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"How do anti-wrinkle ingredients influence cellular behavior? The effects of cosmetic ingredients on cell shape and movement"

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1. Introduction

From the body surface, the skin comprises the epidermis, dermis, and hypodermis. The dermis lies under and is connected to the epidermis by the basement membrane, giving the skin its elasticity and intensity. The dermis also has the important role of protecting the body from external stimuli. The dermis can be broadly divided into two layers: the papillary layer, which is rich in sensory nerve endings [1] and capillary blood vessels [2] ; and the reticular layer, which is made up of fibrous components such as collagen fibers. The reticular layer mainly comprises extracellular matrix (ECM) made up of substances such as collagen and elastin. The ECM is produced by fibroblasts, as the most abundant cells in the dermis [3].

Wrinkles become more apparent with age and represent a skin concern for a great many people. As the word "photoaging" suggests, external stimulation from sources such as ultraviolet radiation is one cause of wrinkles, and disruption of the barrier functions of the skin and decreases in moisture retention triggered by exposure to ultraviolet radiation are known to trigger the gradual formation of fine wrinkles in people around their 30s. On the other hand, the formation of deep wrinkles largely involves the aging of fibroblasts, representing an internal factor [4] . Aging of fibroblasts is accompanied by chronic inflammation, decreased collagen production, breakdown of elastin due to increased levels of neutrophil elastase, and disturbance in the orientation of fibers, all of which are major factors contributing to the formation of wrinkles [5-7]. When the skin overall is in a state of reduced elasticity due to degeneration and reductions in collagen and elastin, as the proteins making up the reticular layer, susceptibility to the effects of ultraviolet radiation is increased, encouraging the formation of deeper wrinkles [8]. This means that to eliminate skin problems such as wrinkles, which become more apparent with age, and to maintain both psychological and physical youthfulness, it is extremely important not just to guard against ultraviolet rays through the daily use of sunscreen [9], but also to prevent the aging of fibroblasts and maintain homeostasis of the dermal ECM. However, much remains unknown about fibroblasts, which play a very big part in wrinkle formation. More research is needed to understand the diversity, functions, morphological characteristics, and fate determination of these cells.

The cosmetic industry has undertaken a great deal of research into wrinkles and other skin concerns related to aging, as well as research and development aimed at preventing and

improving photoaging [10]. For example, niacinamide (NA), a water-soluble vitamin B3, contributes to energy production and oxidation-reduction reactions within the cell and is widely used in the cosmetic industry as one of the main functional ingredients exhibiting anti-wrinkle and other anti-aging effects [11]. Well-known effects of NA that have been reported include reduction of oxidative stress, anti-inflammatory activity, inhibition of melanosome transfer from melanocytes to keratinocytes, and promotion of collagen and elastin production by fibroblasts [12-14].

Understanding wrinkles and other photoaging skin conditions requires a full grasp of individual cells and intercellular communications, as well as of the interactions between cells and ECM. These are closely linked to cell morphology and behavior, and can yield important findings that contribute to the development of functional materials and proposals for appropriate methods of managing wrinkles. However, almost no evaluations have been made from the perspective of cell morphology in the development of materials for cosmetics. Instead, it is more usual to evaluate key factors that change the phenotype of tissues such as collagen, or the effects of a final formulation on the barrier function, viscoelasticity, or external appearance of the skin.

Here, we conducted cell morphological analyses of functional materials that are widely used in cosmetics using time-lapse images from phase-contrast microscopy, to better understand how these materials influence cell morphology and behavior and what effects they exhibit.

2. Materials and Methods

2.1. Cell cultures

Normal human dermal fibroblasts (NHDF) were purchased from PromoCel (Heidelberg, Germany) and normal human skin fibroblast, RCB original (NB1RGB) cells were purchased from RIKEN BRC (Ibaraki, Japan). These cells were maintained in DMEM (NACALAI TESQUE, Kyoto, Japan) culture medium according to the instructions from the manufacturer. NA was added 24 h after seeding cells onto dishes.

