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Immunohistochemical analysis of effects of UVA exposure to the human fibroblasts in the skin equivalent model

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In vitro and *in vivo* studies have reported the induction of matrix metaloproteinase (MMP)-1 in the fibroblasts by ultraviolet (UV) A irradiation. We constructed the skin equivalent model using HaCaT cells as keratinocytes and human neonatal dermal fibroblasts as fibroblasts in the present study. The induction of MMP-1 in the fibroblasts was confirmed immunohistochemically 6 hours after UVA irradiation using this model. This model was simply composed of human keratinocytes and fibroblasts. To our knowledge, there have been a few papers concerning the skin equivalent model in the field of photobiology. The effect of UVA exposure to fibroblasts through keratinocytes was examined using this model. The cross-talk can be examined between keratinocytes and fibroblasts. This model can be a useful tool in the field of photobiology.

Key words: UVA, MMP-1, skin equivalent model, fibroblast

INTRODUCTION

Ultraviolet (UV) A represents high percentage of UV and can penetrate deeply into the dermis, so that its effect on dermal fibroblast is very important. It is not easy to get an informed consent of each person in order to examine the in vivo effect of UVA on human fibroblasts and we tend to use animals. The skin is composed of several kinds of cells such as keratinocytes, fibroblasts, endothelial cells and so on, and the cross-talks are complex among them. Because in vitro experiment is usually performed using one kind of cells in the dish, this condition is simple but make it difficult to consider the effect of cross-talk among composed cells of the skin. We employed the skin equivalent model composed of human keratinocytes and fibroblasts in the present study. HaCaT cells were adopted as keratinocytes and human neonatal fibroblasts as fibroblasts. When the condition was completed, stratified keratinocytes overlay collagen gel containing human fibroblasts. There have been few reports examining the effect of UVA on fibroblasts using this model as far as we know. Previous in vitro studies reported that UVA induced some enzymes such as heme oxygenase-1 and matrix metalloproteinase (MMP)-1 in human fibroblast. We employed MMP-1 as a marker and examined immunohistochemically the effect of UVA exposure to fibroblasts through stratified keratinocytes using this model.

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MATERIALS AND METHODS

HaCaT cells and human neonatal dermal fibroblasts were prepared for the construction of this system.

On the first day, six Cell Culture Inserts (Becton Dickinson 35-3092) were put in one Biocoat Deep Well Plate (Becton Dickinson 35-5467). Human neonatal dermal fibroblasts, which were almost confluent, were trypsinized and neutralized by adding 10 ml of DMEM. Cell suspensions were centrifuged and the supernatant was removed. Cell pellet was resuspended in 2.4 ml of fetal calf serum (FCS). The number of cells was counted and adjusted to $2.5\text{-}3.0 \times 10^6$/ insert. The collagen gel was composed of 24ml of collagen, 3 ml of 10X Hanks buffer and 0.6-1.0 ml of 0.1N NaOH/ PBS. When the color of collagen gel became pinkish, the gel was mixed with 2.4 ml of cell suspension in FCS. Each 4 ml of gel mixture was put into cell insert. Cell inserts were incubated in the Biocoat Deep Well Plate for one hour at 37℃. After that, the glass ring was put onto each gel

mixture in the cell insert. Each cell insert was fed with DMEM from outside in the well plate.

On the second day, confluent HaCaT cells were trypsinized and neutralized by 10ml of DMEM. The number of cells was counted, suspended and adjusted to $5x10^5$/ cell insert. Cell suspension was spin and cell pellet was resuspended in 6 ml of DMEM. Extra volume of DMEM was removed inside the glass ring on the collagen gel mix in the insert. 1 ml of DMEM containing HaCaT cells was put onto each insert. Six inserts set in the Biocoat Deep Well Plate were incubated.

On the third day, medium was removed inside the glass ring so that HaCaT cells became to face the air in the end. Medium was change twice a week. Two weeks later, this system was used for the experiment.

The source of UVA was ATTO model HP-12BLB (ATTO Corporation, Japan) with a glass filter and the dose was 1.8J/cm^2. During UVA irradiation, cell inserts were incubated in PBS and maintained at 37-39℃. Following irradiation, PBS was replaced by fresh DMEM with 10% FCS and cell inserts were incubated at 37℃ for various periods of time. Cell inserts were treated at 0, 2, 4 and 6 hour after UVA irradiation for immunohistochemical analysis.

