Development of an Animal Model of Onychomycosis in Guinea Pigs

Nami Hasegawa and Kazutoshi Shibuya

Department of Surgical Pathology, Toho University Graduate School of Medicine

ABSTRACT

Onychomycosis is a common and intractable superficial mycosis that occurs worldwide. Treatment with both oral and topical drugs is recommended, but the objective evaluation procedure to determine the efficacy of and the appropriate delivery system for the drugs remains controversial. This may be attributed to the lack of a reliable animal model that not only mirrors the pathophysiology of human onychomycosis but is also feasible. Therefore, we attempted to establish an animal model of onychomycosis using immunosuppressed guinea pigs and elucidate the pathophysiology of human onychomycosis. In the present study, we applied Trichophyton mentagrophytes TIMM2789 to the hind limb nails of corticosteroid-treated guinea pigs. The nails were examined macroscopically and histopathologically at 0, 14, and 42 days after a 2-week exposure period to the fungus. A large portion of the experimentally infected nails showed discoloration, which is an important clinical sign, and most infections were confirmed histopathologically in the deep layer of the nail plate at all time points. The infection rates at 0, 14, and 42 days after exposure were 39%, 61%, and 78%, respectively. Thus, we established an animal model of onychomycosis with good reproducibility and that might be appropriate for extrapolation to the pathophysiology of the human disease.

Key words: fungus, histopathology, in vivo, nail, onychomycosis

Introduction

Onychomycosis is a common and intractable superficial mycosis that causes nail discoloration, separation, brittleness, and/or thickening and leads to decreased quality of life. The prevalence of the disease is approximately 5.5% worldwide. Onychomycosis is traditionally classified into the following four clinical forms, as proposed by Zaias: distal and lateral subungual onychomycosis (DLSO), superficial white onychomycosis, proximal subungual onychomycosis (PSO), and totally dystrophic onychomycosis. DLSO is the most common form in humans and is characterized by involvement of the deep layer of the nail plate, nail bed, and hyponychium. Both oral and topical drugs are recommended as treatments for onychomycosis, but the objective evaluation procedure to determine the efficacy of and the appropriate delivery system for these drugs remains controversial. A large part of the problem may be attributed to the lack of a reliable animal model that not only mirrors the pathophysiology of human onychomycosis but is also feasible.

Two animal models of onychomycosis have been reported previously. The first was developed in guinea pigs, but the infection rate and distribution of fungal infection have not been clarified. The second, developed in immunosuppressed rabbits, histopathologically showed fungal invasion into the deep layer of the nail plate. However, it may be difficult to prepare and handle large numbers of rabbits, which are larger than rats and guinea pigs.

Therefore, we attempted to establish an animal model of onychomycosis using immunosuppressed guinea pigs to help elucidate the pathophysiology of the disease in humans.

Material and methods

Animals

Male Hartley guinea pigs (5 weeks old) purchased from Japan SLC Inc. were used in this study. The animals were housed individually and allowed to acclimate for 7 days, after which they were randomly assigned to different experimental groups in accordance with the postinfection period: Day 0, Day 14, and Day 42 groups. Each experimental group
consisted of six guinea pigs.

The experimental schedule is shown in Fig. 1. The nails examined in this study were those of the first to third toes of the right and left hind paws; i.e., six nails were used per animal. All experimental procedures were evaluated and approved in accordance with the Institutional Animal Care and Use Committee of Pola Pharma Inc. (Approval number: PP-2017-25) and Kobe BM Laboratory (Approval number: ma-K1390).

**Fungal strain**

*Trichophyton mentagrophytes* TIMM 2789, a zoophilic dermatophyte\(^{11}\), maintained at Teikyo University Institute of Medical Mycology (Tokyo, Japan), was used in this study. Freeze-dried *T. mentagrophytes* was subcultured on Sabouraud dextrose agar (Becton, Dickinson and Company) at 28°C for 2 weeks to produce microconidia.

Following incubation, microconidia of *T. mentagrophytes* were taken from the fungi and incubated in saline containing 0.05% Tween80. The suspension of microconidia was adjusted to a concentration of 10\(^7\) conidia/mL by counting on a hemocytometer.