2.2. Timelapse phase holographic imaging

The HoloMonitor M4 holographic live cell imaging system (Phase Holographic Imaging PHI, Boston, MA, USA) was employed to acquire multi-point time-lapse imaging data of cells. Using 48-h imaging data, we examined variations in cell morphology, including cell size and thickness, proliferation rate, and motility between NA-treated and untreated cells.

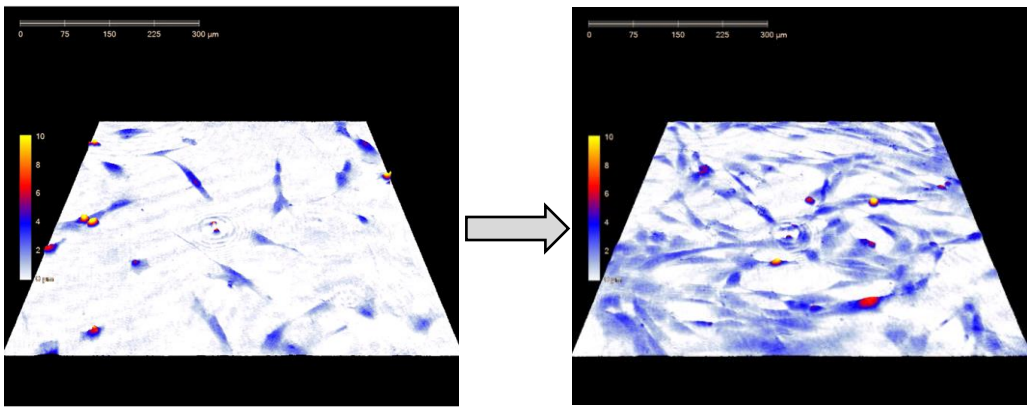
2.3. F-actin staining

F-actin was stained with fluorescent phalloidins (Thermo Fisher Scientific, Waltham, MA, USA) 24 h after NA treatment. Cells were fixed in 4% paraformaldehyde and subjected to F-actin staining according to the instructions from the manufacturer.

