THE STUDY ON WOUND HEALING POTENTIAL OF COSMETIC INGREDIENTS IN CULTURED KERATINOCYTES

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ABSTRACT

MMA-5 and Malva are extracts from a defined seaweed of the Rhodophyceae family and an annual or perenial plant, respectively. They have traditionally a significant stimulating properties such as protein synthesis, astringent, emollient, and et al.. In order to investigate the effect on wound healing, we studied for cell migration, cell attachment, and cell proliferation in vitro. The results of present study demonstrated that MMA-5 and Malva extract had the effect on wound healing, therefore we suggested that they could be effective materials to be applied to Cosmetic products.

1. INTRODUCTION

Tissue response to injury has been divided into three overlapping phases : inflammation, granulation tissue formation, and matrix formation and remodeling (figure-1)(1, 2). The first phase of wound healing is inflammation which can be divided into early and late phases. Injury of the skin and concomitant blood vessel disruption lead to extravasation of blood constituents, followed by platelet aggregation and blood clotting. The macrophage plays a pivotal role in the transition between wound inflammation and repair, since this cell both scavenges tissue debris and releases a plethora of biologically active substances that include growth factors (table-1). The second phase of wound healing is the granulation tissue formation which consists of a dense population of macrophages, fibroblasts, and neovasculative embeded in a loose matrix of collagen, fibronectin, and

hyaluronic acid (table-2).

Fibroblasts proliferate and migrate into the wound space and these cells align themselves and thier newly deposited matrix along the radical axes of the wound, form cell-cell and cell-matrix links, and thereby are able to generate a concerted tension that results in wound contraction. The third and final phase of wound healing is

Table-1. Macrophage activities in wound repair

1. Scavenging a. Pathogenic organisms b. Tissue debris	2. Secretion of biologically active substances a. Vasoactive mediators b. Chemotactic factors c. Growth factors d. Proteases

matrix formation and remodeling. Matrix formation begins simulteneously with granulation tissue formation, and the matrix is constantly altered with the relatively rapid elimination of most fibronectin from the matrix and with the slow accumulation of large bundles of type 1 collagen that provide the residual scar with increasing tensile strength (table-3). Following injury to the epidermis, keratinocytes undergo a series of characteristic changes in behavior including hyperproliferation, cell-matrix attachment and cell migration at the edges of the injury site (3).

Table-2. Major components of late inflammatory and early granulation tissue formation phases of tissue repair

matrix component	cellular component
Fibrin	Epidermal cells
Fibronectin	Macrophages
Type III/I collagen	Endotherial cells
Hyaluronic acid	Fibroblasts

Table-3. Major components of late granulation tissue formation and matrix remodeling of tissue repair

Matrix component	cellular component
Basement membrane	Epidermal cells
Laminin	Endotherial cells
TypeIV collagen	
Interstitium	
TypesI/III collagen	Fibroblasts
Chondroitin sulfate	
Dermatan sulfate	

Keratinocyte migration across the wound bed is an early and critical event in the wound healing process, but re-epithelialization in wound repair has not known and a number of possibilities have been discussed. In vitro and in vivo experimental systems, such as those in which skin explants are placed on matrices, studied in animal models, and examined for keratinocyte outgrowth, migration, and megacolony formation in short term cell culture, have been used to assess the effects of wound healing (4,5,6,7). In order to assess the effects of cosmetic ingredients on wound healing, we studied for cell-matrix attachment, cell proliferation, and cell migration which is modified in order to assess keratinocyte outgrowth system in vitro (8). The object of this study was to investigate the possibility of application at cosmetic products, and to establish modified migration test method which is very simple technique (9). The study showed efficiency and reliability of the modified test method in assessing cell migration. Also we found that the several cosmetic raw materials sustained cell proliferation, attachment, and migration in cultured cell.

2. Methods and Materials

Cell culture and Reagents

Normal human keratinocyte cultures were derived from neonatal foreskin

epidermis using a modification of the method described by Rheinwald and Green (10).

