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New nanotechnology for the guided tissue regeneration of skin – potential of lyotropic liquid crystals

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Tissue in body must quickly recognize injury to response to the rapid pace of epidermal growth. In skin, the epidermal cells must also react to danger signals from the surrounding extracellular lipid of the stratum corneum spaces and immediately participate by initiating the wound repair process. The topical administration of the lyotropic liquid crystal nanocube™ to stratum corneum rapidly broke down the lipid lamella structure which would be recognized as a wound without organ-change. This can activate a variety of biological processes. This study set out to determine whether the phase transition of the lipid to a neighbouring different physicochemical structure can stimulate keratinocyte cells and what mechanism is responsible for this response. Using small angle x-ray scattering (SAXS) analysis, a response to the transient structural change of lipid was detected which might result from the diffusion of oil and/or water from nanocube™ liquid crystal towards the lipid lamella phase. Simultaneously, a significant increase in growth factors and inflammatory cytokines was detected after administration of nanocube™. Not only the excess expression of cytokines but also the extent of TEWL as a barrier marker of skin increased. These observations suggest that a structural change in lipid can stimulate and trigger recognition of a slight injury in the wound defence and a repair response as homeostasis. This method actually succeeded in improving photo-induced hyperpigmentation on a human face.

1. Introduction

Being the barrier between the individual and the environment, the skin prevents the entry of micro-organisms, blocks radiation and prevents water loss. Epidermal cells are regularly exposed to marked variations in ambient temperature, to environmental stressors that affect the cellular redox states and osmotic balance, and to physiological stressors such as inflammatory cytokines. The barrier resides in the outermost skin layer, the stratum corneum. It is already well known that the epidermal extracellular spaces in the stratum corneum play a crucial role as the skin barrier. This is mechanical and anatomical, as well as chemical in nature; laterally overlapping cell multi-layers are sealed by tightly packed, intercellular, lipid multi-lamellae.

Prompt recognition and response to injury is critical for activation of epithelial innate defence mechanisms, recruitment of inflammatory cells, and initiation of the repair process. The response to injury may involve both organic disease and non-organic disease. Tissue damage in organic disease resulting in skin wounds triggers defence and repair mechanisms. However, the mechanisms for recognition of injury in non-organic change are less well described. Our study of skin regeneration focused on the lipid of the epidermal extracellular spaces in the stratum corneum as the outermost layer of the skin structure. Local and transient structural changes in the stratum corneum may correspond to non-organic injury. This should induce stress signals such as endogenous indicators of injury. Due to the role of the epidermal layer in sensing injury and signalling repair, we sought to determine how structural changes in skin lipids correspond to changes following non-organic wounds. Understanding how structural change of the lipid in epidermal extracellular spaces responds to skin regeneration and the mechanisms involved in this signalling pathway will lend perspective as to how tissue damage is recognized.

The recognition of a wound event must switch on homeostasis in the organism, result the repairing mechanism will start spontaneously. Generally, in the case of wound repair with organic change, the use of drugs is necessary. Also, the guided tissue regeneration of skin may be selected by chemotherapy. Our findings suggest that the induction of skin regeneration could be achieved simply by physicochemical structural changes of lipid lamella in extracelluar spaces without any drugs.

The nanostructure of the epidermal extracellular space has been investigated by a newly developed method, cryo-electron microscopy (Al-Amoundi et al. 2005). The extracellular spaces express a rich variety of complex membrane-like structures, including lamellar, lyotropic liquid crystal. Recently, we have focused on skin regeneration using all-trans retinoic acid (atRA), and the novel DDS nanotech-

