Oral Candidiasis Deteriorated by Local Application of a Glucocorticoid-Containing Film in a Mouse Model

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In order to estimate predisposing activity of oral application of beclomethasone dipropionate (BDP)-containing mucoadhesive films for oral candidiasis, the effects of BDP on growth of Candida albicans were examined in vivo and in vitro. Murine neutrophils inhibited the mycelial growth of C. albicans in vitro, but this anti-Candida activity was clearly suppressed by the presence of 10⁻⁷ M of BDP. In vitro, a BDP-release test showed that the amount of BDP released from BDP-containing films into the fluid phase increased in a time- and concentration-dependent manner and reached about 10—15% of the total amount of BDP in the film within 30 min. When the BDP-containing film was attached to the tongues of mice orally infected with C. albicans, oral infection by C. albicans deteriorated, but not as severely as in mice systemically immunosuppressed with prednisolone. Based on these findings, we also discuss the problems associated with the clinical application of BDP-film as an anti-inflammatory tool.

Key words glucocorticoid; mucoadhesive film; oral candidiasis; mice; anti-inflammatory agent

The anti-inflammatory agent glucocorticoid is one of the most effective and widely-used drugs for various kinds of inflammatory diseases. However, it has serious side effects, including suppression of host defense mechanisms against microbial infections. Therefore, more efficient delivery methods of glucocorticoids are required for various types of inflammatory diseases. For clinical use films containing glucocorticoids have been developed for oral inflammatory diseases, including mucosal lesions in the oral cavity. Very recently we succeeded in preparing mucoadhesive hydroxypropyl cellulose (HPC)-film containing more than 100 mg/cm² of beclomethasone dipropionate (BDP).

Before conducting clinical research on this film, the effects of application of this film on oral infection were examined in an animal model, since it is well known that glucocorticoids predispose to oral or pharyngeal Candida infection. In our previous papers, a murine oral candidiasis model or an esophageal candidiasis model were used, allowing the examination of the effects of topical application of mucoadhesive films.

In the present study, we examined the effects of BDP- and neutrophil-Candida interactions in vitro, as well as the level to which oral application of BDP-containing HPC-film predisposes towards oral candidiasis in this murine model.

MATERIALS AND METHODS

Film Preparation The film was composed of one layer of HPC (150—400 cP; Wako Pure Chemical Industries, Ltd., Osaka, Japan) as the base component. To prepare BDP-containing film, BDP (Wako) was first dissolved in ethanol, HPC added, and then ethanol added to give a final volume of 30 ml per 0.9 g HPC. A 10-ml aliquot of this solution was cast using a graduated pipette at a rate of 10 ml/4 min into a flat round Teflon dish of diameter 75 mm and then dried on a clean bench overnight. These dried films were then cut into circles of diameter 30 mm for the release study or into rectangles of 0.6 cm×0.5 cm for the oral candidiasis study in mice.

Release of BDP from the Film Measurement of BDP-release from the BDP-containing films was performed as follows. A dissolution apparatus was modified from the original construction of Okamoto et al. Briefly, a 10-ml beaker was used as the receptor compartment and a piece of sample film (3-cm diameter) attached to the inner wall of the beaker using double-sided tape, to produce the donor side. An 0.1 M phosphate buffer (PB) (pH 7.0) was then added to the beaker, which was maintained at 37 °C and stirred at a constant speed using a magnetic bead. Aliquots (100 μl each) were withdrawn at preset times (1, 2, 3, 4, 5, and 30 min), and then the amount of BDP released estimated using an HPLC system (Shimadzu Co., Ltd., Kyoto, Japan) equipped with a UV detector (wavelength=254 nm). To maintain a constant volume and sink conditions in the experiment, the removed solution was replaced each time with 100 μl of 0.1 M PB prewarmed to 37 °C. The cumulative amount of drug release per 1 cm² of BDP film was then calculated. Each experiment was performed in triplicate.

C. albicans The C. albicans TIMM1768 strain, which is a clinically isolated serotype A strain, maintained at the Institute of Medical Mycology, Teikyo University, Japan, was used. This Candida strain, which had been stored at −80 °C in YPD broth and 10% glycerol, was initially grown on Sabouraud Dextrose Agar (Eiken Chemical Co., Ltd., Tokyo, Japan) at 37 °C for 20 h. The yeast cells were then harvested using a microspatula, suspended in RPMI1640 medium with 2.5% heat-inactivated fetal calf serum (complete medium), and then finally resuspended in the same fresh medium at a concentration of 2×10⁶ cells/ml for in vivo Candida inoculation.