Cells were cultured in KGM(keratinocyte growth medium, Clonetics, USA) supplemented with BPE(Bovine pituietry extract), and kept at 37^{0}C in a 5% CO_2 incubator. Keratinocytes were used between passages 2 and 4 for all assays.

All of the supplements in cell culture were purchased from Gibco(U.S.A). Cosmetic raw materials which were tested, were MMA-5(Sodichimic, France), Melva extract(Alban Muller international, France), Aloe extract(Terry, U.S.A), and Royal jerry(Taiyo KAGAKU Co. Ltd.).

Total cell protein assay

The media at multi-well plates grown cells were removed and wells were washed twice with PBS. Each well then received 50ul of 0.1N NaOH and the plate was incubated for at least 15min at 37^{0}C and then 200ul of diluted dye reagent (Bio-Rad reagent : D.D.W = 1 : 3) was added to each well. The plate was agitated on a shaker after dye addition. After 30min of incubation at room temperature, the plate was scanned in a Micro-plate Reader(Bio-Tek instruments, EL340) using a 405nm filter for the reference wavelength and a 630nm filter for the absorption wavelength (11).

Cell attachment assay

The effects on cell-plastic attachment of keratinocytes were measured by a previously described assay method (7). Briefly, viable keratinocytes released from T-flasks were loaded in 96 well plates at a concentration 5x10^4/well, incubated for 2hr at 37^{0}C in a 5% CO_2 incubator and gently washed with PBS(phosphate buffered saline, pH 7.4) to remove unattached cells. The amount of total cell protein was determined by microplate reader at 630nm.

Cell proliferation assay

To assess the proliferative potential of human keratinocytes, 10^3 keratinocytes were seeded onto 96 well plates, and the plates were incubated for 16hr at the same condition, and the medium at each well in the plates was replaced with fresh BPE free medium containing test materials or not. Positive control medium was added 3% serum in BPE free medium and the medium without serum was used as negative control. Each micro plate was incubated for 2, 4, and 6 days at 37^{0}C in a 5% CO_2 incubator. The amount of total cell protein was determined by microplate reader.

Cell migration assay

Label tapes of 4mm diameter were adhered on the bottom of each well in 24 well plate, and one milliliter of 70% ethanol to make aseptic condition was poured into each well, and then 70% ethanol was removed and each well was washed twice with PBS to remove remaining Ethanol. 10^5 keratinocytes were seeded into each well of 24 well plates and label tapes were removed from the plates after incubation for 24hr. Medium was replaced with fresh medium containing each test materials, negative control and positive control medium. The micro plates were incubated at 37^0C in a 5% CO_2 incubator. After 48hr the number of migrating cell layers or migration distance toward inner empty space were counted or estimated under inverted Microscope.

3. Results and Discussion

In cell plastic attachment assay, the OD values represented attachment rate of keratinocytes adhered to plastic surfaces of 96 well micro plates.

The result of cell attachment rate was illustrated in figure-2. As showed in the graph, the attachment rate was greatly improved in the test groups containing MMA-5 and Malva extract when it was compared with any other test groups. But the increasing rates of cell attachment was lower than those of positive controls containing 3% serum. Cell population at the group of serum free medium containing cosmetic ingredients or not was gradually decreased. Although cell population was gradually decreased in all serum free test groups, the level of decrease in the test groups of MMA-5 and Malva extract was lower than negative control. In the case of positive control and cosmetic ingredients containing 3% serum, the cell population was gradually increased in all test groups. The each result from cell proliferation experiment indicated that MMA-5 and Malva extract were able to maintain cell activity at serum free conditions and this function depended on concentration of cosmetic ingredients as illustrated in figure-3. The results of cell migration assay were represented in table-4. As shown in table-4, the number of migrating cell layer was 7-8 layers for MMA-5 and Malva extract, whereas the migrating ability for other samples was as low as

negative control. But the cells of positive control were migrated to 9-10 layers toward inner empty space. Although the result from positive control showed the highest migrating effect among all test groups, MMA-5 and

Table-4. The number of migrating cell layer of several cosmetic ingredients which were applied at 0.5% concentration.

