Localization of Keratinolytic Proteinase in Skin Tissue Sections of Guinea Pigs with *Microsporum canis* Infection by Immunoperoxidase Technique in Electron Microscopy

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An immunoperoxdase technique in electron microscopy was used to investigate the ultrastructural site of keratinolytic proteinase (KPase) of *Microsporum canis* in sections of skin from guinea pigs infected with the same organism. Ultrastructurally, the KPase was present only in the cell walls of the invading dermatophytes as a continuous deposition of the electron-dense reaction product on the inner and outer aspects of the cell wall of the fungal hyphae without deposition in the keratin surrounding the invading hyphae. Our results suggest that the KPase may not play an absolute role in the invasion of dermatophytes into keratinized tissue in vivo. (Ann Dermatol 3:(1) 1—4, 1991)

Key Words: Dermatophytosis, Electron microscopy, Immunoperoxidase technique, Keratinolytic proteinase

The keratinolytic proteinase (KPase) from dermatophytes may play a role in the invasion into keratin layers of dermatophytes[1,2]. We have previously demonstrated that KPase was present at the horny layer and at the level of the outer and inner root sheaths of hair follicles in guines pigs infected with *Microsporum(M) canis* using an immunoperoxidase technique and light microscopy[3]. We suggested that KPase may be produced only during active infection with *M. canis* after the development of erythema and before desquamation of the crust. However, it is still unclear whether the KPase is located at the cell wall of the infecting organism or is actively secreted into the surrounding tissues.

The purpose of this investigation is to determine the localization of KPase on the invading dermatophyte and the surrounding keratinocytes in situ using an immunoperoxidase technique in electron microscopy.

MATERIALS AND METHODS

Antiserum to KPase

The KPase was derived from *M. canis* isolated from a child's scalp lesions and was maintained at Yonsei University. The enzyme was prepared from culture filtrates of *M. canis* by ion-exchange chromatography and gel filtration as described previously[4,5]. The purified KPase solution (2mg/ml) was emulsified with equal volume of Freund's complete adjuvant. The emulsion, 1.0ml for each animal was injected intradermally into

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the footpads and midback of rabbits, and 0.2mg of the purified enzyme in 28mM phosphate buffer (pH 7.8) was given intramuscularly into the buttock as a booster three times at 10-day intervals. Sera from the guinea pigs immunized with the KPase were analyzed for precipitating antibodies to the KPase by immunodiffusion analysis.

Tissue specimens

Guinea pigs of both sexes, weighing between 350 and 500g, were used in all experiments. Regular sampling prior to inoculation revealed all animals to be free of dermatophyte spores. Inocula were made from cultures of *M. canis* grown at 27°C on rice agar media. The aerial growth was harvested using sterile saline containing 0.01% Tween 80. The suspension was shaken in a vortex mixer for 5 min and centrifuged at 2,500×g for 5 min. The deposited spores were then washed once in phosphatebuffered saline (PBS), pH 7.2, containing 50μg per ml chloramphenicol and 500μg per ml cycloheximide and twice in PBS. A dilution of 10^5 macroconidia per ml was then used for experimental infections. Experimental infections were produced without occlusion of the inoculated area[5]. The skin biopsies taken from the back were obtained from 9 noninfected pigs and 9 guinea pigs infected with *M. canis*.

Electron microscopy employing an immunoperoxidase technique

Fifteen-μm sections of infected skin fixed overnight with 2% glutaraldehyde in 0.1 M sodium cacodylate were mounted on glass slides coated with formal gelatin. After air drying for 30 min, the immunohistochemical reaction using the avidin-biotin-peroxidase complex (ABC) method was performed as suggested by Hsu et al.[6]. The sections were treated with 3% H_2O_2 and 3% NH_4OH followed by normal swine serum (DAKO Chemicals, Denmark). Sections were incubated with rabbit antibody to KPase at 1:1000 dilution followed by biotinylated swine anti-rabbit immunoglobulin (DAKO Chemicals, Denmark) at 1:200 dilution. The ABC at 1:100 dilution was added to the sections. Each step lasted 20 minutes and was followed by a 10-minute wash in PBS. The ABC was made by mixing 10μl of avidin (DAKO Chemicals, Denmark) with 10μl of biotinylated horseradish peroxidase in 1 ml of 0.05 M Tris buffer, pH 7.6 at 30 minutes before use. After the final wash in cacodylate buffer, the sections were post-fixed for 2 hours with 1.33% osmium tetroxide followed by a 10-minute wash with cacodylate buffer before alcohol dehydration. Each section was subsequently covered with propylene oxide and a gelatin capsule containing Epon mixture was inverted over each section. The specimens were then polymerized at 60°C for 36 hours. The blocks separated from the slide were sectioned on a Richert-Jung ultramicrotome. Zones suitable for ultrastructural examination were selected after inspection of semi-thin (0.5μm) sections. Ultra-thin sections were examined in a Hitachi H-500 electron microscope at 75KV.