Measurement of in Vitro Activity of BDP against Mycelial Growth of Candida albicans To determine the...
extent of mycelial growth of *C. albicans*, the crystal violet (CV) staining assay was performed as described previously.\(^{111}\) Briefly, *C. albicans* suspensions \((1 \times 10^4 \text{ cells/ml})\) in 200 \(\mu\)l of complete medium with or without BDP samples and/or neutrophils were incubated at 37°C for 16 h and then the medium in the wells discarded by inverting the microplates. The *Candida* cells were sterilized by immersion of the plates in 70% ethanol, and the neutrophils were washed out with 100 \(\mu\)l of 0.25% sodium dodecyl sulfate (SDS). Each well was washed twice with 100 \(\mu\)l of 0.25% SDS and mycelia attached to the wells were stained using 0.02% CV in 100 \(\mu\)l phosphate-buffered saline (PBS) for 15 min. After washing with saline, mycelia-binding CV were solubilized by 200 \(\mu\)l of 0.25% SDS in 0.04 \(\text{N} \) HCl-isopropanol, and the absorbance of the samples at OD \((590—620 \text{ nm})\) was then measured photometrically. The relative growth percent of *Candida* was calculated as follows:

\[
\text{[absorbance (tested)/absorbance (Candida alone)]} \times 100 \%
\]

**Preparation of Murine Neutrophils** All animal experiments were performed according to the guidelines for the care and use of animals approved by Teikyo University. Murine neutrophils were prepared from peritoneal exudate as described previously.\(^{121}\) ICR mice (Charles River Japan, Inc., Yokohama, Japan) were injected intraperitoneally with 3 ml of 8% casein sodium (Tokyo Kasei, Tokyo, Japan) 1 d prior to oral infection. Anesthetized by intramuscular injection of 100 \(\mu\)g of prednisolone (Mitaka Pharmaceutical Co., Tokyo, Japan) \(1 \text{ d prior to oral infection.}\)

For microbiological evaluation, the oral cavities of the mice were swabbed using a cotton bud on day 3, and *Candida* cells collected on the bud were then suspended in 3 ml saline. After a series of 10-fold dilutions in saline, 50 \(\mu\)l of each cell suspension was incubated on a *Candida* GS plate at 37°C for 24 h. The colony forming units (CFU) of *C. albicans* were calculated by counting the number of *Candida* colonies.

For histological examination, specimens of tongue mucosa of mice were taken from sacrificed animals, fixed in 0.1 M PB containing 4% paraformaldehyde solution and embedded in O.C.T. compound. Specimens 8-\(\mu\)m thick were sectioned using a cryostat and stained with periodic acid-Schiff (PAS).

**Statistical Analysis** Body weight changes were analyzed using non-repeated ANOVA with Bonferroni correction. Statistical analysis of the log CFU of *C. albicans* isolated from the tongues of the mice in the experimental groups was performed using the Kruskal Wallis H-test and the Mann–Whitney U-test with Bonferroni correction. \(p\) values of \(<0.05\) were considered statistically significant. All calculations were performed using a statistical software program (ystat2006: Igaku Tosho Shuppan, Tokyo, Japan).

**RESULTS**

**Release of Beclomethasone** The efficiency of BDP release from our preparation of BDP-containing HPC-film into the fluid phase was examined *in vitro*. The amount of BDP released from 1 side surface of the BDP film linearly increased in the period from 1 to 30 min (Fig. 2). BDP release from films containing various concentrations of BDP after a 30 min incubation was as follows: BDP=\(25 \mu\text{g/cm}^2\): \(3.75 \pm 0.03 \mu\text{g/cm}^2\); \(50 \mu\text{g/cm}^2\): \(6.84 \pm 1.01 \mu\text{g/cm}^2\); \(100 \mu\text{g/cm}^2\): \(11.87 \pm 3.18 \mu\text{g/cm}^2\). This indicates that the absolute amount of BDP released was proportional to the concentration of BDP in the film and was 10—15% of the total amount of BDP in the film by the end of a 30-min incubation.
Effects of BDP on Mycelial Growth with or without Murine Neutrophils

In order to examine possible interactions of BDP with the host defense against \textit{C. albicans} growth, the effects of BDP on the mycelial growth of \textit{C. albicans} with or without murine neutrophils were examined. Table 1 shows that BDP at various concentrations up to $10^{-6}$ M did not affect the mycelial growth of \textit{C. albicans} without the presence of murine peritoneal neutrophils. Table 1 also shows that neutrophils at an effector-target ratio of 120 strongly inhibited \textit{Candida} growth and that this anti-\textit{Candida} activity of neutrophils was significantly suppressed in the presence of $10^{-6}$ M of BDP.