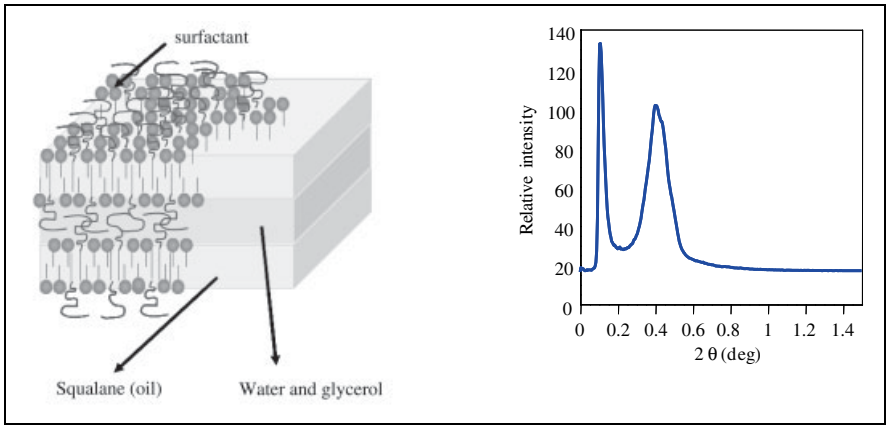

Fig. 1:
(a) Schematic representation of lyotropic liquid crystal with lamellar structure, nanocube™ Surfactant, squalane as oil, glycerol and water as a polar solvent ternary system can form a lamellar structure. (b) Small angle x-ray scattering (SAXS) shows the sheet structure

nology has been developed (Yamaguchi et al. 2005). In this study, we evaluated the efficacy of a liquid crystal which corresponds to a similar structure of skin lipid, causing spontaneous skin regeneration on *in vivo* topical administration, as a new concept for skin whitening.

2. Investigations, results and discussion

2.1. Preparation of lyotropic liquid crystal, nanocube™

The mechanical properties and the symmetry properties of a certain phase are intermediate between those of a liquid and those of a crystal. This phase has often been called a liquid crystal. A more accurate name is mesophase. A number of surfactants and other colloids in suitable solvents give a mesophase in concentrated solution systems (Robinson et al. 1958). In the concentrated region, the surfactant, glycerol, oil and water ternary system spontaneously forms a remarkable mesophase which corresponds to a lamellar phase with successive sheets of water and lipid. This structure is similar to biological membranes with thin sheets (8 nm) made of lipids and proteins (Gulik-Krzywicki et al. 1972). It is hoped that each individual sheet will have some analogy with the membrane. Since a lyotropic liquid crystal has a 3D structure in space, a viscoelastic property (gel-like) is indicated. This physicochemical property is rather useful in the case of topical administration.
Here we describe the development of a novel nanotechnology based on mesophase formation. Fig. 1 shows the schematic structure of the lyotropic liquid crystal, the so-called nanocube™ and a structure analysis by small angle x-ray scattering (SAXS). SAXS data indicate a lamellar structure with repeating texture, giving a highly ordered self-assembly system on the nanometer scale.

2.2. Topical administration of nanocube™ in vivo

Histologically, when skin regeneration and/or guided tissue regeneration take place, thickening of the epidermis will be observed to appear. The thickened epidermal layer may be related to the accelerated turnover of keratinocyte cells. The proliferation of basal cells and the respective differentiation of spinous cells, granular cells and corneocytes would accompany this, and as a result skin regeneration and/or wound repair takes place. Furthermore, it is already known that a relative reduction in melanin content should accompany the process, simulteneously (Klingman et al. 1984; Klingman et al. 1986).

Fig. 2 shows histological and tissue studies of the epidermis of ddY mice (male, 5-week) at 2-days after topical administration. The nanocube™ treated tissue was obviously thickened in the epidermal layer, while single treatments of squalane and the surfactant, which is one of components in nanocube™, respectively, indicated almost no response.
The role hyaluronen (HA) may play in the wound repair process has recently been studied (Taylor et al. 2004). HA is synthesized at the cell surface and is a uniform glycosaminoglycan since its disaccharides are not sulfated or epimerized. Recently, small molecular weight HA has been implicated in several biological processes including angiogenesis, cell proliferation, maturation, migration, activation of protein tyrosine kinase cascades, and inflammatory gene expression (Chen 1999; Termeer 2003). HA fragments may activate repair processes and signal that injury has occurred. Thus, in this experiment, the skin specimens treated were prepared using colloidal iron staining to retain hyaluronan (HA). Blue colored areas correspond to the presence of HA in Fig. 2. Only the dermal layer in Fig. 2(a) treated with nanocube™ showed the presence of HA.