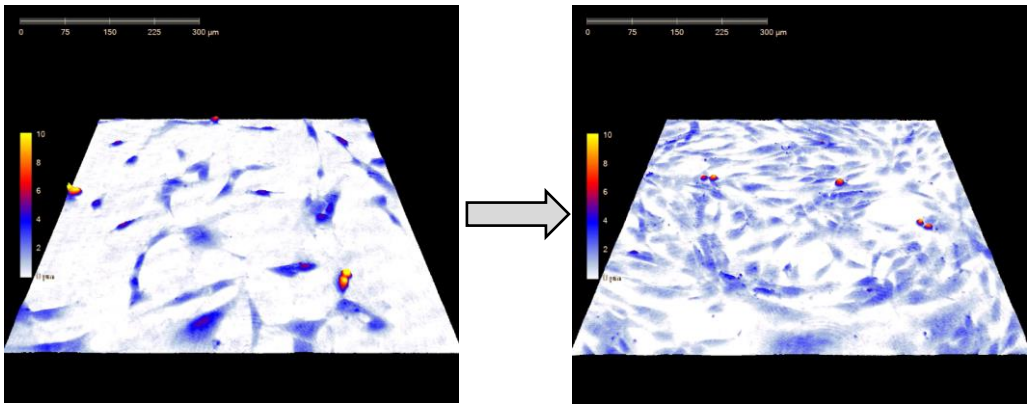
3. Results

3.1. NA promotes dermal fibroblast proliferation

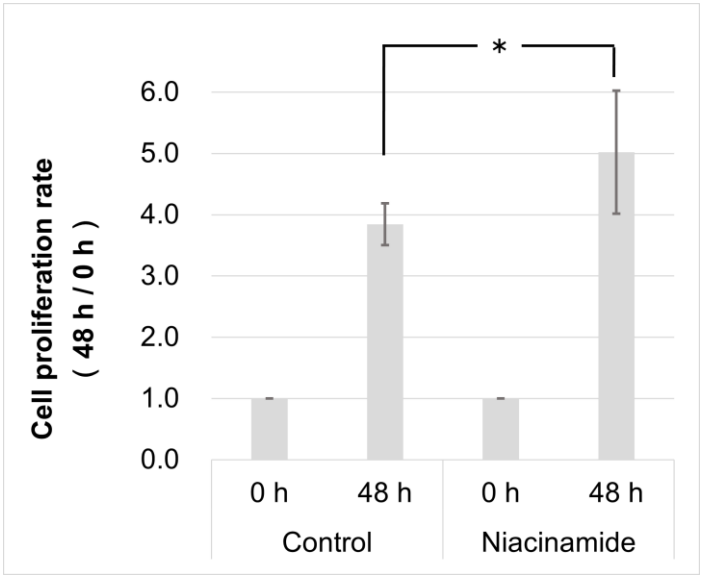
Phase-contrast time-lapse imaging, as a non-invasive imaging technique that does not involve labeling, was used to examine changes in the morphology and behavior of cells for 48 h after NA was added, to study the effects of NA from the perspective of cell morphology. The proliferative ability of fibroblasts is generally considered to decrease with aging [15]. Since NA is known to be largely involved in the production of energy (in the form of adenosine triphosphate) as a component of the coenzymes nicotinamide adenine dinucleotide and nicotinamide adenine dinucleotide phosphate [16], this protein is likely to be involved in cell proliferation. Promotion of cell proliferation as a result of adding NA was examined, and the number of fibroblasts after 48 h was found to be 1.25 times greater than in controls. This confirms that NA promotes fibroblast proliferation (Figure 1).



(**a**) Control 0 h (**b**) Control 48 h



(**c**) NA 0 h (**d**) NA 48 h

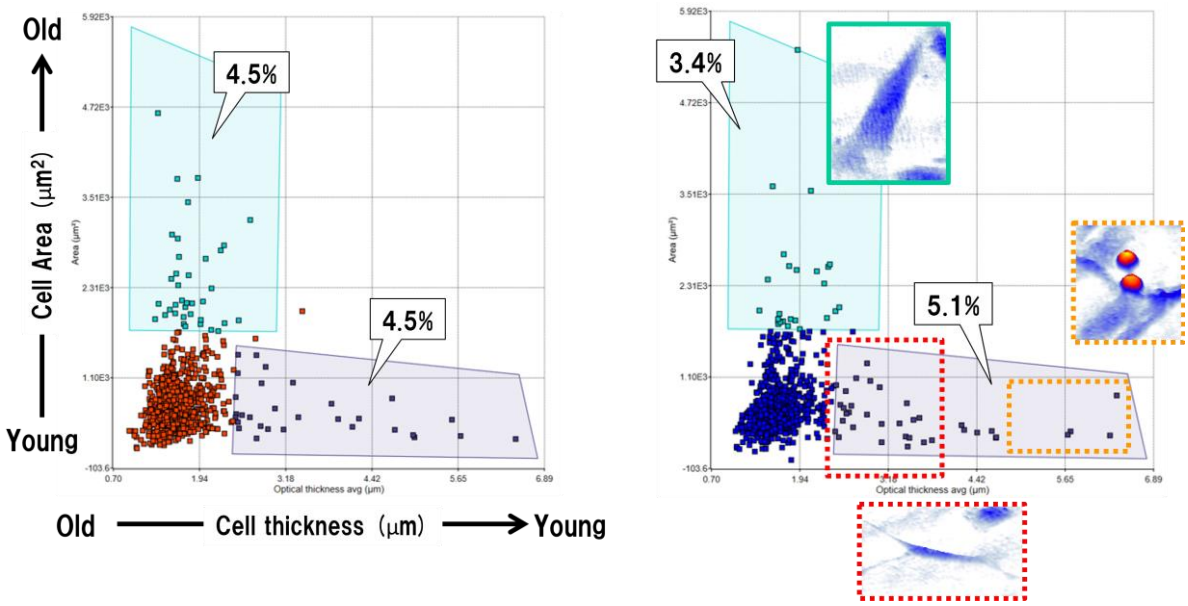


(**e**)