	The number of migrating cell layer
1) Negative control(W/O serum)	3-5 cell layer
2) Positive control (conteining 3% serum)	9-10 cell layer
3) Malva extract	7-8 cell layer
4) MMA-5	7-8 cell layer
5) Aloe extract	1-2 cell layer
6) Royal jerry	4-5 cell layer

Malva extract had the predominent ability of cell migration. According to Maker's claim about MMA-5 and Malva extract, it was known that Mallow had astringent, demulcent, emollient and expectorant properties and but they might delay the healing of wounds upon which they are placed. The MMA-5 had a significant stimulating effect upon the total protein synthesis, particularly the synthesis of collagen, the production of collagenase by normal human fibroblasts, and retraction of a collagen gel. But the results of present tests showed that Malva ectract and MMA-5 had effects on cell plastic attachment and cell migration in vitro besides their native properties. Especially MMA-5 was considered to have effect on wound healing because of its native properties such as collagen synthesis, protein synthesis, coolagenase activation, et al. Recently, L.B. Nanney(1990. JID) had reported that cell proliferation, attachment, and migration were deeply related with each other(12). But more recently, Sergei A. Grando et al.(1993. JID) have reported that cell-matrix attachment, spreading and locomotion of human keratinocytes, but not mitosis, mediate the earlist stages of skin re-epithelization(7).

MMA-5 and Malva extract are not cytotoxic and have very low effect on

them ultiplication rate of keratinocytes in normal human epidermis as illustrated in figure-4. The results of present study showed that cell matrix attachment and cell migration have a close relation with wound healing, but proliferation do not. Through various experiments such as cell attachment, proliferation, and migration assay, we concluded that MMA-5 and Malva extract was useful materials to wound healing. The important problem, however, remaining to be solved is animal test or any in vivo test to clarify the effect on wound healing. For the further study, to give proof about effect on wound healing and to investigate biochemical phenomenon of various enzyme such as plsminogen, collagenase et al., we will try several experiments.

As a conclusion, the modified migration assay method is a simple and reliable method allowing accurate measurement of keratinocyte migration in vitro, and MMA-5 and Malva extract might be useful materials to cosmetic products.

국문요약

MMA-5와 Malva는 각각 해조류(Rhodophyceae)와 일년 또는 다년생 풀에서 추출되었으며 이들은 과거부터 보습, 수렴, 단백질 합성등의 효과를 가지는 것으로 구전되어왔다. 상처치유에 대한 이들의 효과를 조사하기 위해서 우리는 배양된 사람의 표피 세포를 이용하여 세포증식, 세포부착, 그리고 세포이동에 미치는 영향을 조사하였고 실험결과 MMA-5와 Malva추출물들은 상처치유에 유효한 효과를 나타낼수 있음을 밝혀냈다.