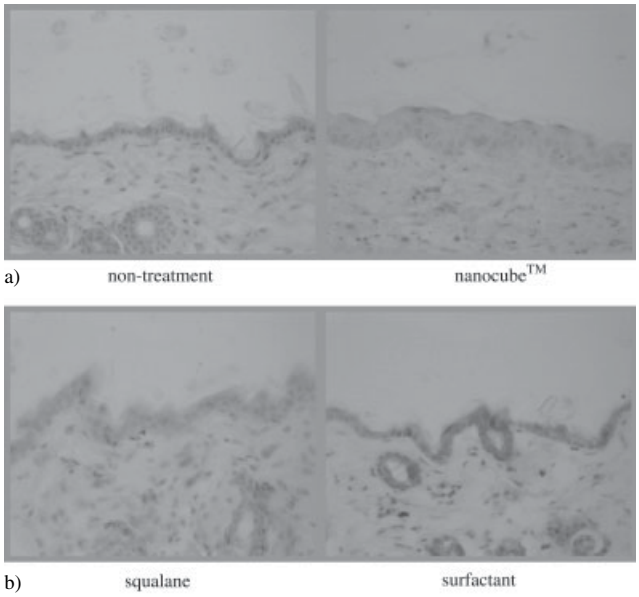

Fig. 2: Histological researches on topical administration of nanocube™. The dorsal areas of ddY mice (male, 5-week) were used *in vivo*. (a) non-treatment (b) nanocube™ treatment (c) squalane (d) surfactant

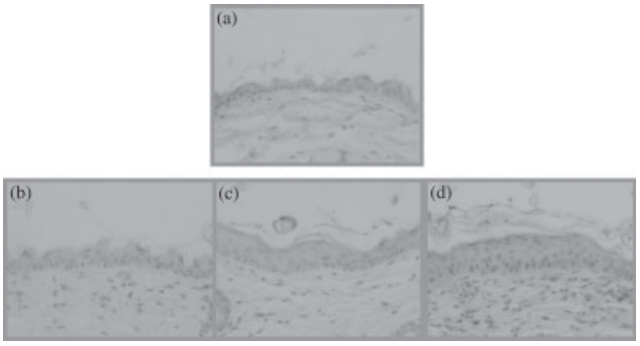

Fig. 3: Dose dependence of nanocube™. The dorsal areas of ddY mice (male, 5-week) were used *in vivo*. (a) nanocube™ 0% (b) nanocube™ 10% (c) nanocube™ 20% (d) nanocube™ 30%

2.3. Dose dependence of the effect of nanocube™ on proliferation and differentiation of epidermis

To determine whether the action of nanocube™ is dose – dependent, emulsions including nanocube™ 10%, 20% and 30% were prepared for topical administration. Nanocube™ clearly induced an increase in epidermal thickness according to its dosage (Fig. 3). 30% nanocube™ emulsion was quite effective with regard to thickened epidermis, and the presence of HA not only in the dermis but also in the spaces between the basal and spine cells in the epidermal layer.