RESULTS

Application of immunoperoxidase staining to skin sections of guinea pigs with *M. canis* infection resulted in an intense and specific labeling of cell walls of invading hyphae in keratinized tissue (Fig. 1). Electron-dense reaction product was irregularly distributed in a linear pattern along the outer and inner wall of invading hyphae (Fig. 2a). Dissolving features, or lacunae, and positive deposition of KPase in the keratin surrounding the

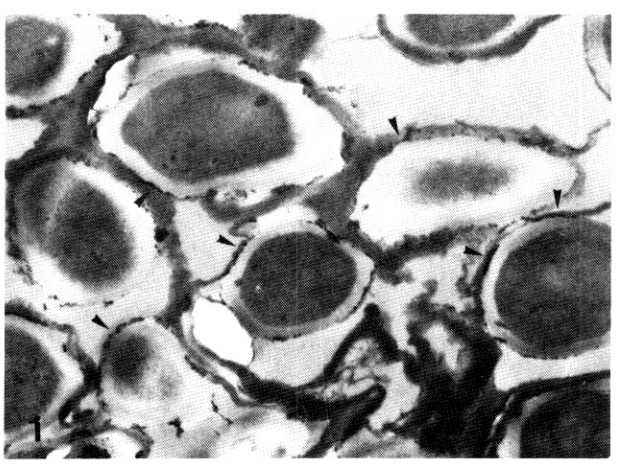

Fig. 1. Transmission electron micrographs (TEM) of invading M. canis in tissue treated with immunoperoxidase staining 3 weeks after the inoculation of M. canis on the skin of guinea pig. Electron-dense reaction products deposited in the cell walls (arrowheads) (×20,000).

