Role of autophagy and apoptosis in atrophic epithelium in oral submucous fibrosis

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Abstract: Oral submucous fibrosis (OSF) is a serious, potentially malignant oral disorder. It is histopathologically characterized by chronic inflammation and atrophic epithelium accompanied by the accumulation of collagen fibers in the lamina propria. The molecular mechanisms leading to atrophic epithelium remain poorly understood. Therefore, the present study investigated the role of autophagy and apoptosis in atrophic epithelium in OSF. The expression of Caspase-3 and autophagy-related proteins (LC3 and P62) in OSF epithelial tissues was quantified by immunohistochemistry. In addition, the influence of apoptosis and autophagy marker proteins in OSF epithelial and normal oral mucosal tissues using immunohistochemistry. In addition, the influence of apoptosis and autophagy on human oral keratinocytes (HOKs) treated with arecoline and role of autophagy in arecoline-induced apoptosis were investigated.

Keywords: apoptosis, arecoline, autophagy, oral submucous fibrosis

Introduction

Oral submucous fibrosis (OSF) is a serious, potentially malignant oral disorder [1]. The malignant conversion rate of OSF in India was 7.6% in a 17-year longitudinal study [2]. The common signs and symptoms of OSF include a burning sensation, dry mouth, and progressive inability to open the mouth. OSF is histopathologically characterized by chronic inflammation and atrophic epithelium with the accumulation of collagen fibers in the lamina propria [3]. Although OSF is typically a disease of fibroblasts, epithelial changes could be important in these processes.

Epidemiological studies provide overwhelming evidence that areca nut chewing is a major etiological factor for OSF and carcinomaogenesis [4]. An areca extract-induced oral mucosa fibrosis rat model showed progressive changes in epithelial thickness (ET); this leads to atrophy and cytokine infiltration into the subepithelium layer to promote fibroblast proliferation and collagen formation [5,6]. Recent studies have shown that arecoline, the main areca alkaloid, is cytotoxic to epithelial cells and fibroblasts. Arecoline-induced epithelial cell apoptosis could contribute to atrophic epithelium in OSF [7,8]. However, the exact mechanisms underlying arecoline-induced epithelial cell apoptosis remain poorly understood.

Autophagy is a process of self-digestion in which abnormal cytoplasmic macromolecules and organelles are encapsulated in autophagosomes of double-membrane structures [9]. Then, autophagosomes fuse with lysosomes to form autophagosomes, which degrade and recover their contents. Therefore, autophagy is essential for maintaining cellular metabolism and homeostasis in eukaryotic cells. Autophagy and apoptosis are two forms of programmed cell death, and many studies have shown a complex relationship between them [10]. Autophagy activation has been proposed to function as a cell survival mechanism under metabolic stress [11]. Conversely, excessive autophagy can promote apoptosis by actively or non-selectively degrading essential cellular components [12]. Oxidative stress has been implicated as a major contributor to the pathogenesis of arecoline-induced human keratinocyte apoptosis [13]. Multiple lines of evidence indicate that elevated reactive oxygen species or oxidative stress can trigger autophagy [14]. Thus, autophagy may be involved in the progression of arecoline-induced epithelial cell apoptosis.

To further study the possible mechanism underlying atrophic epithelium in OSF, the present study investigated the expression of autophagy and apoptosis marker proteins in OSF epithelial and normal oral mucosal tissues using immunohistochemistry. In addition, the influence of apoptosis and autophagy on human oral keratinocytes (HOKs) treated with arecoline and role of autophagy in arecoline-induced apoptosis were investigated.

Materials and Methods

Ethics approval and consent to participate

This study was approved by the Medical Ethics Committee of Xiangya Hospital, Central South University (IRB No.201609053), and informed consent obtained from participants.