Figure 1. NA promotes dermal fibroblast proliferation. Cell proliferation rate was calculated using 48-h timelapse phase holographic imaging data. a) Control, 0 h; b) Control, 48 h; c) NA, 0 h; d) NA, 48 h; e) cell proliferation rate. Data are presented as mean ± SEM. *p<0.05.

3.2. Senescent fibroblasts decrease with NA treatment

Prior studies have shown that senescent fibroblasts are larger and flatter than young cells and show a distorted shape [17, 18]. Here, cell morphology was observed for 48 h following the addition of NA by measuring cell area and cell thickness. The proportion of cells with a relatively large area decreased from 4.5% to 3.4% after adding NA. The proportion of thick cells with proliferative potential also increased from 4.5% to 5.1% (Figure 2). Adding NA thus resulted in cells becoming smaller and thicker, suggesting that NA has the effect of reducing senescent fibroblasts.

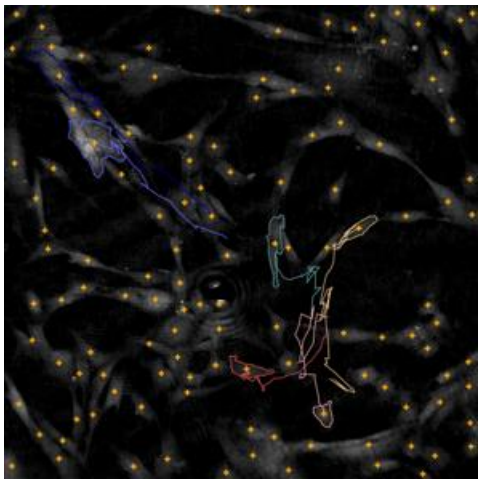


(**a**) Control (**b**) NA

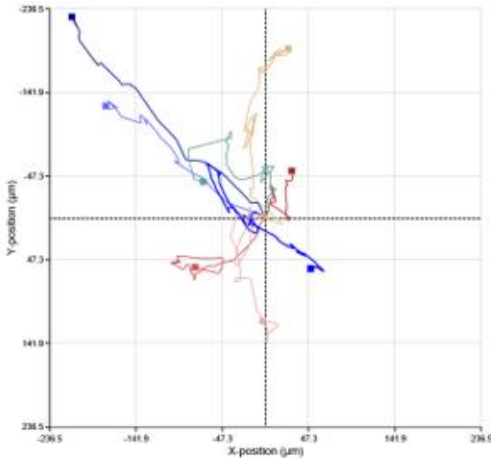
Figure 2. NA decreases senescent dermal fibroblasts. Cell area and cell thickness were measured using 48-h timelapse phase holographic imaging data. a) Control; b) NA.

3.3. Holographic timelapse imaging reveals that NA enhances cell motility

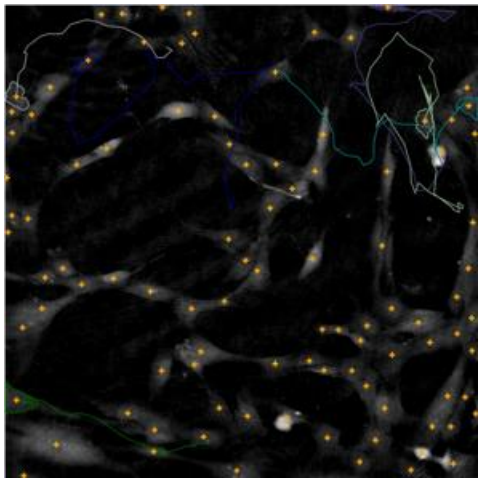
Next, the effects of NA on cell behavior were examined. Cell movements were tracked for 48 h after adding NA, showing that movement was more rapid and cells covered a wider area than control cells (Figure 3).