**Induction of onychomycosis**

For induction of the disease model, 0.2 mL of the microconidia suspension was dripped onto 8-mm paper discs (Advantec), which were inserted between the first and second toes and between the second and third toes of the hind paw. After the paper discs were inserted with self-adherent bandages, self-adhering foam pad (3M, Reston) was placed on the sole of each foot and fixed using self-adherent bandages. These conditions were maintained for 2 weeks to facilitate infection. The paper disc, foam pad, and bandages were removed after 2 weeks of exposure, and the animals were housed for an additional 0, 14, or 42 days without the paper discs, foam pads, and bandages; this was termed the postinfection period.

**Immunosuppression**

For immunosuppression, 30 mg/kg triamcinolone acetonide (KENACORT-A; Bristol-Myers Squibb, NY, USA) was administered intramuscularly into the hind limb of each guinea pig daily for 28 days until Day 14 postinfection.

**Clinical and histological evaluation**

After macroscopic evaluation of discoloration in the nails on Days 0, 14, and 42 postinfection, the toes were examined histopathologically on the same days. For histopathological examination, whole toes were fixed in 10% (v/v) buffered neutral formalin solution for 1 week and decalcified in 5% (v/v) formalin solution containing 5% (v/v) buffered formic acid for 1 day. Samples were then neutralized in 5% (v/w) sodium sulfate aqueous solution for 1 week and embedded in paraffin. Paraffin sections of the nails were observed by light microscopy after staining with Periodic acid-Schiff (PAS) stain. For observations, sections were divided into 14 areas comprising nine nail plate areas (3 × 3), the cuticle, the dorsal matrix, the intermediate matrix, the nail bed, and the hyponychium (Fig. 2). The nail plate was divided into three sections depthwise from the dorsum of the nail plate to the nail bed and into three sections widthwise from the proximal to...
The distal ends of the nail plate, excluding the free edge of the nail plate. The infection rate (number of infected toes / total number of toes examined) was calculated for each area.

Results

The prevalence of discoloration in the nail increased with time, similar to the pathophysiology of human onychomycosis (Fig. 3 and Table 1). Most of the experimentally infected nails showed discoloration in the Day 14 and Day 42 groups (0% [0/36] in the Day 0 group, 67% [24/36] in the Day 14 group, and 78% [28/36] in the Day 42 group). On histopathological examination, 94% [17/18] of animals were confirmed to be infected. In the Day 0 group, fungal spores and hyphae with strong PAS staining were observed in the cuticle, matrix, and proximal nail plate; in particular, the hyphae were concentrated in the stratum corneum, cuticle, and dorsal matrix and ran parallel to the direction of growth of the nail (Fig. 4a and 4b). In the Day 42 group, fungi were spread distally in the nail plate (Fig. 4c and 4d). Fungal elements with decreased PAS staining were observed in the nail deep layers and ran parallel to the direction of growth of the nail (Fig. 4d).

Table 2 shows the infection rates in each nail area. The highest rate of infection in Day 14 group was found in the cuticle, whereas that in the Day 42 group occurred in the superficial areas of the nail plate. The infection rates in the hyponychium and nail bed were only 0-8.3% and 0-11%, respectively, in the three groups over the entire observation period. The total infection rate was higher in the Day 42 group than in the Day 0 group. Fungal infection was confirmed histopathologically in more than 90% of discolored nails (92% [22/24] in the Day 14 group, and 93% [26/28] in the Day 42 group).

Discussion

Two animal models of onychomycosis, one using guinea pigs and the other rabbits, have been reported previously\(^9,\ 10\). Although both models were used to evaluate the fungal infection and postinfection periods, the rabbit model involved an immunosuppression procedure requiring corticosteroid administration. Although the guinea pig model, without an immunosuppression procedure, was easier to handle and simpler to establish, changes in the detailed pathophysiology, gross appearance, infection rate, and fungal infection distribution were not clarified\(^6\). In addition, in our preliminary experiment using guinea pigs in which no immunosuppression procedure was used, fungal infection in the nail plate and/or bed was not detected by histological examination. This result is in line with previous experimental studies indicating that immunosuppression might be essential for deep fungal invasion\(^12,\ 13\). Further, rabbits, which are classified in a group of medium-sized experimental animals, can be somewhat difficult to handle and prepare in large numbers, and the nails of these animals may become fragile as a result of the fungal infection. For these reasons, we chose to use guinea pigs to establish a reproducible model of onychomycosis using an immunosuppression procedure with corticosteroid administration, and this model potentially displays a similar pathophysiology to that of the human disease.