Materials and reagents

Chloroquine (CQ) and methyl thiazolyl tetrazolium (MTT) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Rabbit anti-P62, mouse anti-β-actin, and horseradish peroxidase (HRP) goat anti-rabbit IgG were purchased from Proteintech (Chicago, IL, USA). Fetal bovine serum (FBS) and penicillin-streptomycin were purchased from Gibco (New York, NY, USA). Further, arecoline was purchased from Abcam (Cambridge, MA, USA). Rabbit anti-LC3 and rabbit anti-cysteine-aspartic acid protease (Caspase)-3 were purchased from Cell Signal Technology (Danvers, MA, USA).

Tissues

This study included a total of 80 total samples: 20 normal oral mucosal tissue samples and 60 OSF epithelial tissue samples (including samples from 20 patients with early-, 20 with moderate-, and 20 with advanced-stage OSF). All 60 patients were clinically and histopathologically diagnosed with OSF and showed no comorbidity with other oral mucosal diseases. OSF samples were graded using the Fndborg and Sirsat histopathological classification system as well as by measuring the interincisal mouth opening (stage I, ≥3 cm; stage II, 2-3 cm; and stage III, <2 cm) according to the report by Haider et al. [15,16]. The normal oral mucosal tissue samples were obtained during surgical removal of third molars. All samples for this study were obtained from the Centre of Stomatology, Xiangya Hospital, Central South University (Changsha, P. R. China). The clinical characteristics of all samples are listed in Table 1.

Assessment of epithelial changes by hematoxylin and eosin (H&E) staining

Epithelial changes in H&E-stained sections were assessed based on ET following the method reported by Rajiv et al. [17]. Epithelial layers were counted in the thinnest and thickest areas of each section and averaged.

Immunohistochemistry

The collected tissue samples were fixed in 10% formalin, embedded in paraffin, and sectioned at 4-μm thickness. The sections were dewaxed with paraffin, and rehydrated in a series of graded alcohol. The sections were then incubated in 3% H2O2 for 10 min to block endogenous peroxidase activity. Non-specific binding was blocked with 10% PBS containing bovine serum albumin (BSA) for 30 min. The sections were incubated with primary antibodies overnight at 4°C. After washing, the sections were incubated with secondary antibodies (goat polyclonal antibody against rabbit IgG) for 1 h. The sections were then incubated with biotinylated secondary antibody for 30 min, followed by incubation with peroxidase-conjugated streptavidin (HRP) for 30 min. The sections were stained with diaminobenzidine tetrahydrochloride (DAB) and counterstained with hematoxylin. The slides were dehydrated and mounted with neutral gum. For positive control, the sections were incubated with primary antibodies diluted in PBS. For negative control, the sections were incubated with PBS instead of primary antibodies.

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xylene, rehydrated using gradient alcohol, and incubated in 3% H2O2 for 10 min. Subsequently, the sections were incubated with LC3B (1:50, ab51520, CST), P62 (1:50, 18420-1-AP, Proteintech), and Caspase-3 (1:50, ab4051, CST) primary antibodies overnight at 4°C, followed by incubation with goat-antibody conjugated secondary antibody for 30 min at 37°C. Then, the samples were stained with 3,3-diaminobenzidine, counterstained with Harris hematoxylin, mounted with Permount TM Mounting Medium, and observed under a microscope. PBS was used instead of the primary antibody as a negative control, and normal oral mucosal tissues were used as a positive control. The staining results were quantitatively evaluated using the Image-Pro-Plus 6.0 Image analysis software (Media Cybernetics, Rockville, MD, USA) to measure the mean optical density (MOD) of OSF epithelial tissues.

**Cell culture**

HOKs (ATCC) were grown on Dulbecco’s modified Eagle’s medium (HyClone, Logan, UT, USA) containing 10% heat-inactivated certified FBS, 1,000 IU/mL penicillin, and 1,000 IU/mL streptomycin. Cells were cultured in a humidified incubator containing 5% CO2 at 37°C.

**MTT assay**

Cells were seeded in 96-well culture plates at a density of 1×104 cells per well and exposed to various concentrations of arecoline for 24 h. Thereafter, 0.5 mg/mL MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] was added to each well and incubated for 4 h. Then, 200 μL DMSO was added to each well to dissolve formazan crystals, and the absorbance was measured at 490 nm using a microplate reader (Bio-tek, Winooski, VT, USA).