To confirm the increase of epidermis with nanocube™ treatment suggested by light microscopy, immunostaining of Ki-67 and cytokeratin 1, 5 and loricrin (K1, K5, lor) was carried out for each specimen (Fig. 4). Qualitative staining supported an apparent increase in proliferation of basal cells and differentiation of spine, granular cells and

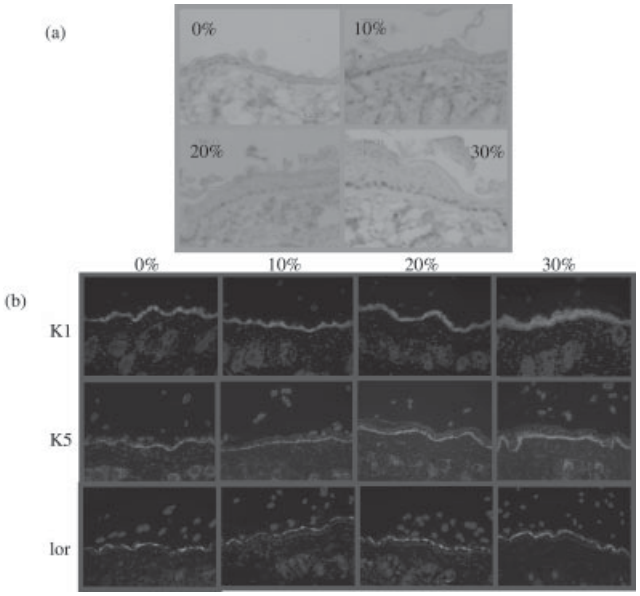

Fig 4: Expression of molecular markers in epidermal layer of ddY mice. In Figure (a) Ki-67 immunostaining, brown colors correspond to expression of Ki-67. (b) green corresponds to antibody staining, and red corresponds to nuclear staining. Dorsal skin sections from ddY mice at 2-day after the external treatment were stained with antibody against cytokeratin 1 (K1), cytokeratin 5 (K5), and loricrin (lor). The treatments were carried out with nanocube™ emulsions 10%, 20% and 30%, respectively. The dorsal sections ($2.0 \times 2.0\ cm^2$) were administrated 30 mg emulsion. K1 is marker from spinous layer to stratum corneum, K5 basal cell, and loricrin granular layer to stratum corneum

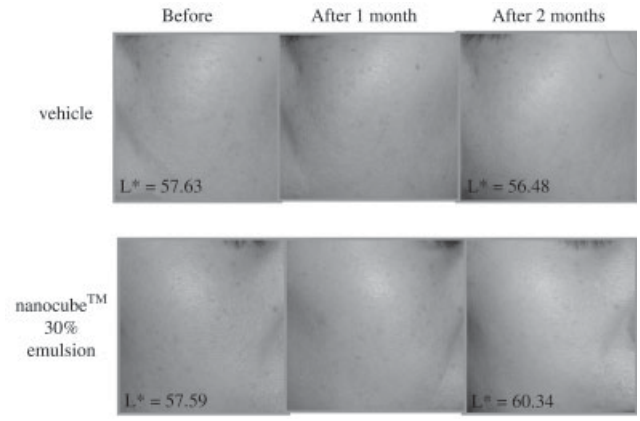

Fig. 5: Pictures of photoaged hyperpigmentation on human faces (female, 42 years old). The pictures showed before treatment and after treatment with vehicle and nanocube™ 30% emulsion. Daily external administration was carried out once at night, continuously

corneocyte in the treated specimen by nanocube™ emulsion. This increase clearly depended on the dosage of nanocube™.

2.4. Trial of nanocube™ 30% emulsion on human face skin

As a preliminary trial for in vivo treatment of skin regeneration, nanocube™ therapy was performed for two months on human faces. To confirm the increase of epidermis thickness in the experiment in mice, improvement of brown spots on the human face would be expected due to the accelerated turnover of epidermis. The face treated with emulsion vehicle was not markedly improved, while 30% nanocube™ emulsion treatment achieved a decrease of hyperpigmentation for two months, as is evident in Fig. 5. The brightness L^* value also increased from 57.6 to 60.3 during treatment with nanocube™, the increase of L^* value indicating an improvement of whiteness in skin color. It is noteworthy here that not only the mouse tissue but also human skin would regenerate by the topical administration of nanocube™.