As an important symptom of human onychomycosis\(^8,\ 14,\ 15\), discoloration was commonly observed in our experimental model with a prevalence rate of 78% (28 of 36 nails) on Day 42 postinfection, and 26 of the 28 nails showing discoloration were confirmed histologically for fungal infection in the nail.
plate. Thus, the discoloration in our model may be considered a sign of fungal infection in the nail plate, and the pathophysiology of the discoloration is similar to that in human onychomycosis.

On the other hand, although the current study showed higher infection rates in the cuticle, proximal nail plate, and dorsal matrix in the Day 0 group, the infection rates in the nail bed and hyponychium were low (0-11%). This result suggests that our model is similar to the clinical subtype of PSO on Day 0 postinfection. The causative organisms of PSO are thought to penetrate via the proximal nail fold\(^{16}\), which was classified as the cuticle and dorsal matrix in this study. PSO is an uncommon subtype that occurs most frequently in patients with acquired immunodeficiency syndrome\(^{17,18}\). In individuals with human immunodeficiency virus infection, the presence of onychomycosis generally predicts that the number of helper T cells is less than 100 cells/mm\(^3\)\(^{17,19}\). The number of helper T cells decreases following steroid administration\(^{19}\); thus, this may explain why our model has characteristics similar to clinically diagnosed PSO in humans.

The most common subtype of onychomycosis is DLSO, which is characterized by fungal invasion of the nail bed and underside of the nail plate beginning at the hyponychium\(^{16,20,21}\). In contrast, fungal invasion in our model developed on the surface of the nail plate, after which the dermatophyte invaded the deep nail plate. The progression pattern of this model mimics the clinical features of PSO but not DLSO.

The infection rate in the distal nail plate was higher in the Day 14 and Day 42 groups than in the Day 0 group. In addition, fungal invasion was observed in the profound areas of the nail plate at all three postinfection time points, similar to the findings in the previous rabbit model\(^{10}\). The rabbit model showed a high rate of subungual abscesses, which are rarely

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Fig. 4. Histological findings in the infected nails on Days 0 (a, b) and 42 (c, d) postinfection. (b and d) Higher-magnification images of the red dashed regions in (a) and (c), respectively. Fungi were observed in the cuticle, matrix, and deep layer of the proximal nail plate (b) and showed distal spreading along the nail plate (d). On Day 0 postinfection, fungal spores and hyphae with strong PAS staining were observed in the stratum corneum, cuticle, dorsal matrix, and proximal nail plate (a, b). The hyphae ran parallel to the direction of nail growth (b). On Day 42 postinfection, fungi showed distal spreading along the nail plate (c, d). Fungal elements with decreased PAS staining were observed in the deep layers of the nail and ran parallel to the direction of nail growth (d).
observed in humans and are thought to be an artificial event in rabbits\(^{22}\). However, the rate of subungual abscesses was lower in our model (44\% versus 93.3\% in the rabbit model\(^{10}\) on Day 42 postinfection)\(^{22}\). These findings may be explained by differences in the susceptibility to corticosteroids between rabbits and guinea pigs. The latter are corticosteroid-resistant, similar to humans, whereas rabbits are corticosteroid-sensitive\(^{23}\). Moreover, Shewell et al. reported that administration of cortisone acetate markedly decreases body and thymus weights in corticosteroid-sensitive species\(^{24}\).

The body weights of the guinea pigs in our study increased in a time-dependent manner (data not shown), suggesting minimal effects of steroids in our model. Thus, the guinea pig may be a more appropriate rodent for establishing a highly reproducible animal model of onychomycosis induced by an immunosuppression procedure using corticosteroids.