**Transmission electron microscopy (TEM)**

HOKs were treated with arecoline (15 μg/mL) for 0, 3, 6, 12, and 24 h. Cells were harvested by centrifugation and prefixed with 2.5% glutaraldehyde. After post fixing with 1% osmium tetroxide for 1 h and washing with PBS, cells were dehydrated in a graded series of ethanol and embedded in Epon 812. Ultrathin sections (60-70 nm) were cut with an ultramicrotome and post-stained with 1% uranyl acetate and lead citrate. Finally, the sections were observed and photographed using TEM.

**Western blotting**

The harvested cells were washed twice with PBS, lysed on ice for 10 min with RIPA lysis buffer, and centrifuged at 12,000 rpm for 15 min. The supernatant was collected and protein concentration was determined using a BCA protein assay kit (Thermo Fisher Scientific, MA, USA). Equal amounts of protein were separated by 12% SDS-polyacrylamide gel electrophoresis and transferred onto a polyvinylidene difluoride membranes. The membranes were blocked with 5% non-fat milk for 1.5 h at room temperature and then incubated with primary antibodies against LC3B (1:1,000, CST), Caspase-3 (1:1,000, CST), P62 (1:1,000, Proteintech), and β-actin (1:5,000, Proteintech) overnight at 4°C. Then, the membranes were washed thrice with TBST and incubated with HRP-conjugated secondary antibodies for 1 h at room temperature. Finally, protein bands were visualized using an enhanced chemiluminescence kit (Thermo Fisher Scientific). The relative quantity of proteins was analyzed using the Quantity One software, and β-actin was used as the loading control.

**Statistical analysis**

All data represent at least three independent experiments and are presented as mean ± standard error of the mean. Statistical differences were determined using one-way analysis of variance. Data analysis was performed using the SPSS 22.0 software (IBM, Armonk, NY, USA) and GraphPad Prism 5 software (GraphPad, San Diego, CA, USA). P < 0.05 was considered statistically significant.

**Results**

ET of normal oral mucosal and OSF epithelial tissues

The ET of normal oral mucosal and OSF epithelial tissues was analyzed to assess changes in epithelial tissues (Table 2). The ET of OSF epithelial tissues significantly reduced compared with that of normal oral mucosal tissues. Across the three stages of OSF, the mean ET gradually decreased and reached the lowest level at the advanced stage. Epithelial dysplasia was not observed in OSF.

**Expression of LC3, P62, and Caspase-3 in normal oral mucosal and OSF epithelial tissues**

H&E and immunohistochemical staining were performed on normal oral mucosal and OSF epithelial tissues. H&E staining showed that OSF epithelial tissues were characterized by collagen hyalinization, obliterated or narrowed blood vessels, and atrophic epithelium unlike normal oral mucosal tissues (Fig. 1A). Immunohistochemical staining showed that the positive expression of LC3, P62, and Caspase-3 was localized in the cytoplasm and nucleus in OSF epithelial tissues. In normal oral mucosal tissues, LC3-and Caspase-3-positive cells were mainly present in the spinous and granular layers and were minimally expressed in the basal layer. However, LC3 and Caspase-3 expression levels were elevated, and they were expressed in the basal layer in OSF epithelial tissues (Fig. 1B, D). P62-positive cells were found in the entire epithelial layer of the normal mucosa.