2.5. Why is nanocube™ so effective for skin regeneration?

Stoll et al. reported that mRNA expression of heparin-binding epidermal growth factor (HB-EGF), a member of the EGF family of growth factors, is induced when skin regeneration takes place in human keratinocytes and skin (Stoll and Elder 1998; Yamaguchi et al. 2005). Transforming growth factor (TGF)-β is a multifunctional cytokine that regulates cell proliferation, differentiation and extracellular matrix production (Jennings and Pietenpol 1998). Furthermore, the expressions of tumor necrosis factor (TNF)-α and interleukin in the epidermal layer seem to be related to the repair of the epidermal barrier (Hatano et al. 2005).

Fig. 6 shows mRNAs of HB-EGF, TGF-β1, IL-β and TNF-α expressions in mouse dorsal areas treated nanocube™. One-time application was performed and the tissues were taken at 2 days after the administration. Excess expressions of all mRNAs were detected compared to those of wild-type. This result correlates well with both the increased epidermal thickness in mice and the whitening of human skin by nanocube™ treatment.

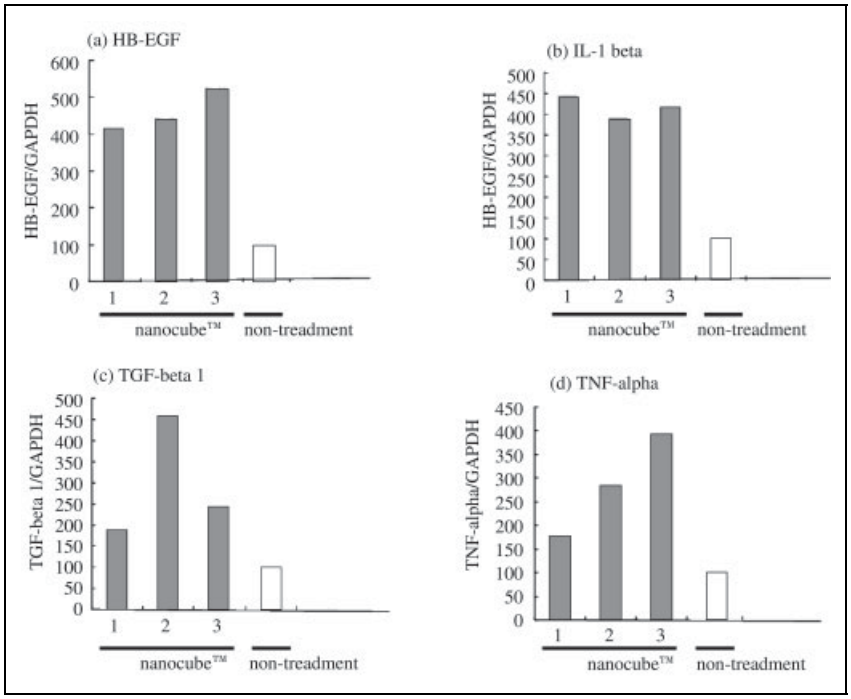

Fig. 6:
Expression of mRNAs of several cytokines in mouse dorsal areas at third day after one-day treatment with nanocube™. (a) HB-EGF (b) I-1β (c) TGF-β1 (d) TNF-α