Immunohistochemistry

Labeled streptavidin biotin (LSAB) method was adopted (DAKO LSAB 2 System, peroxidase) and anti-human metalloproteinase (MMP)-1 antibody (rabbit immunoglobulin) was purchased from SIGMA (#M-4177, USA). The antibody is polyclonal and does not cross-react with other MMP family members (MMP-2, -3, -9 etc). (data from the manufacturer)

Five micrometer tissue sections were deparaffinized in xylene. Endogenous peroxidase was blocked with 3% hydrogen peroxide in distilled water for 5 minutes at room temperature (RT). After washing in 0.05M TBS for 5 minutes, each tissue section was immersed in 0.05M Tris-buffered saline (TBS) containing 5% bovine serum albumin for 20 minutes at RT to diminish the non-specific binding of the secondary antibody. The sections were treated with anti-human MMP-1 antibody (1: 200) at 4°C overnight. The next day, the sections were washed and treated with biotinated secondary antibody at RT for 10 minutes. After rinsing, the sections were treated with streptavidin-HRP at RT for 10 minutes. After washing, the slides were treated with substrate solution (DAB) for about 10 minutes at RT. After washing, the slides were counterstained with hematoxylin solution. The tissue of squamous cell carcinoma was used as a positive control[1], while negative control staining was obtained by the replacement of the primary antibody with nonimmunized rabbit serum.

RESULTS

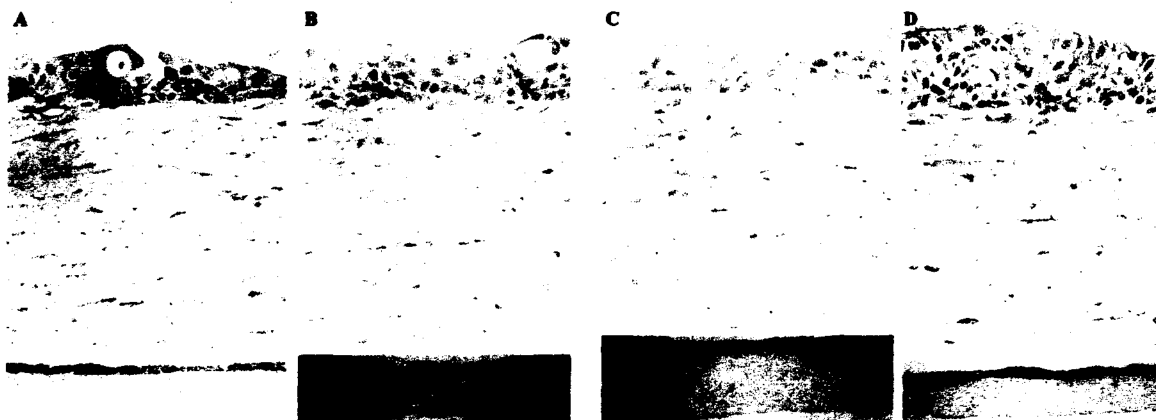
We constructed the skin equivalent model using HaCaT cells as keratinocytes and human neonatal dermal fibroblasts as fibroblasts in the present study.

The induction of MMP-1 protein was confirmed in fibroblasts immunohistochemically 6 hours after UVA irradiation using a skin equivalent model. (Fig 1)

DISCUSSION

In the present study, we constructed a skin equivalent system using HaCaT cells and human dermal fibroblasts. To our knowledge, there have been a few papers concerning the skin equivalent model in the field of photobiology. *In vitro* studies have reported the induction of MMP-1 in the fibroblasts by UVA irradiation[2]. We also confirmed the induction of MMP-1 in fibroblasts by UVA irradiation through keartinocytes using this system.

The commercially available equivalent model is composed of human normal keratinocytes and fibroblasts from young Caucasian foreskins. The present study is a preliminary experiment for us and our goal is the establishment of an artificial system composed of human normal cutaneous keratinocytes and dermal fibroblasts from all ages and all sites. This model was simply composed of human keratinocytes and fibroblasts. The effect of UVA through keratinocytes was examined in the fibroblasts using this model. Furthermore, we can also examine the cross-talk between keratinocytes and fibroblasts using this system. This system can be a very useful tool in the field of photobiology.



(Fig 1)

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Legends

Fig 1 Immunostainings of MMP-1 at 4 time points

The induction of MMP-1 protein was confirmed in fibroblasts 6 hours after UVA irradiation.

A: No UVA irradiation
B: 2 hour after UVA irradiation
C: 4 hour after UVA irradiation
D: 6 hour after UVA irradiation

Lower panels show corresponding figures with low power magnification.(HE)