**Table 1** Clinical characteristics of the samples included in the study

<table>
<thead>
<tr>
<th></th>
<th>NOM</th>
<th>OSF1</th>
<th>OSF2</th>
<th>OSF3</th>
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</thead>
<tbody>
<tr>
<td>Number of samples</td>
<td>20</td>
<td>20</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>Acrea nut chewing</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Intercinal mouth opening</td>
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<td>&gt;3 cm</td>
<td>2-3 cm</td>
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<tr>
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<td>20</td>
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<td>2</td>
<td>0</td>
</tr>
<tr>
<td>Range</td>
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<td>20-60</td>
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<tr>
<td>Mean ± SD</td>
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<td>39.5 ± 15.1</td>
<td>43.3 ± 12.7</td>
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NOM, normal oral mucosa; OSF1, early stages of OSF; OSF2, moderate stages of OSF; OSF3, advanced stages of OSF

**Table 2** Epithelial thickness in normal oral mucosal and OSF epithelial tissues

<table>
<thead>
<tr>
<th></th>
<th>NOM</th>
<th>OSF1</th>
<th>OSF2</th>
<th>OSF3</th>
</tr>
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<td>Number of samples</td>
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<tr>
<td>P values</td>
<td>—</td>
<td>&lt;0.05</td>
<td>&lt;0.001</td>
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</table>

NOM, normal oral mucosa; OSF1, early stages of OSF; OSF2, moderate stages of OSF; OSF3, advanced stages of OSF; a Compared with normal oral mucosa
oral mucosal tissues, whereas P62 expression was restricted to the basal layer in advanced-stage OSF epithelial tissues (Fig. 1C). Quantitative analysis of LC3, P62, and Caspase-3 expression is shown in Fig. 1E-G. Compared with normal oral mucosal tissues, OSF epithelial tissues had increased LC3 expression and decreased P62 expression. Moreover, there was a significant difference between the average expression levels of LC3 and P62 at all OSF stages in OSF epithelial tissues (Fig. 1E, F). All results indicate that autophagy is activated in OSF, and greater autophagy activity occurs in advanced-stage OSF. Caspase-3 expression levels were significantly higher in OSF epithelial tissues than in normal oral mucosal tissues, and they further increased with the progression of OSF (Fig. 1G). LC3, P62, and Caspase-3 expression levels were not significantly different between normal oral mucosal and OSF epithelial tissues in the early stages of OSF.

**Relationship among the expression of LC3, P62, and Caspase-3 in normal oral mucosal and OSF epithelial tissues**

The relationship among the expression of LC3, P62, and Caspase-3 was determined using two-tailed Pearson’s correlation between normal oral mucosal and OSF epithelial tissues. Linear regression lines were fit to analyze the predictive relationships of the immunohistochemical staining cores. Caspase-3 expression was positively correlated with LC3 expression ($P < 0.001$, $r = 0.6036$, $n = 80$) and negatively correlated with P62 expression ($P < 0.001$, $r = -0.4601$, $n = 80$) (Fig. 1H). Moreover, LC3 expression was negatively correlated with P62 expression ($P < 0.001$, $r = -0.4395$, $n = 80$) (Fig. 1I). These results indicate that autophagy and apoptosis play important roles in OSF epithelial tissues.

**Caspase-3 expression in normal oral mucosal tissues (NOM, $n = 20$), early-stage OSF epithelial tissues (OSF1, $n = 20$), moderate-stage OSF epithelial tissues (OSF2, $n = 20$), and advanced-stage OSF epithelial tissues (OSF3, $n = 20$). Magnification: 100× and 400×. (E) Quantitative analysis of LC3 expression (MOD = 0.0297 ± 0.0062 in NOM, 0.0406 ± 0.0085 in OSF1, 0.0588 ± 0.0106 in OSF2, and 0.0819 ± 0.0153 in OSF3). (F) Quantitative analysis of P62 expression (MOD = 0.0872 ± 0.0100 in NOM, 0.0771 ± 0.0090 in OSF1, 0.0618 ± 0.0120 in OSF2, and 0.0459 ± 0.0087 in OSF3). (G) Quantitative analysis of Caspase-3 expression (MOD = 0.0194 ± 0.0043 in NOM, 0.0275 ± 0.0065 in OSF1, 0.0427 ± 0.0093 in OSF2, and 0.0966 ± 0.0102 in OSF3). Data were analyzed using the GraphPad Prism 5 software. Data are presented as mean ± standard error of the mean. **$P < 0.001$; *$P < 0.05$, NS, no significance. (H) Caspase-3 expression was positively correlated with LC3 expression ($P < 0.001$, $r = 0.6036$, $n = 80$) and negatively correlated with P62 expression ($P < 0.001$, $r = -0.4601$, $n = 80$) based on two-tailed Pearson’s test.