The excess expression of HB-EGF and TGF-β1 appears to play a crucial role for keratinocyte proliferation and differentiation, while interleukin 1 and TNF may be produced because of slight inflammation. These are generally classified as the inflammatory cytokines. Due to the effect of the expression on the levels of genes for these inflammatory cytokines towards the barrier function, the extent of the transepidermal water loss (TEWL) was measured. The degree of TEWL increased with time after the administration of nanocube™, and finally after 6 hours its level reached a peak (Fig. 7). In general the cutaneous TEWL level in mice is around 0.02 mg/cm^2/min. Thus, the collapse of the barrier function of the extracellular lipid bilayer of the stratum corneum seems not to be remarkable.
Why did a slight collapse of the epidermal barrier occur by topical administratin of nanocube™? It may be suggested here that the phase transition of the extracellular lipid bilayer of the stratum corneum would take place transiently. Nanocube™ is composed of chemical materials which are similar to the lipid of the epidermal extracellular spaces. One would thus expect the spontaneous diffusions of each material in to lipid due to the coverage of nanocube™ on the stratum corneum in topical administration. The change of material balance in the lipid may induce destruction of the structure from lamellar to bi-continuous cubic phase and/or transient spinodal-type phase separation. The transient structure change of the lipid will be recognized as a particular wound without organchange. As a result, repair should be started following the homeostasis mechanism, which needs several inflammatory cytokines and growth factors from keratinocytes. Therefore, expressions of cytokine mRNAs in Fig. 6 were enhanced with nanocube™ treatment.
Small angle X-ray scattering (SAXS) is a useful tool for the detection of nano-order structure. In Fig. 8 the lipid in stratum corneum and the lipid after administration of nanocube™ were measured by the tape-stripping method. The black line for lipid shows the typical lamella liquid crystal structure. The lamellar phase may be visualised as having been constructed by stacking together bilayers in exactly the same manner with the detected peaks at the same interval. However, the red line for nanocube™ treatment showed no continuous peaks. This probably indicates a structural change of the lipid lamella phase. It is

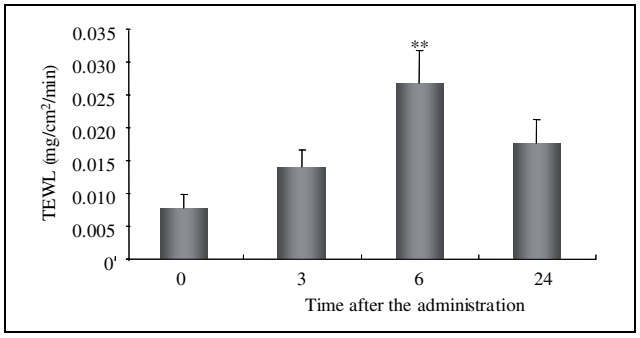

Fig. 7: Change of TEWL after topical administration of nanocube™ to mouse dorsal area 2 × 2 cm^2. 50 mg of nanocube™ was administered one time (n = 5)

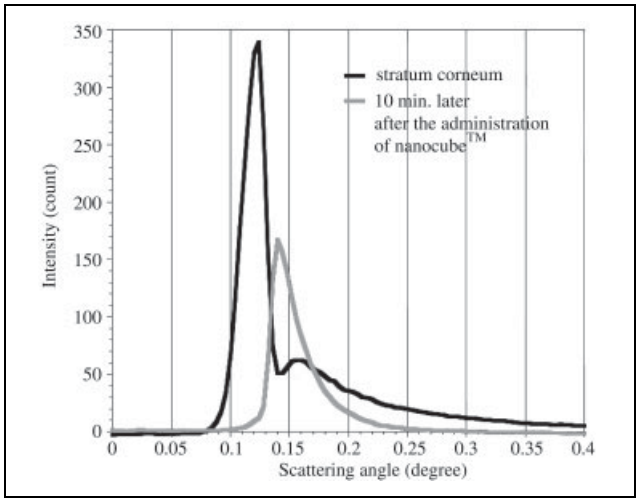

Fig. 8: Small angle X-ray scattering of lipid in stratum corneum and after administration of nanocube™ extracted by the tape-stripping method. Topical administration was carried out on the dorsal area of hairless mice (HR-1, male, 10-week), extraction by tape-stripping was carried out 10 min. after treatment

generally recognized that the lamellar phase plays a central role in the evolution of the liquid crystal phase structure, and that the geometric features of the other liquid crystal phases may be viewed as perturbations which bend and reshape the lamellar phase towards either the water side or the oil side (Fontell, K 1990). Therefore, the phase transition to the neighboring phase (bi-continuous cubic phase) and/or transient spinodal phase separation might occur by the diffusion of water or oil from nanocube™. Neither structures can show the marked sharp peaks in SAXS measurement. Fig. 8 would support the above hypothesis that phase transition takes place from lamella to another phase.