As depicted in Fig. 3, microscopic observation indicates that in the presence of neutrophils, BDP at a concentration of $10^{-6}$ M appeared to increase the number of mycelial colonies of \textit{C. albicans} and to enlarge the size of the mycelial colonies, whose growth was prevented by neutrophils. The mean diameter of \textit{Candida} hyphae decreased in the presence of murine neutrophils (E/T ratio 120: diameter 250—400 $\mu$m, E/T ratio 0: 300—500 $\mu$m), whereas in presence of $10^{-6}$ M BDP the hyphae were apparently not suppressed (E/T ratio 120: diameter 300—500 $\mu$m; E/T ratio 0: 300—500 $\mu$m).

Effect of BDP Film

The effects of BDP on \textit{Candida} infection were kinetically checked in mice that had been orally treated with BDP-containing film formulation on the tongue surface mucosa or with BDP suspension. As shown in Fig. 4, attachment of BDP-containing film increased by 10 times the number of viable \textit{C. albicans} cells recovered from the oral cavity of orally infected mice at day 3. The viable cell number of the BDP-film group was significantly different from that of film formulation control group ($p<0.05$). The viable cell number from the oral cavity also tended, although not significantly, to be increased in the group of mice treated with BDP suspension compared with its control group. Figure 4 also shows that \textit{Candida} cell number in the mice immunosuppressed by subcutaneous administration of prednisolone markedly increased to 100 times the level in the BDP-treated mice.

Table 2 presents the changes in mouse body weight of

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<th>Beclomethasone (mg)</th>
<th>Relative \textit{Candida} growth (%)</th>
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<tr>
<td></td>
<td>E/T=0</td>
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<tr>
<td>0</td>
<td>100.0±16.1\textsuperscript{b}</td>
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<tr>
<td>$10^{-9}$</td>
<td>107.4±11.9</td>
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<tr>
<td>$10^{-8}$</td>
<td>106.6±12.7</td>
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<tr>
<td>$10^{-7}$</td>
<td>107.9±10.6</td>
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<td>$10^{-6}$</td>
<td>108.1±12.2</td>
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\textit{C. albicans} was cultured for 14 h at 37$^\circ$C with or without neutrophils at the indicated E/T ratio. \textit{a)} of the number of effecter cells (neutrophils) to the number of target cells (\textit{C. albicans} cells). Each concentration was tested six times. \textit{b)} mean±S.D.

\textsuperscript{*}p<0.05.
each treated mouse. The body weight of each group of orally-infected mice decreased with progression of *Candida* infection, and markedly decreased in the prednisolone-treated mice until day 3. Weight loss was significantly greater in the prednisolone-treated mice compared to the other groups.

The dose dependence of the increase in *Candida* cell number was also checked against film formulation. Figure 5 shows that the increase in cell number of *C. albicans* in the high dose group of mice was 3 times that of the lower dose group.

Next, we histologically analyzed the tongue mucosa of control mice, film-treated mice and BDP-film treated mice on day 3. PAS-positive fungi was observed on the tongue surface of the mice of groups B and C. The tongues were obtained on day 3 and sections were stained with PAS. Arrows represent hyphae of *C. albicans*. Bar=50 μm.

The dose dependence of the increase in *Candida* cell number was also checked against film formulation. Figure 5 shows that the increase in cell number of *C. albicans* was dependent on the dose of BDP in the BDP-films, *i.e.*, the CFU of *C. albicans* in the high dose group of mice was 3 times that of the lower dose group.

Next, we histologically analyzed the tongue mucosa of control mice, film-treated mice and BDP-film treated mice on day 3. PAS-positive fungi was observed on the tongue surface of the mice of groups B and C. The tongues were obtained on day 3 and sections were stained with PAS. Arrows represent hyphae of *C. albicans*. Bar=50 μm.
faces of the BDP-film treated mice in areas near or covered by the mucoadhesive BDP film (Fig. 6). However, almost no hyphal growth of *C. albicans* was observed in the control or film-treated mice.

