)

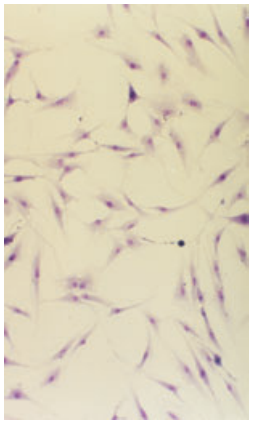

Figure 4 Oral normal fibroblasts at passage 3 (Giemsa stained, light microscope, ×100)

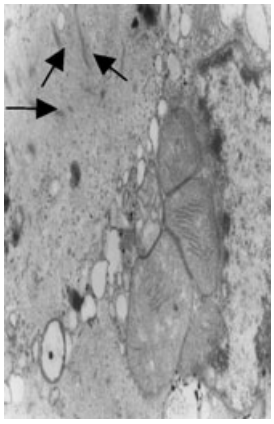

Figure 5 Oral carcinoma-associated fibroblasts at passage 3. Arrows indicate myofilaments and dense patches (TEM, ×12 000)

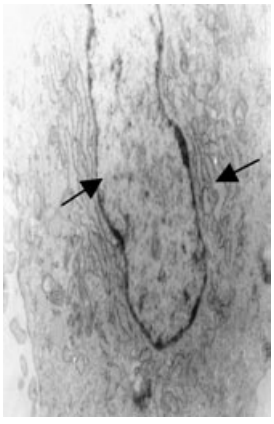

Figure 6 Oral normal fibroblasts at passage 3. Arrows indicate irregular nuclei and endoplasmic reticulum (TEM, ×8000)

adipose tissues were pruned away from the specimens. The residual connective tissue was cut into small pieces (1 mm × 1 mm × 1 mm) and the tissues were placed in synthetic DMEM medium and incubated at 37°C. The medium was replaced every 2–3 days. When the cells fully covered the bottom of the culture bottle, areas in which epithelial cells had grown in groups were wiped with small amount of trypsinase to remove them. The remaining cells were digested with trypsinase for 2 min. Once shrinkage of the cytoplasm of cells and increased refraction were

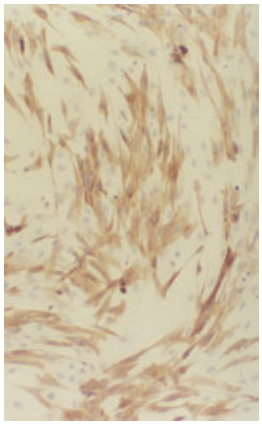

Figure 7 Oral carcinoma-associated fibroblasts at passage 3, positive staining with α-smooth muscle actin antibody (SP, ×100)

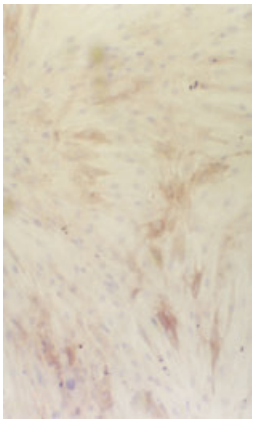

Figure 8 Oral carcinoma-associated fibroblasts at passage 3, positive staining with matrix metalloproteinase-2 antibody (SP, ×100)

Table 1 Cell count ($\times 10^4$)

Cell type	*Day 1*	*Day 2*	*Day 3*	*Day 4*	*Day 5*	*Day 6*	*Day 7*	*Day 8*
Carcinoma-associated fibroblasts	5.50	5.15	12.00	25.00	33.00	36.00	37.00	38.00
Normal fibroblasts	6.15	6.00	8.50	16.00	24.00	26.00	27.00	28.00

found under phase-contrast microscope, digestion was terminated with 2 ml of medium containing serum. Cell suspensions were prepared by gently blowing cells repeatedly with a pipette. Cells were then counted and inoculated in fresh medium. Cultures at passage 3 were used for the studies for cell identification and determining the biological characteristics of the oral CAFs.

Normal fibroblasts (NFs) from the oral mucosa were also cultivated using the methods described above for CAFs.

Identification of oral CAFs

Cell morphology and ultrastructure

Comparison of the purified passage 3 CAFs and NFs was performed by phase-contrast, light and transmission electron microscopy.