As a signal of danger or wound, it would be important for normal tissue homeostasis that the transient change of lipid structure by phase transition is regulated. Existing information regarding the turnover of the epidermis layer supports this hypothesis, and it is proposed that the transient phase transition of lipid by the administration of nanocube™ is such a guide for tissue regeneration. In the case of wound repair without organ change, the structural change of lipid appears to be a conserved "danger" signal, communicating to the host the presence of tissue damage through a pathway shared with identification of the cell challenge. Furthermore understanding of this response system may be a useful approach to penetration of drugs across the skin.

3. Experimental

3.1. Nanocube™ preparation

The raw materials of squalane and glycerol for nanocube™ were commercially obtained from Wako Pure Chemical Industries Ltd. Surfactant Emulgen 2020HA was purchased from Kao Corporation in Japan. All chemical materials were used with no further purification and were of reagent grades. Emulgen 2020HA 27.1 wt%, glycerol 36.8 wt%, Squalane 19.6 wt%, and water 16.5 wt% were slowly mixed in a glass beaker, and finally the stirring was stopped when the viscoelasticity of the mixed solution became gel-like. Each material was mixed thoroughly by hand for approximately 20 min.

3.2. Small angle X-ray scattering (SAXS) measurements

Small angle X-ray scattering (SAXS) was carried out with a X-ray diffractometer with imaging plate (Rigaku Denki, Nano-Viewer IP, Tokyo, Japan) for nanocube™ structure and lipid structure in the stratum corneum of hairless mice. The scattering angle was between $0.01^\circ < 2\theta < 5^\circ$. Empirical scattering results were obtained by putting the samples in a sealed capillary. A Rigaku X-ray generator with a rotating copper anode, and maximum power over 20.7 kW/mm^2 was used for the SAXS measurements.

3.3. Measurement of growth factor and inflammatory cytokine mRNAs

To detect the mRNA of each cytokine, a real-time PCR system was utilized. ddY mouse (5-week, male) dorsal areas in liquid nitrogen were used as samples for the extraction of RNA. After grinding the mouse skin well, RNA was extracted by the Isogen method. The measurements of mRNA HB-EGF for each sample were carried out with a LightCycler® 2.0 Instrument (Roche Diagnostics GmbH, Germany) with six detection channels and 20 μL capillaries. For the measurements, 1st strand cDNA synthesis kit and Light Cycler SYBR Green I were obtained from Roche Diagnostics Japan. Light Cycler Software 4.0 was installed as a quantification method which automates grouping, melting curves and analysis.

3.4. Immunostaining and colloidal iron staining

Biopsy specimens were obtained after the treatment with each material of the dorsal area of ddY mice. All biopsy specimens were taken from the same area in all subjects, i.e., the lateral region on the back of the ddY mouse, to ensure uniformity of sample sites and collection of specimens from the external adhesion area. All biopsy specimens included both epidermal and dermal tissue. All the formalin-fixed specimens of dorsal area of ddY mouse (5 ∼ 6 weeks, male) were stained as follows: (1) Colloidal iron for the detection of hyaluronan (HA) (2) immunostaining of Ki-67 and cytokeratin 1, 5 (K1, K5) and loricrin (lor). Ki-67 was obtained from Dako Cytomation Japan, and K1, K5 and loricrin were purchased from CRP Inc. After the samples were dehydrated, 1-μm sections were cut from the paraffin-embedded specimens and stained with colloidal iron staining (Yamaguchi et al. 2005).

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