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Original Article

In Vitro Effect of Antigenic Extract of *Trichophyton verrucosum* on Fibroblast Proliferation and Matrix Metalloproteinase -2 Activities

AR Salehinodeh [1], S Rezai [2], H Javanmard-Khamene [2], P Ekhtiyari [1], *A Mirshafiey [1]

1. Dept. of Medical Immunology, School of Public Health, Tehran University of Medical Sciences ,Tehran, Iran
2. Dept. of Medical Mycology, School of Public Health, Tehran University of Medical Sciences, Tehran, Iran

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Abstract

Background: *Trichophyton verrucosum* is a zoophilic fungus with a worldwide distribution. Our aim was to investigate the proliferative effect of antigenic compounds of *T. verrucosum* on dermis fibro blasts and endothelial cells.

Methods: *T. verrucosum* was cultured in SCC medium and was then transferred to a broth medium. Surface antigens of this fungus were separated using the freeze and thaw method. The sample was centrifuged and the supernatant was taken. The supernatant was homogenized and purified. The prepared antigenic extract was added to fibro blast cell lines according to a regular timetable. Cytotoxicity and cell proliferation were evaluated using zymography and densitometry in order to assay MMPs activity.

Results: Statistical analyses showed that this antigenic extract is able to enhance the MMPs activity

Conclusion: *Trichophyton verrucosum* increases the proliferation of dermis germinal layer and MMP-2 activity, which has a direct relation with wound healing process.

Keywords: *Trichophyton verrucosum*, Proliferation, Matrix metalloproteinase

Introduction

Trichophyton verrucosum is a zoophilic dermatophyte, inducing bovine dermatophytosis. It is transmitted to human through direct contact with contaminated cattle or its products in infected patients with inflammatory lesion in head, face etc (1). *Trichophyton verrucosum* invades hair and is one of the causes of tinea capitis especially in children (2). This organism can be cultured in sabouraud media containing glucose enriched with thiamine; inositol, pyridoxine, and yeast extract (2). Skin colonization of this dermatophyte could lead to an inflammatory reaction with different severities (3). On the other hand, it can reactivate the specific and nonspecific immunity reactions including cellular and humoral immunity (4).

The most important mechanism of active nonspecific responses to dermatophytes is macroglobulin keratinase, unsaturated transferrine and scaling of epidermis. Moreover, the accumulation of neutrophils has also been reported in inflamed epidermis in acute stages of disease (5).

Trichophyton verrucosum has a variety of surface antigen, which can raise different responses, such as immunostimulatory property and proliferative response on skin layers. It causes a severe scaling in infected region. This characteristic can imply a possible role for superficial antigenic determinants in stimulating the proliferation of fibroblasts and endothelial cells. The role of fibroblasts which are a less specialized group of connective tissue are secretion of extra cellular matrix rich in collagen type I & II. When a tissue lesion occurs, the surrounding fibroblasts proliferate and immigrate to the lesion and secret the collagenic matrix. In the

***Corresponding Author:** Tel: +98 21 88954913, E-mail address: mirshafiey@tums.ac.ir

process of wound healing, TGF-β plays a significant role. This cytokine promotes converting the fibroblasts to fibrocytes and formation of tissue rich in collagen (6). In addition, the endothelial cells, which are the covering cells of vessels, can generate the new vessels in the process of angiogenesis (7). As mentioned above, the immigration of fibroblasts and endothelial cells to lesion region is necessary for tissue repair.

Matrix metalloproteinase (MMPs) are a large group of proteolytic enzymes responsible for degradation of intracellular matrix. The activity of these enzymes are mandatory for some of biological processes such as wound healing, angiogenesis etc, this will happen transiently. MMPs family in human comprises 18 members (8, 9). Endogen inhibitors control the expression and function of MMPs continuosly. Tissue inhibitors of MMPs and α_2 macroglobulin are two kinds of such inhibitors, which are secreted by connective tissue cells, hepatocyte, macrophages, and fibroblasts (10). Nowadays, it has been demonstrated that the over expression of tissue inhibitors of matrix metalloproteinases can inhibit immigration of different cells including fibroblasts and endothelial cells (11). It seems that, surface antigenic determinants of *T. verrucosum* can stimulate MMPs for tissue repair phenomenon; since the compounds of this fungus probably enhances the tissue repair process by stimulating MMPs, proliferation and immigration of fibroblasts and endothelial cells.