**Effects of aracoline on the cell viability of HOKs**

The ability of aracoline to reduce the cell viability of HOKs was detected using the MTT assay. Cells were treated with different concentrations of aracoline (0, 7.5, 15, 30, 60, and 120 μg/mL) for 24 h. As shown in Fig. 2A, aracoline decreased the cell viability of HOKs in a concentration-dependent manner. Because cells treated with 15 μg/mL aracoline showed approximately 50% inhibition, this concentration was used for further experiments.

**Arecoline induced apoptosis in a time-dependent manner**

To investigate the influence of arecoline on apoptosis, cell apoptosis was assessed using an annexin V-FITC/PI staining assay after treatment with 15μg/mL arecoline for 0, 3, 6, 12, and 24 h. Flow cytometry showed that arecoline induced apoptosis in HOKs in a time-dependent manner (Fig. 2B). Next, the protein expression of the apoptosis executioner Caspase-3 was analyzed using Western blotting. Consistent with annexin V-FITC staining results, aracoline increased Caspase-3 expression and cleaved it in a time-dependent manner (Fig. 2C).

**Arecoline induced autophagy in a time-dependent manner**

The present results demonstrate autophagy in OSF epithelial tissues. To investigate whether aracoline can induce autophagy in HOKs, TEM was used to observe the presence of autophagic vacuoles (Fig. 3A). HOKs treated with aracoline for 6, 12, or 24 h demonstrated a greater number of autophagic vacuoles than HOKs treated with aracoline for 0 or 3 h. LC3 is the most widely used autophagy marker in current research. Western blotting showed that the LC3-II/LC3-I ratio increased significantly in a time-dependent manner (Fig. 3B). Similarly, P62 (an autophagy marker) expression increased in a time-dependent manner (Fig. 3C). These results suggest that aracoline induces autophagy in HOKs and P62 was not completely degraded and accumulates moderately in cells.

**Arecoline-induced autophagy promotes the action of apoptosis**

To evaluate whether autophagy affects arecoline-induced apoptosis, HOKs were pretreated with CQ before treatment with aracoline. Western blotting showed that CQ inhibited the LC3-I to LC3-II conversion and increased P62 expression (Fig. 4A). These results indicate that CQ can inhibit arecoline-induced autophagy. Moreover, the expression of Caspase-3 and cleaved Caspase-3 decreased following co-treatment with CQ and arecoline compared with that following treatment with arecoline alone.
a deeper understanding of OSF pathogenesis. The present study used Therefore, the study of the response of HOKs to arecoline could provide are atrophic, which is more pronounced in the advanced stages of OSF. 20]. The present study supports the findings that OSF epithelial tissues aggravating atrophic epithelium and thereby forming a vicious cycle [18- decreased vascularity lead to epithelial ischemia and hypoxia, further lagen fibers in the lamina propria in OSF. Connective tissue fibrosis and cytokines that result in fibroblast proliferation and accumulation of col -tion in OSF epithelial tissues was significantly higher than that in normal expression is reduced in OSF epithelial tissues and autophagy is induced which is a crucial regulator of autophagy [33]. It was speculated that Bcl-2 acts as the key executioner [21]. In the present study, Caspase-3 expres -specific mechanism underlying apoptotic induction will be elucidated in well understood. LC3 is an autophagy marker protein that is a mamma- linear homologue of Atg8 in yeast [26]. After autophagy activation, ubiquitinated LC3-I is covalently linked to phosphatidylethanolamine to form LC3-II on the autophagosome membrane [27]. P62 is also an autophagy marker protein. During autophagy, P62 is associated with different ubiquitin-tagged cargos and is degraded in autophagosome/ lysosome fusion [28]. The present results confirmed that LC3 expression increased and P62 expression decreased in OSF epithelial tissues compared with their expressions in normal oral mucosal tissues. These changes were significantly correlated with the pathological stage of OSF.