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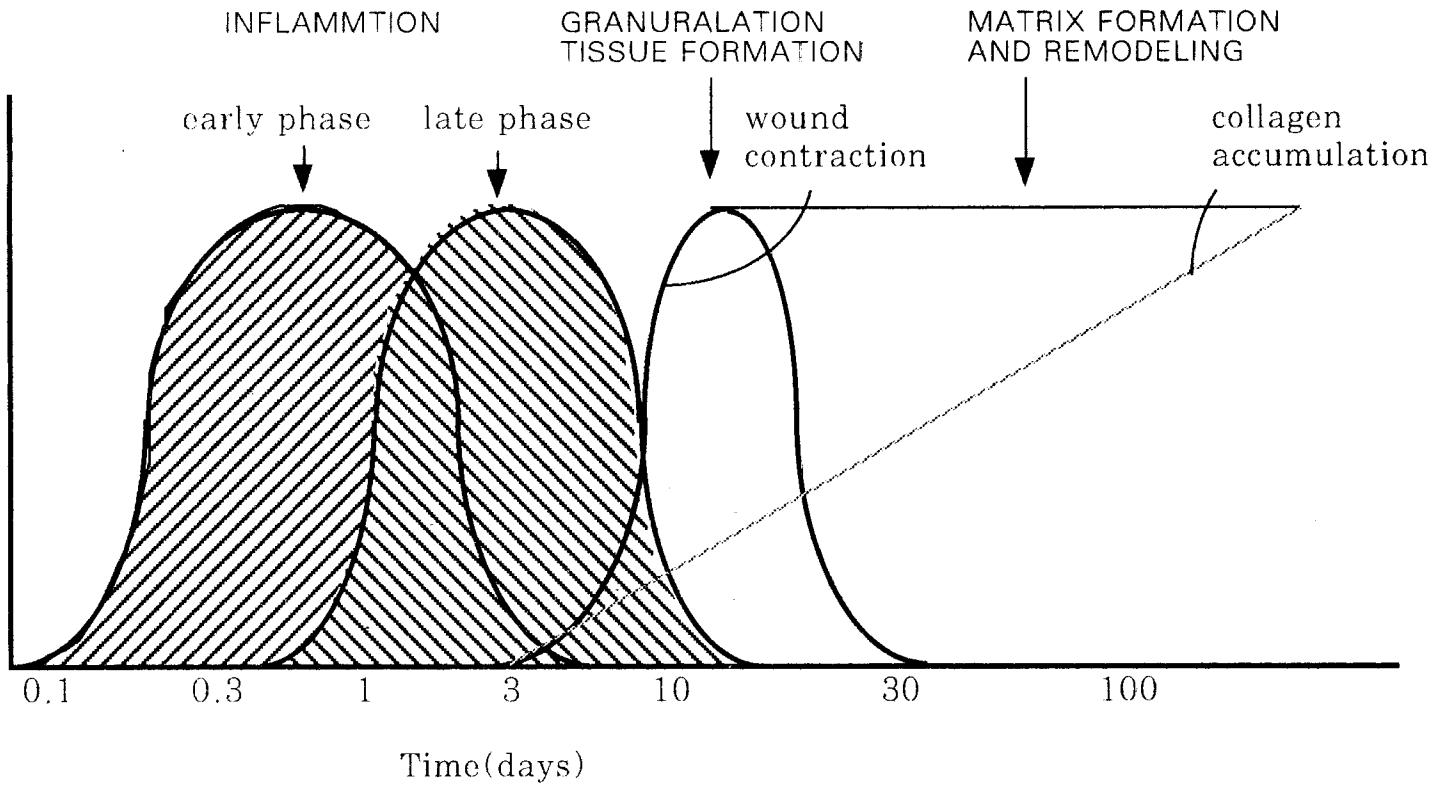
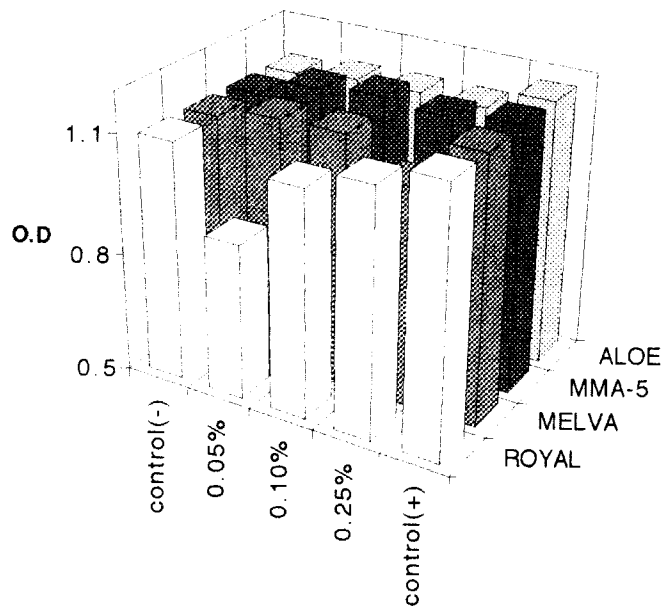