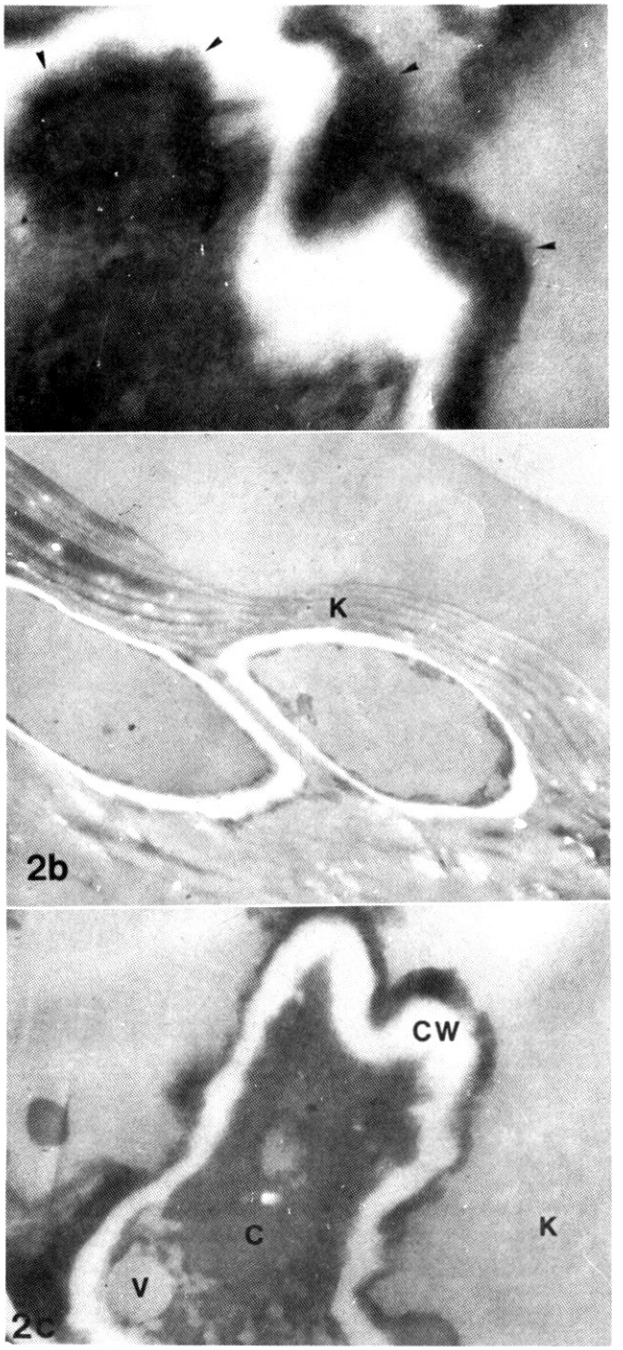

Fig. 2. TEM of invading M. canis in tissue treated with immunoperoxidase staining. (a) Linear deposition of the electron-dense reaction product along the cell wall (arrowheads) (×112,500). (b) No positive deposition of KPase and dissolution of the surrounding keratin(K) were seen (×25,000). (c) Cytoplasm, nuclei, and organelles of fungal hyphae are free of labeling. CW, cell wall; V; vacuole; K, keratin; C, cytoplasm (×62,500).

invading hyphae were not seen (Fig. 2b). Cytoplasm and organelles of fungal hyphae were free of labeling (Fig. 2c).

DISCUSSION

It is interesting to investigate the mechanism of dermatophyte invasion and utilization of keratinized tissue which is typically strongly resistant to most keratinolytic enzymes[7]. The keratinolytic dermatophytes show a peculiar morphology when growing in vivo on the keratinized tissue. They produce flattened fronds of hyphae and a perforating organ develops from these fronds to penetrate the keratinized tissue.[8, 9] However, during in vivo infection, hyphae of the dermatophytes do not have this specific structure at the invading tips[10, 11]. Several investigators suggested that a lytic enzyme may be secreted by the dermatophytes and that this is an important factor in the pathogenesis of dermatophytosis. Holden et al.[12] reported that the keratin immediately surrounding invading hyphae shows positive staining in an immunoperoxidase study using rabbit antiserum to crude extracts. Kanbe and Tanake[13] and Choi et al.[10] also observed a clear space between the cells and Tanaka[13] and Choi et al.[10] also observed a clear space between the cells and the hair cortex, which may be formed by KPase digestion, by electron microscopy.

Recently, KPase was isolated and purified from different dermatophytes[4, 14-16]. The keratinized portion of hair[17] and human epidermis, especially the stratum corneum[18] can be dissolved in vitro by the fungal keratinase. Lee and Park[19] also observed that the KPase of *M. canis* digested human scalp hair, stratum corneum, and nail in vitro. The study for the localization of dermatophytes. antigens has not been heavily investigated. One component of dermatophytes, a glycoprotein, has been demonstrated on fungal cell walls using immunofluorescence microscopy[20]. In an earlier study using rabbit antiserum to crude mycelial extracts, the cell wall was shown to be a major antigenic site by immunoperoxidase technique in light and electron microscopy[12]. It was observed that the surrounding margins of the lacunae invaded by dermatophyte hyphae were

coated with dermatophyte-derived antigen. These results suggested that a diffusible substance, such as a lytic enzyme, was secreted by the dermatophyte.

The immunoperoxidase technique is useful for detection and localization of antigen, especially extracellular antigen, by light and electron microscopy, and can be employed in the study of the relationship of the hyphae to the corneocytes[21, 22]. In the present study, the use of immunoperoxidase technique in electron microscopy has allowed the in situ localization of fungal KPase in the cell walls. Unfortunately, we did not find evidence that KPase was secreted by dermatophytes causing subsequent digestion of the surrounding keratin tissue. Our results suggest that KPase may not be directly responsible for dermatophytic invasion of keratinized tissue in vivo, but plays a role as an antigen in dermatophytosis. Growth of filamentous fungi was highly polarized at their hyphal tips, where a large number of vesicles were seen. These vesicles were thought to carry lytic and synthetic enzymes[13]. Therefore, the presence of selective hyphal tips which can secret KPase into the surrounding tissue was postulated but unsubstantiated. The possibility that KPase is not an exo-enzyme, but rather a cell membrane-bound enzyme can not be completely excluded at the present time.

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