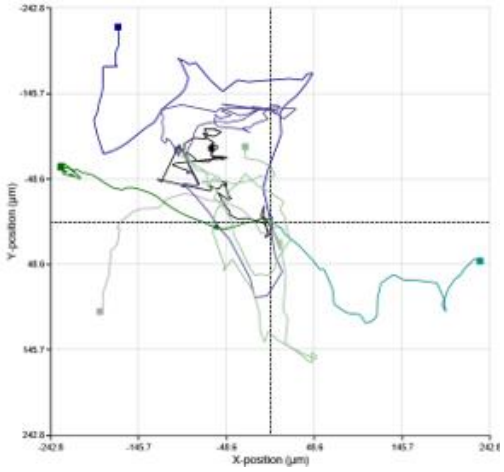
(**a**) Control (Phase image)



(**b**) Control (Cell motility trajectories)



(**c**) NA (Phase image)

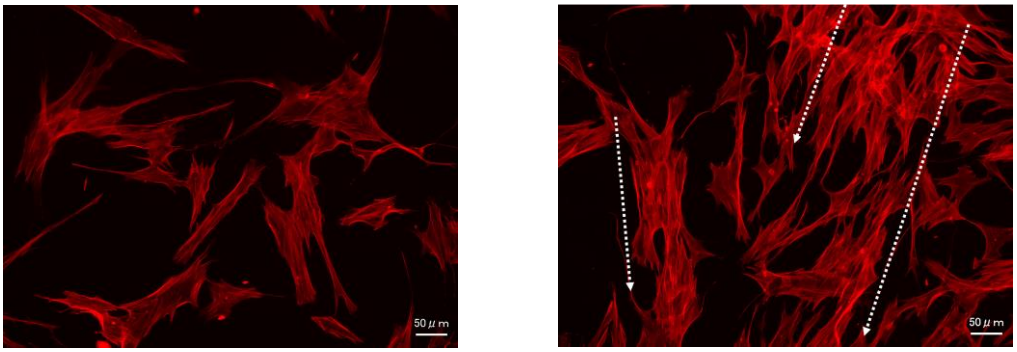


(**d**) NA (Cell motility trajectories)

Figure 3. NA promotes dermal fibroblast motility. Cell tracing was performed using time-lapse data from 0 to 48 h. a) Phase image of control; b) cell motility trajectories of control; c) phase image of NA-treated cells; and d) cell motility trajectories of NA-treated cells.

3.4. NA affects cell polarity

Since cell motility changed as a result of adding NA, expression of actin, a protein that controls the movement and polarity of cells, was examined using phalloidin staining. A greater number of actin stress fibers were observed in cells with the addition of NA than in control cells. Also, cells treated with NA were observed to form numerous cell groups resembling colonies earlier than control cells, and cells in these groups showed better alignment of polarity (Figure 4).



(**a**) Control (**b**) NA

Figure 4. NA affects cell polarity. F-actin staining was conducted by Alexa fluor 546 phalloidin. a) Control; b) NA.

4. Discussion

In skin with wrinkles and other manifestations of photoaging, collagen fibers show damage and overall skin elasticity is reduced. In this condition, the skin appears even more susceptible to damage from ultraviolet radiation, promoting the formation of deep wrinkles [8]. In addition to chronic exposure to ultraviolet rays, internal factors are known to be associated with cell aging. Such factors include oxidative stress and increased expression of MMP1, which promotes the breakdown of ECM, causing damage to collagen and other components of the reticular layer and thus exacerbating wrinkles [5, 19].