**DISCUSSION**

Oral application of mucoadhesive films containing anti-inflammatory steroidal drugs has attracted attention as a drug-delivery system for oral inflammatory diseases, because these films may make it possible to minimize the undesirable side effects of steroidal agents.\(^3\)

Several formulations were used for the application of the most widely used topical glucocorticoids in oral medicine. Orabase with triamcinolone acetonide (TRCA) is an adhesive paste (KENALOG\(^6\)) widely used as a vehicle in topical glucocorticoids therapy, but orabase losses of 85—90% of the total dose applied have been reported, and another vehicle of spray-type therapeutic agent with BDP (Salcoat\(^7\)) is for extensive stomatitis. We selected BDP as main medicament, because inflammatory effect of BDP was reported five times stronger than that of TRCA,\(^5\) and prepared film formulation for more topical application than orabase or spray.

Here, we showed that attachment of film containing BDP to tongues of *Candida*-infected mice aggravated oral candidiasis in a manner dependent on the BDP-dose. The aggravation was indicated by the increase in the number of viable *Candida* cells in the oral cavity and histopathological observation of the dorsal surface of tongues. As far as we know, this is the first report to demonstrate the microbiological side effects of mucoadhesive films containing steroidal drugs.

We previously reported that systemic or oral administration of anti-inflammatory steroidal compounds remarkably deteriorated the pathogenesis of oral or pharyngeal candidiasis in mice.\(^7\) Here, we compared the effects of the attachment of BDP-containing film, topical application of BDP-suspension and subcutaneous administration of prednisolone. As shown in Fig. 4, 150 μg of BDP in films caused a significant increase in *Candida* cell number in the oral cavity, whereas 200 μg of BDP in suspension did not. This difference may explain by the longer retention time of BDP in the case of films, as noted below. We wish to emphasize that systemic administration of prednisolone (100 mg/kg s.c.) made the *Candida* infection much worse than that of BDP-film, as monitored by the increase in number of *Candida* cells and loss of body weight. Thus, deterioration of oral candidiasis due to BDP-film is not as severe as that caused by prednisolone.

When analyzing the bio-pharmacological activity of the BDP-containing film, the retention time of the films in the oral cavities and the release kinetics of BDP from the films appeared to be critical factors in their activities in vivo. In our animal experiment, the films were adhered to the tongues of chlorpromazine-treated mice. We found that this sedative treatment allows attachment of the films for at least for 2 h.

Measurement of BDP concentration of the surface of BDP-film-attached mucosal membrane is important to understand the possible in vivo role of BDP. At present, we can only estimate the concentration of BDP present; the results of the in vitro release test suggested that about 10—15% of BDP enclosed in the BDP-films was released from the film during a 30 min incubation. Thus, we can assume that attachment of BDP-film containing 150 μg BDP (0.3 cm\(^2\)) may supply more than 10 μg BDP for 0.3 cm\(^2\) area of tongue surface. Addy\(^{10}\) reported that the glucocorticoid TRCA, which was topically administered onto dog oral mucosa, can be absorbed and is located in an area of 0.3—0.4-mm thickness. If this is true in the present model, the local concentration of BDP on the BDP-film-attached tongue surface may rise to the level of 10 μg BDP/0.012 ml (0.15 mw). This concentration is about 1000 times the BDP concentration necessary for suppression of anti-*Candida* activity of neutrophils (1 μM), as described below.

The cellular mechanisms of the aggravation of oral candidiasis by the application of BDP-films remain to be clarified. We can assume that BDP exuded from the BDP-film facilitated colonization, perhaps through suppression of host defense mechanisms, based on our previous report\(^7\) indicating that oral inhalation of BDP allows elongated deterioration of experimental pharyngeal candidiasis in mice. However, a glucocorticoid may suppress various types of host defense mechanisms against microbial infections; in this study we examined the effects on neutrophils. As shown in Fig. 3, BDP did not enhance the mycelial growth of *C. albicans in vitro*, but 10\(^{-6}\) M of BDP clearly suppressed the growth inhibitory activity of neutrophils. Therefore, we believe that aggravation of oral candidiasis by BDP may be explained by suppression of the anti-*Candida* activity of neutrophils or other effector cells including epithelial cells.\(^7\)

Although film formulation for application of glucocorticoids was originally developed in order to suppress local inflammation without side effects, our present results suggest that attachment of films containing glucocorticoids may inhibit the local defensive functions and predispose to oral candidiasis. Therefore, when we promoting trials to develop the BDP-film as a clinical method of applying glucocorticoids we must pay attention to the possible development of local microbial infections, including candidiasis, as a side effect, although these effects are not as severe as those produced with systemic administration of high dose glucocorticoids. In order to resolve these problems, we are currently preparing a new film formulation that does not have significant predisposing activity to microbial infection.

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**REFERENCES**