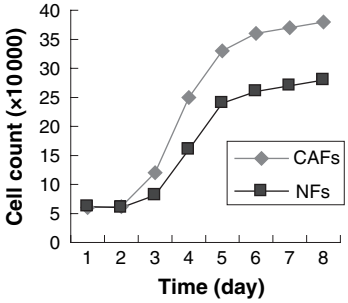

Figure 9 Cell growth curves

Table 2 Karyokinesis count (cells per thousand cells)

Cell type	*Day 1*	*Day 2*	*Day 3*	*Day 4*	*Day 5*	*Day 6*
Carcinoma-associated fibroblasts	17	45	75	20	3	0
Normal fibroblasts	10	26	49	15	1	0

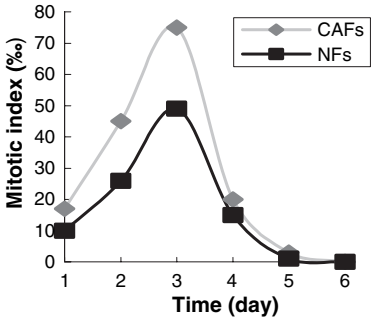

Figure 10 Cell mitotic index curves

Immunohistochemistry

Coverslips with attached cells were fixed with formaldehyde for 5 min. Staining procedures using the SP kit (Zymed, San Francisco, CA, USA) were performed according to the manufacturer's specifications. The following primary antibodies were used: anti-human cytokeratin (antibodies to all types of cytokeratins; Dako, Carpinteria, CA, USA), anti-human vimentin (Dako), anti-human α-smooth muscle actin (α-SMA; Dako), anti-human matrix metalloproteinase-2 (MMP-2; Dako). PBS was used as the negative control, in place of the primary antibody (both blank and isotype control could be used as negative control). To validate the antibody-binding specificity of these commercialized antibodies and also to choose one effective means of negative control, we first took preliminary test and both PBS and mouse or rabbit non-immune immunoglobulin G were used as the negative control, in place of the primary antibodies. The results of the preliminary test were satisfactory. In our experiments, we chose the blank control. Paraffin-stained sections of normal oral mucosa, leiomyosarcoma, and breast carcinoma were used as positive controls. Cells showing light brown or yellow brown grains in the cytoplasm were classified as positively stained.

Table 3 Cell viability (MTT absorbance values)

Cell type	*Day 1*	*Day 2*	*Day 3*	*Day 4*	*Day 5*	*Day 6*	*Day 7*
Carcinoma-associated fibroblasts	0.083 ± 0.003	0.080 ± 0.004	0.156 ± 0.019	0.319 ± 0.034	0.383 ± 0.017	0.421 ± 0.022	0.478 ± 0.025
Normal fibroblasts	0.083 ± 0.001	0.076 ± 0.006	0.084 ± 0.001	0.162 ± 0.006	0.179 ± 0.009	0.204 ± 0.007	0.220 ± 0.010

Figure 11 Karyotype analysis of the oral carcinoma-associated fibroblasts

Figure 12 Karyotype analysis of the oral normal fibroblasts

Proliferative characteristics of oral CAFs
Growth curves and population doubling time
The purified passage 3 CAFs and NFs (6×10^4 ml^{-1}) were mixed with equal amounts of medium respectively and seeded in 24-well plates. Three wells were classified as a group. Cell numbers in a group were recorded every 24 h and the mean values were calculated. The basic principle and method of cell record is just the same as that of hemocyte record and the results were expressed as cell numbers in per unit volume (n ml^{-1}). Cells were monitored for seven consecutive days. The growth curves and population doubling time (DT) were obtained, where $DT = (t-t_0) \log 2/(\log N - \log N_0)$ (t and t_0 represent the final and initial time of cultivation, respectively; N and N_0 represent cell numbers at t and t_0, respectively).

Mitotic index curves
Well-growing cells were inoculated into culture dishes containing coverslips. One coverslip was removed every 24 h, fixed with formaldehyde for 5 min, and stained with Giemsa stain. Regions with appropriate cell density were selected and mitotic figures were identified by light microscopy using high magnifications. The number of mitotic cells in 1000 cells was recorded and the mitotic index calculated. Mitotic index curves were drawn from the data of seven consecutive days.

Viability (MTT method)
Cells were seeded (4×10^3 per well) into 96-well plates in 200 μl of medium. MTT (10 μl) was added to three wells every 24 h. Incubation was terminated 4 h after the addition of MTT; the supernatant fluid was removed, 50 μl of DMSO was added to each well, and the plate was gently shaken for 10 min. Three wells per day were examined for seven consecutive days. Absorbance values were measured at 490 nm using an enzyme-linked immunosorbent assay reader.