In general, it takes a long time to cure onychomycosis because of the rate of nail outgrowth\(^{25}\). However, the rate of subungual abscesses was lower in our model (44\% versus 93.3\% in the rabbit model\(^{20}\) on Day 42 postinfection)\(^{22}\). These findings may be explained by differences in the susceptibility to corticosteroids between rabbits and guinea pigs. The latter are corticosteroid-resistant, similar to humans, whereas rabbits are corticosteroid-sensitive\(^{23}\). Moreover, Shewell et al. reported that administration of cortisone acetate markedly decreases body and thymus weights in corticosteroid-sensitive species\(^{24}\).

The body weights of the guinea pigs in our study increased in a time-dependent manner (data not shown), suggesting minimal effects of steroids in our model. Thus, the guinea pig may be a more appropriate rodent for establishing a highly reproducible animal model of onychomycosis induced by an immunosuppression procedure using corticosteroids.

In conclusion, we developed an animal model of onychomycosis using immunosuppressed guinea pigs, which showed high reproducibility and a similar pathophysiology to human onychomycosis and was relatively easy to handle. Therefore, our model may be advantageous over the previously reported rabbit model, because guinea pigs may not be excessively affected by steroid treatment and may be easier to manage. Further investigations using our model may be required to improve simulation of the clinical features of DLSO and to evaluate antifungal drug efficacy.

**Acknowledgments**

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**Declaration of interest**

Conflicts of interest: K.S. received research grants from Pfizer Inc., Dainippon-Sumitomo Pharma, and Astellas Pharma Inc.; he also received payments for lectures from Dainippon-Sumitomo Pharma and for consulting from Miraca Holdings Inc. N.H. was an employee of Pola Pharma Inc.

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**Table 2. Localization of fungi, total infection rate, and frequency of subungual abscess in an experiment to establish a model of onychomycosis**

<table>
<thead>
<tr>
<th>Group</th>
<th>Nail Plate</th>
<th>Cuticle</th>
<th>Matrix</th>
<th>Nail bed</th>
<th>Hyponychium</th>
<th>Total infection rate</th>
<th>Subungual Abscesses</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Proximal</td>
<td>Mediate</td>
<td>Distal</td>
<td>Dorsal</td>
<td>Intermediate</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 0</td>
<td>Superficial</td>
<td>33%</td>
<td>14%</td>
<td>0</td>
<td>39%</td>
<td>31%</td>
<td>17%</td>
</tr>
<tr>
<td></td>
<td>Medium</td>
<td>22%</td>
<td>0</td>
<td>0</td>
<td>61%</td>
<td>36%</td>
<td>8.3%</td>
</tr>
<tr>
<td></td>
<td>Profound</td>
<td>19%</td>
<td>0</td>
<td>0</td>
<td>58%</td>
<td>17%</td>
<td>0</td>
</tr>
<tr>
<td>Day 14</td>
<td>Superficial</td>
<td>47%</td>
<td>17%</td>
<td>0</td>
<td>50%</td>
<td>64%</td>
<td>28%</td>
</tr>
<tr>
<td></td>
<td>Medium</td>
<td>25%</td>
<td>14%</td>
<td>2.8%</td>
<td>25%</td>
<td>14%</td>
<td>2.8%</td>
</tr>
<tr>
<td></td>
<td>Profound</td>
<td>17%</td>
<td>14%</td>
<td>2.8%</td>
<td>25%</td>
<td>14%</td>
<td>2.8%</td>
</tr>
<tr>
<td>Day 42</td>
<td>Superficial</td>
<td>50%</td>
<td>64%</td>
<td>28%</td>
<td>50%</td>
<td>64%</td>
<td>28%</td>
</tr>
<tr>
<td></td>
<td>Medium</td>
<td>2.8%</td>
<td>31%</td>
<td>28%</td>
<td>25%</td>
<td>19%</td>
<td>42%</td>
</tr>
<tr>
<td></td>
<td>Profound</td>
<td>0%</td>
<td>19%</td>
<td>42%</td>
<td>25%</td>
<td>19%</td>
<td>42%</td>
</tr>
</tbody>
</table>

\(^{a}\) Localization of infection rate = (number of histologically fungus-positive nails in each region / number of nails tested in each region) × 100.

\(^{b}\) Total infection rate = (number of histologically fungus-positive nails / number of nails tested) × 100.

\(^{c}\) Appearance rate = (number of nails with subungual abscesses / number of nails tested) × 100.
Competing interests
All authors declare that they have no competing interests.

References