In this study, our aim was to assess the effect of antigenic determinants of *T. verrucosum* on proliferation of fibroblast and endothelial cells under in vitro condition. Moreover, we evaluate the rate of MMPs activity in vitro. To do this purpose, treatment of fibroblast and epithelial cells with antigenic extract of *T. verrucosum* (AETV) was carried out to determine its effect on processes of tissue repair.

Materials and Methods

Cell culture

The fibro sarcoma (WEHI-164) cell line was seeded at density range of 5-40×10^3 cells/well in 96-well tissue culture plates. Cells were maintained in RPMI-1640 medium supplemented with 5% fetal calf serum, penicillin at 100 units/ml, and streptomycin at 100 µg/ml, with 5% CO_2, 37ºC and saturated humidity. To assess the proliferative effect of antigenic extract of *T. verrucosum* on wehi cells, these cells were incubated with various concentration of AETV (2.5, 5, 10, 20) µg/ml. The proliferative effect of AETV was studied with colorimetric method

T. verrucosum culture filtrate

Lyophilized isolate of *T. verrucosum* was reconstituted with 0.5 ml of sterile water, inoculated onto Sabouraud agar, and then expanded into larger (250ml and 5 lirers) volumes of Sabouraud liquid culture medium (Sabouraud-2% dextrose broth with concentration of 30g/L in demineralized water).

Trichophyton verrucosum was cultured in a liquid defined medium on a gyratory shaker (120 rev/min) for 15 days at 30°C. The cells were removed by centrifuging at 9200g for 20min and filtering the supernatant through a 0.2µm sterile filter and then dialyzed in the cold vs. deionized water. Moreover, the suctions (16KH, with10 min, interval for 8 stages) for removed cells were performed in ice. The antigenic extract of *T. verrucosum* (AETV) used in this study was concentrated 3-fold, resulting in a protein concentration of 0.4mg/mL. Carbohydrate concentration was determined by the phenol-sulfuric acid assay. The concentrated AETV solution was then used immediately or stored at 20°C for no longer than 30 days.

Dose – response analysis

Triplicate, two-fold dilutions of AETV at concentrations of 2.5-20 µg/ml were transferred overnight to cultured cells. Non-treated cells were used as controls. Cells were cultured overnight and then subjected to colorimetric assay. A sample of the media was used for zymography.

Colorimetric assay

After each experiment, cells were washed three times with ice-cold phosphate-buffered saline (PBS), followed by fixation in a 5% formaldehyde

solution. Fixed cells were washed three times and stained with 1% crystal violet. Stained cells were then washed and solubilised with 33.3% acetic acid solution. The density of developed purple color was read at 580 nm.

Zymography

This technique was used for determining gelatinase (collagenase type IV or matrix metalloproteinase type 2, MMP-2) activity, in conditioned media according to the modified Heussen and Dawdle method. Briefly, aliquots of conditioned media were subjected to electrophoresis in (2m/ml) gelatin containing polyacrylamide gels, in the presence of sdium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under non-reducing conditions. The gels underwent electrophoresis for 3 hours at a constant voltage of 80 volts. After electrophoresis, the gels were washed and gently shaken in three consecutive washings in 2.5% triton X100 solution to remove SDS. The gel slabs were then incubated at 37ºC overnight in 0.1 M tris HCl gelatinize activation buffer (pH 7.4) containing 10 mM $CaCl_2$ and were subsequently stained with 0.5% Commission blue. After intensive distaining, proteolysis areas appeared as clear bands against a blue background. Quantitative evaluation of both surface and intensity of lyses bands, based on grey levels, were compared relatively to non-treated control wells, and expressed as "Relative Expression" of gelationlytic activity.

Statistical analysis

All data are expressed as the mean ± SEM. The significance of differences was evaluated with *t*-test. *P*-values less than 0.05 were considered significant.