Statistical analysis
Statistical analysis was performed using SPSS 11.0 software (SPSS Inc., Chicago, IL, USA). Values were compared using paired-design t-tests.

Karyotype analysis of oral CAFs
Cells in log phase were cultivated with 50 μl of colchicine for 4 h, and mitotic cells were gently blown with a pipette. Cells were centrifuged at 235 g for 10 min and the supernatant fluid removed. KCl (0.075 mol l^{-1}; 37°C) was added to the cells and the mixture was incubated at 37°C for 30 min. Then the cells were fixed with fresh fixative. Cell suspensions were dropped onto slides at −10°C from a distance of 15 cm. The slides were air dried and then stained with Giemsa stain for 20 min. The slides were observed by oil immersion light microscopy. Analysis was performed using Visus 2.1 software (Visus Imaging, Foresthill, CA, USA).

Results

Separation, cultivation and purification of oral CAFs
Primary cells
After 2 days of incubation, cancerous epithelial cells and CAFs had grown from the tissue pieces: the former grew in groups while the latter grew radially. The cells fully

covered the bottom of the culture dish after about 5 days. In contrast, NFs grew slowly: sparse NFs were seen around the tissue after 4 days of incubation, and cells fully covered the bottom of the culture dish after about 10 days. Few normal epithelial cells were seen.

Passage of cells
No epithelial cells were found in passage 3 cells. There were no obvious differences before and after passage of CAFs; however, the growth speed increased. CAFs were distinguished from NFs in their morphology and growth pattern.

Identification of oral CAFs
Observations under the phase-contrast microscope
Carcinoma-associated fibroblasts were long-spindle shaped cells with small cytoplasmic protrusions. The cell size was variable, and the cells were crowded together in a disorderly arrangement. Overlapping of cells was observed, and both contact and density inhibition had been lost (Figure 1). In contrast, NFs were flat-star shaped cells with more pronounced cytoplasm protrusions. The cell size was consistent, and the cells were arranged in an orderly manner. No overlapping of cells was seen, and contact and density inhibition were preserved [Figure 2].

Observations under the light microscope (Giemsa staining)
Carcinoma-associated fibroblasts had a large cell volume and irregular morphology, and the cytoplasm and nuclei exhibited light and dark purple staining, respectively (Figure 3). Apocytes with two or three nucleoli were seen (apocyte means the cell that has more than one nucleus in its cytoplasm. It suggested that the proliferation ability of CAFs increased). In contrast, NFs had regular morphology. The nuclei were oval and the cytoplasm stained homogeneously (Figure 4).

Observations under the transmission electron microscope
Carcinoma-associated fibroblasts presented homogeneous staining in the nuclei. The nucleoli were obvious. Many endoplasmic reticula, mitochondria and parallel bundles of myofilaments were seen in the cytoplasm. Electron-dense patches resembling those in smooth muscle were dispersed within the cells (Figure 5). NFs showed irregular nuclear morphology, and the endoplasmic reticulum and Golgi complex were abundant; myofilaments and dense patches were not found (Figure 6).

Immunohistochemical staining results
Besides cytokeratins, CAFs showed expression of vimentin, α-SMA and MMP-2 (Figures 7 and 8). In contrast, NFs showed positive staining for vimentin only.

Proliferation of oral CAFs
Growth curves and population doubling time
With the same culture conditions, number of seeded cells and observation time, the proliferation speed of the CAFs was significantly higher compared with the NFs ($P < 0.05$) (Table 1, Figure 9). The population DT of the CAFs and the NFs were 60.2 and 76.6 h, respectively.

Mitotic index curves
The mitotic index of the CAFs was higher than that of the NFs, and the proliferation ability of the CAFs was significantly increased compared with the NFs ($P < 0.05$) (Table 2, Figure 10).

Viability
The CAFs showed higher proliferation and viability compared with the NFs ($P < 0.05$) (Table 3).

Karyotype analysis of oral CAFs
There were no obvious differences in karyotype between CAFs and NFs (Figures 11 and 12).

Discussion

Separation, cultivation and purification of oral CAFs
We have successfully separated and cultured oral CAFs. From our research, we have noted that (a) the squamous carcinoma specimen should be fresh and infiltrating the adjacent connective tissue; (b) the connective tissue being cut should be as close as possible to the epithelium; and (c) because epithelial cells and fibroblasts have different tolerance to trypsinase, we should pay special attention to the digestion time, so as to collect the cells needed at the appropriate time. With careful observation under the phase-contrast microscope, once the shrinkage of cytoplasm and increased refraction were found in most of the fibroblasts, digestion was terminated with 2 ml of medium containing serum. When the wall of the bottle was gently blown with a pipette, the fibroblasts were shed from the wall while epithelium cells were still attached to the wall. Thus the suspension of fibroblasts were obtained and inoculated.