Figure-1. The phases of wound repair. Healing of a wound has been arbitrarily divided into three phases: inflammation(early and late), granulation tissue formation, and matrix formation and remodeling

Figure-2. Cell attachment rate of Human keratinocytes on the bottom of micro-plates in serum free medium containing several cosmetic ingredients.



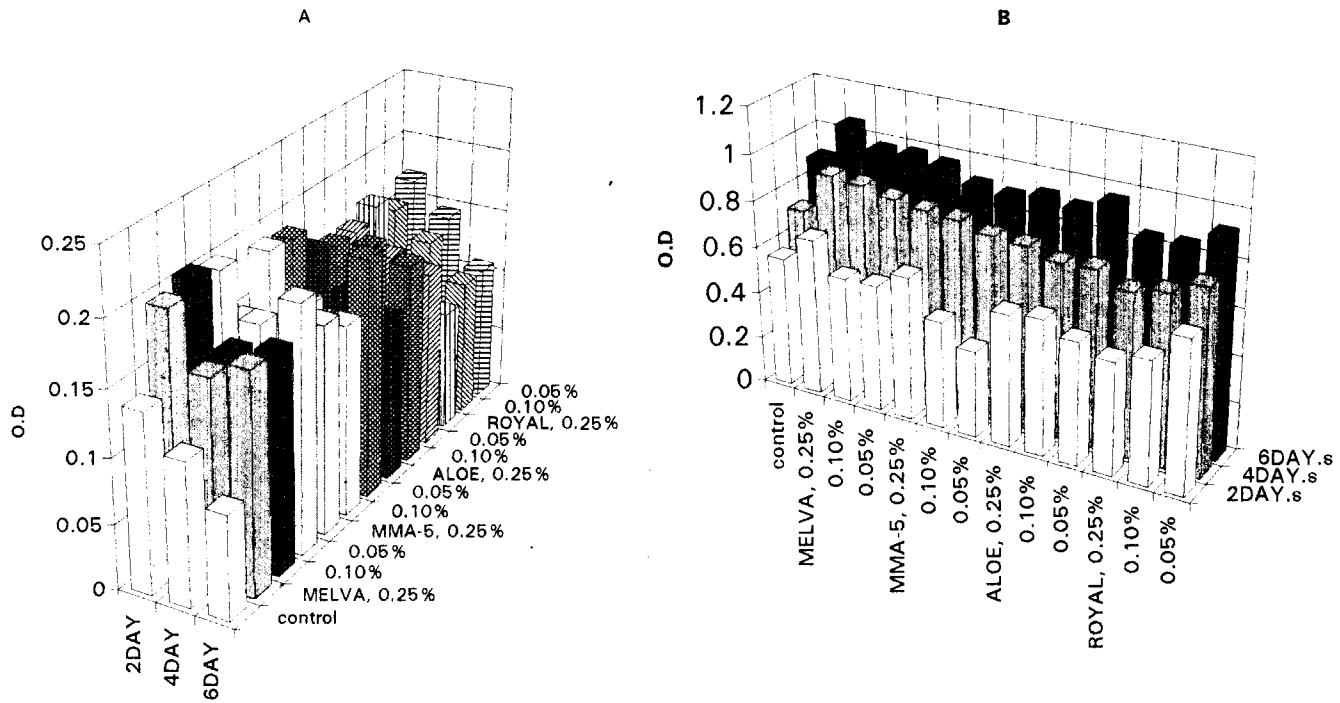


Figure-3. A) Cell population at the group of serum free medium containing cosmetic ingredients.
B) Cell proliferation at the group of medium with 3% serum and cosmetic ingredients.