Result

1- Effect of AETV on cell proliferation: the antigenic extract of *T. verrucosum* showed a significant prolifarative effect on wehi cells culture (Fig.1).

2- Effect of AETV on MMP-2 activity. The results, as shown in Fig. 2 indicate that AETV increases the activity of MMP2.

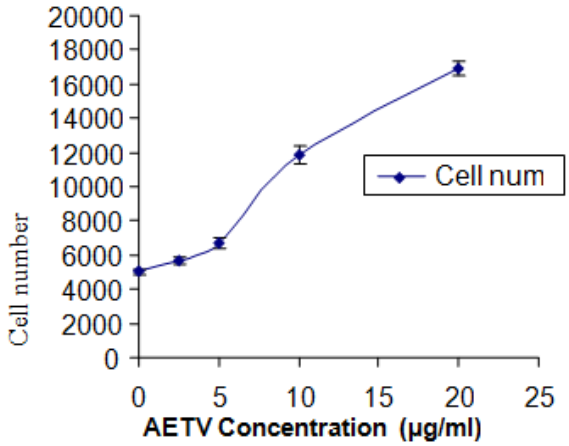

Fig. 1: The proliferative response of fibrosarcoma (WEHI-164) as a sensitive cell line to antigenic extract of *T. verrucosum* (AETV) at different doses (2.5-25) (µg/ml)

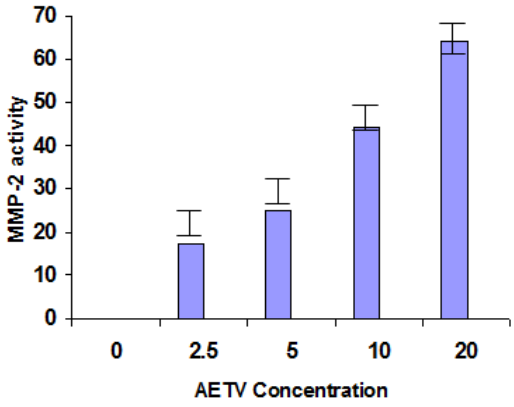

Fig. 2: The stimulatory effect of antigenic extract of *T. verrucosum* (AETV) on MMP-2 activity at different doses (2.5-25) µg/ml

Discussion

Trichophyton verrucosum is a worldwide spread and zoophilic fungus. It causes tinea in cattle. This fungus is transmitted to human through direct contact with infected animal or its products. It can stimulate cellular and humeral immunity of host. Therefore this strain can be used for anittinea vaccines (1, 3). The MMPs are group of proteolytic enzymes, which are responsible to degradation of intracellular matrix.

Degradation happens transiently in a physiological process such as wound healing and angiogenesis. These proteinasesare usually expressed following the stimulation by proinflammatory cytokines, and some metal cofactors such zinc and calcium. In normal situation, their secretion is controlled by endogen inhibitors. Proteinases allow fibroblasts and endothelial cells to immigrate to injured region by degradation of intracellular matrix. Among various dermatophytones, *T. verrucosum* has more immunostimulatory effects on proliferative layers of skin (5).

Therefore, its antigenic determinants seem to be able to stimulate proliferation of fibroblast and endothelial cell in dermis. Regarding the MMP2 plays a significant role in the process of tissue repair based on its proliferation ability and cellular migration, this enzyme was selected. The biological property of antigenic extract of *T. verrucosum* and its effects on immune system are presently being investigated, but a similar study with our research has not yet been done.

Therefore, it seems that AETV can do its function through increasing of fibroblasts proliferation and immigration to injured region. Based on this property, and the role of MMPs in the immigration of endothelial cells and fibroblasts to inflamed tissue, the extract seems to be effective in wound healing. In this connection, future studies could be conducted in such a way for determining the main factor of this fungus in increasing the activity of MMPs.

Ethical considerations

Ethical issues (Including plagiarism, Informed Consent, misconduct, data fabrication and/or falsification, double publication and/or submission, redundancy, etc) have been completely observed by the authors.

Acknowledgments

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