The present results indicate that NA, an anti-aging component, promotes the formation of actin stress fibers in fibroblasts (Figure 4). Decreased levels of intracellular actin fibers reportedly upregulate expression of MMP1 via the c-Jun/AP-1 pathway [20], and formation of actin fibers is reported to regulate collagen production via TGF-β type II receptors [21]. This suggests that NA may contribute to the regulation of collagen fiber expression by modulating the amount and degree of polymerization of actin fibers in fibroblasts. In addition, actin fibers tended to align in the same direction after adding NA (Figure 4). Such findings suggest that NA contributes to the proper structural formation of collagen and other fibers in the reticular layer, imparting elasticity to the skin.

Further, photoaged skin has been reported to show reduced adhesion of fibroblasts to collagen due to fragmentation of collagen, while decreased density of the ECM promotes fibroblast senescence, in turn reducing collagen production [22]. This means that regulation of fibroblast intracellular actin fibers by NA may also regulate cell senescence. In our analysis using phase-contrast microscopy time-lapse imaging, fibroblasts treated with NA not only showed greater proliferative potential, but also a decreased proportion of cells with a senescent morphology (Figure 1 and 2). It is currently unclear whether this change in proportion was simply a change to a smaller, thicker morphology as a result of increased tensile force within the cell from

accelerated actin fiber formation, or whether it was due to the removal of senescent cells through senolysis mediated by the inhibition of proteins such as Bcl-2, as a characteristic of senescent cells [23]. We aim to further our understanding of this in the future.

In addition, analysis of the time-lapse images we obtained suggested that NA influences cell behavior (Fig. 3). The finding that both distance and speed of movement were greater than in control cells is similar to the findings of prior studies regarding the effectiveness of NA in wound healing [24]. The finding that cells treated with NA formed colonies earlier than controls and that these colonies tended to be oriented in the same direction (Figure 4) is of great interest. Esfahani et al. showed that NA increased the densities of fibroblasts and collagen fibers in the wound healing region [25]. NA thus appears to alter the behavior of fibroblasts and promote local fiber formation as needed. However, is not yet known what factors regulate cell motility and polarity, and how collagen and elastin structures are formed, and we aim to address these questions in future studies.

We hope to link the results of this study to future image analysis technologies that will be capable of using the morphological characteristics of fibroblasts to estimate the amount of senescence-associated secretory phenotype (SASP) factors derived from senescent cells or predict their effectiveness when screening for functional components, but the hurdles remain extremely high. Tohgasaki et al. developed a technology capable of performing AI analysis on the morphological characteristics of normal human epidermal keratinocytes (NHEKs) obtained from live imaging, then using the analysis results to estimate expression levels of SASP factors such as IL-1α and NF-κB [26]. In the future, levels of gene or protein expression should also be able to be estimated from morphological characteristics of fibroblasts. However, fibroblasts in the human dermis reportedly comprise a heterogenous population made up of at least four cell types, each with different roles and generating ECM with different characteristics [27]. This means that in order to use live imaging to estimate amounts of proteins derived from senescent cells or to predict their roles in the dermis and apply this to functional component screening technology, an understanding of the diverse cells and the ability to identify them must first be gained. We intend to carry out further research to establish the same sort of technologies already reported for NHEKs.

This study evaluated NA from the perspective of cell morphology. The results suggest that NA may comprehensively regulate internal factors that contribute to wrinkle formation not only through the known effect of promoting collagen production, but also by removing senescent cells to increase the proportion of cells in proper condition. We hope to continue this research in greater depth to contribute to the wellbeing of the great many individuals troubled by wrinkles.

5. Conclusion

Our findings suggest that NA may exert anti-wrinkle effects not only by promoting collagen production, but also by influencing cellular orientation. The cell morphological assessment methodology applied in this study proved useful and cost-effective for screening functional materials, and further research in this area is anticipated.

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