The primary CAFs are usually mixed with epithelial cells, and the latter may grow either in groups or scattered among the CAFs. The method of curettage combined with trypsinization was chosen to remove epithelial cells Zhang and Yu (2002). The technique were operated as described in Materials and methods: separation, cultivation and purification of oral CAFs. Briefly, epithelial cells grown in groups were first wiped with small amount of trypsinase, then the remaining cells were digested by trypsinase with careful observation under a phase-contrast microscope. Compared with previous studies using trypsinization alone, our procedure was more effective in eliminating the epithelial cells. Using immunohistochemistry for vimentin and cytokeratins – with positive and negative staining respectively – the fibroblastic features of the CAFs were further verified.

Identification of oral CAFs
Qualitative analysis of oral CAFs was performed by observing their morphology and ultrastructure and by studying the expression of certain proteins. There were

marked differences between CAFs and NFs. The CAFs are long spindle-shaped cells with decreased cytoplasmic protrusions; some characteristics of smooth muscle cells were also apparent (myofilaments and electron dense patches). The CAFs also have acquired the proliferative ability at the edge of tumors, losing density-dependent growth inhibition and directed mobility.

Both oral CAFs and NFs were positive for vimentin and negative for cytokeratins, which suggested that some fibroblast traits were preserved in the CAFs. Importantly, the CAFs presented some characteristics of smooth muscle cells, with the expression of certain proteins. Some reports have shown that α-SMA and MMP-2 could be detected in prostate and breast CAFs, but not in NFs (Olumi *et al*, 1999; Chrenek *et al*, 2001). Our results are similar to those of the previous studies. Overall, the oral CAFs presented features of both fibroblasts and smooth muscle cells, being fibroblasts with phenotype transformation – myofibroblasts (Wang and Tetu, 2002). Thus, CAFs could be identified preliminarily by their special features.

Proliferative characteristics of oral CAFs

Proliferation is one of the most important biological characteristics of cells. In our studies, multiple indexes such as population DTs, growth curves, mitotic index curves and viability were used to estimate different aspects of proliferative ability. The general results demonstrated that the CAFs acquired stronger ability in growth speed, proliferation and viability compared with the NFs.

Several reasons may have contributed to the above changes in the CAFs. (a) The CAFs acquired the characteristics of both fibroblasts and smooth muscle cells, which made them more proliferative (Olumi *et al*, 1999). (b) The expression of peripheral myelin protein (PMP22) and mRNA was significantly reduced in CAFs compared with NFs (these areas were not dealt with in our study, but they could be the subject of future research). PMP22 has been described as protein that inhibits the G_0 phase of the cell cycle and the downregulation of PMP22 leads to a shortened cell cycle and enhanced proliferative ability (Kunz-Schughart *et al*, 2001). If CAFs are located in a tumor–host microenvironment, their proliferation would also be affected by the following factors: (a) the large amount of growth factors secreted by tumor cells could promote clonal growth of CAFs (Elenbaas and Weinberg, 2001); (b) the extracellular matrix may modulate the morphology, proliferation and differentiation of the CAFs by stimulating specific receptors at the cell surface (Tuxhorn *et al*, 2001); (c) other host cells, such as activated macrophages, endothelial cells and mast cells secrete various cytokines to regulate the proliferation of the CAFs, directly or indirectly (Lieubeau *et al*, 1999).

The karyotype of CAFs

Cells possess a certain number, shape and structure of chromosomes, which are kept very stable in the normal cell cycle. When malignant transformation occurs, the karyotype of the cells undergoes obvious changes. Therefore, karyotype analysis is one of the major methods to distinguish normal cells from malignant cells Zhang and Yu (2002). We observed no obvious differences in karyotype between the oral CAFs and the NFs, which suggests that the basic characteristics of the normal cells were maintained in the CAFs, and there were no malignant transformations in the CAFs. This may explain why CAFs alone grafted into nude mice could not form tumors.

Conclusions

The tumor–host microenvironment might be the main factor contributing to phenotype transformation in oral fibroblasts. The above-mentioned changes in the biological characteristics of the oral CAFs may provide us an experimental foundation for further studies on the regulating roles of CAFs in the oral tumor–host microenvironment.

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