To further examine whether arecoline induced autophagy in HOKs, TEM was used to investigate autophagic vacuole structures. LC3 and P62 expression was quantified to further confirm arecoline-induced autophagy. The results showed that the LC3-II/LC3-I ratio increased significantly in a time-dependent manner, suggesting that arecoline induced autophagy in HOKs. Interestingly, another study revealed that cigarette smoke extract transiently induces autophagy activation in bronchial epithelial cells followed by the accumulation of P62 and ubiquitinated proteins, resulting in increased cell death [32]. Because autophagy acts as a double-edged sword in apoptosis, the relationship between autophagy and apoptosis was explored in HOKs treated with arecoline. CQ was used as an inhibitor of autophagy. The anti-apoptotic protein Bcl-2 can inhibit autophagy by binding to Beclin-1, which is a crucial regulator of autophagy [33]. It was speculated that Bcl-2 expression is reduced in OSF epithelial tissues and autophagy is induced by the release of Beclin-1 from Bcl-2, resulting in increased apoptosis. The specific mechanism underlying apoptotic induction will be elucidated in further studies. The present results also demonstrated that autophagy activity in OSF epithelial tissues was significantly higher than that in normal (Fig. 4B). Flow cytometry showed that co-treatment with CQ and areco -line decreased the apoptosis rate compared with treatment with arecoline alone (Fig. 4C). These results indicate that arecoline-induced autophagy promotes the action of apoptosis.

Discussion

The outermost layer of the oral mucosa is a protective barrier composed of epithelial tissues. Long-term areca nut chewing stimulation first causes epithelial changes before damaged keratinocytes release a variety of cytokines that result in fibroblast proliferation and accumulation of col -lagen fibers in the lamina propria in OSF. Connective tissue fibrosis and decreased vascularity lead to epithelial ischemia and hypoxia, further aggravating atrophic epithelium and thereby forming a vicious cycle [18-20]. The present study supports the findings that OSF epithelial tissues are atrophic, which is more pronounced in the advanced stages of OSF. Therefore, the study of the response of HOKs to arecoline could provide a deeper understanding of OSF pathogenesis. The present study used in vitro methods to demonstrate the effects of arecoline and confirmed the mechanism of pathogenesis in OSF epithelial tissues.

The Caspase family plays a crucial role in apoptosis, and Caspase-3 acts as the key executor [21]. In the present study, Caspase-3 expres -sion in OSF epithelial tissues was significantly higher than that in normal oral mucosal tissues, and its upregulation was greater in the presence of advanced-stage OSF pathology. Arecoline-induced apoptosis was also investigated in HOKs. Flow cytometry and Caspase-3 expression measured by Western blotting showed that arecoline induced apoptosis in HOKs in a time-dependent manner. Cheng et al. also demonstrated that arecoline induces apoptosis by Caspase-3 activation and decreased the expression of anti-apoptotic proteins such as Bcl-2 and Bcl-XL [22]. Besides induc -ing apoptosis, arecoline also disrupts the cell cycle. Previous research has shown that arecoline inhibits the proliferation of epithelial cells by arresting cells in S and G2/M phases, thereby damaging the repair process [23]. These data suggest that increased epithelial apoptosis and inhibition of cell proliferation is a potential pathogenesis of atrophic epithelium and that this process is associated with arecoline cytotoxicity.

Autophagy is a lysosome-dependent degradation process that widely exists in eukaryotic cells and causes the digestion of damaged proteins and organelles and recycling [24]. Recent studies have demonstrated that autophagy plays an important role in OSF, and the inhibition of autophagy promotes apoptosis of fibroblasts while reducing collagen fiber synthesis [25]. However, the role of autophagy in OSF epithelial tissues has not been
oral mucosal tissues. Further, LC3 was mainly expressed in the epithelial and early forms of oral submucous fibrosis. Oral Surg Oral Med Oral Pathol 50, 40-44.


