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Comparative study of the physiological properties of collagen, gelatin and collagen hydrolysate as cosmetic materials

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Synopsis

The cell biological properties of collagen, gelatin and collagen hydrolysate (<15 000 Da) were studied using murine keratinocytes. Keratinocyte culture experiments demonstrated that only collagen had significant effects on cell attachment and proliferation, but the results of cells cultured on gelatin and collagen hydrolysate showed the rates of adhesion and proliferation were similar to those of cells cultured on plastic as a control. It is concluded that collagen has better physiological effects than those of gelatin and collagen hydrolysate as skin-care cosmetic materials.

Résumé

Les propriétés biologiques cellulaires du collagène, de la gélatine et d'un hydrolysat de collagène (de masse inférieure à 15 000 Da) ont été étudiées sur des kératinocytes de souris. Les études sur culture de kératinocytes ont montré que seul le collagène possède des effets significatifs sur l'adhérence et la prolifération cellulaire. Les études portant sur les cellules cultivées sur gélatine ou hydrolysat de collagène font ressortir des taux d'adhésion et de prolifération identiques à ceux des cellules cultivées sur le plastique témoin. On en conclue que le collagène possède de meilleurs effets physiologiques que la gélatine et l'hydrolysat de collagène en tant que matière première pour le soin cutané.

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Introduction

Collagen is the primary structural protein in vertebrates. About half of the total body collagen is in the skin, and about 70% of material other than water present in the dermis of skin and tendons is collagen. Twenty-six types of collagen have been reported [1], but about 85% of collagen in the dermis is type I. Type I collagen molecules are characterized by a triple helix formation. The triplex consists of two α_1 chains and one α_2 chain each of over 1000 amino acid residues in length. Each collagen chain adopts a left-handed helical conformation, and the three strands intertwine with a right-handed superhelical twist exhibiting an apparent molecular weight of 300 000 Da [2]. Gelatin is obtained by thermal denaturation of collagen. Its molecules contain repeating sequences of glycine-X-Y triplets, where X and Y are frequently proline and hydroxyproline amino acids. These sequences are responsible for the triple helical structure of gelatin and its ability to form gels by formation of helical regions in the gelatin protein chains that results in immobilization of water. Gelatins are a heterogeneous protein mixture of polypeptide chains with molecular weights ranging from a few thousand up to several hundred thousand daltons.

Collagen hydrolysate is a mixture of polypeptides with a distribution of molecular weights that is a function of the degree of digestion.

Various proteins are now used as standard raw materials in the cosmetic industry. The effectiveness of collagen or collagen hydrolysate as a cosmetic material has already been established [3, 4]. The purpose of proteins in cosmetic formulations is to improve the appearance and to protect the structure and function of skin. There have been several studies on the mechanisms underlying the effectiveness of collagen in cosmetic formulations. Nagelschmidt and Struck found only traces of [^{14}C]-collagen in the epidermis and none in the dermis [5]. Huc, A. *et al.* [6] studied the penetration of biosynthetically [^{14}C]-labeled acid-soluble collagen and collagen tagged with [^{125}I] and found that [^{14}C]-collagen did not penetrate the stratum corneum but that [^{125}I]-labeled collagen was absorbed by the dermis within 15 min.

The objective of this study was to determine the biological effects of collagen, gelatin and collagen hydrolysate on skin keratinocyte attachment and proliferation.

Experimental methods

Preparation of collagen

Calf skins were limed with 1.5% Na_2S and 5% lime powder at 25°C for 18 h to remove hair. The limed skins were then fleshed and split. The splits were neutralized with 1.5% HCl, based on the weight of limed splits. The pH of splits was reduced from 12 to 5–6. After washing with water, the splits were cut into pieces and pulverized with a mill (puluterisette 14 FRITSCH, Germany). Twenty grams of split pieces was digested in 1000 mL of 0.5 M acetic acid solution containing 0.4 g pepsin (1:10 000; Nacalai Tesque Co., Ltd., Kyoto, Japan) at 4°C for 24 h. The soluble collagen solution thus obtained was centrifuged at 10 000 rpm for 15 min to remove insoluble substances and salted out at 3 M NaCl, and it was then centrifuged at 12 000 rpm for 30 min. The precipitate was dissolved in 0.5 M acetic acid. The solution was then salted out at 0.7 M NaCl and centrifuged at 11 000 rpm for 30 min again, and the precipitate was dissolved in 0.5 M acetic acid again. Collagen concentration was determined indirectly from the hydroxyproline concentration, which was determined by the method of Bergman and Loxley [7].

Preparation of gelatin and hydrolysate

Twenty grams of gelatin (from bovine bone; Wako Pure Chemical Industries, Ltd., Osaka, Japan) was solubilized in water, and half was digested with 0.025 g trypsin 250 (Becton Dickinson and Company, Sparks, NV, U.S.A.) at 40°C. The hydrolysate protein content was determined by the method of Biuret [8]. SDS-PAGE analysis demonstrated that the molecular weight of the hydrolysate peptide was <15 000 Da.

Culture of keratinocytes

Murine keratinocyte cultures were maintained in serum-free keratinocyte basal KGM-2 BulletKit medium (BioWhittaker, Takara-Bio Co., Otsu, Japan) containing bovine pituitary extract, gentamicin sulfate amphotericin-B, human epidermal growth factor (recombination in a buffered BSA saline solution), insulin, hydrocortisone, transferrin and epinephrine in an atmosphere of humidified air and 5% CO_2 at 37°C. About 3 or 4 days later, once the cells had become subconfluent, the cells were generated and used for cell attachment and proliferation studies on different protein coatings. Experiments were performed in compliance with the guidelines of the Graduate School of Agriculture, Hokkaido University.

Cell adhesion assay

Thirty-five millimeter culture dishes (Becton Dickinson and Company, Franklin Lakes, MA, U.S.A.) were coated with either collagen, gelatin or hydrolysate at the same concentration of 10 μg mL^{-1} in phosphate-buffered saline (PBS) for 2 h at room temperature. Keratinocyte cells were added to each dish in the same KGM-2 medium at a cell seeding density of 4×10^4 cm^{-2}. To measure the rate of cell attachment on different protein coatings, the cells were cultured for 8, 12, 16 and 20 h and then examined using a microscope (IMT-2; Olympus, Tokyo, Japan). After a single wash with PBS, adhered cells were digested with 0.05% (w/v) trypsin to obtain a cell suspension and counted using a hemacytometer (Burker-Turk, Tokyo, Japan). All experiments were performed in four cultures.

Cell viability and proliferation assay

The surface of each microplate (96 wells; Becton Dickinson and Company, Franklin Lakes, MA, U.S.A.) was coated with collagen, gelatin or hydrolysate (10 μg mL^{-1} in PBS). Cells were seeded at various concentrations (0.875, 1.75 and 3.5×10^4 per well) in a final volume of 100 μL per well culture medium and cultured for 1, 2, 3, 4, 5 and 6 days at 37°C in an atmosphere of 5% CO_2. The quantification of cell proliferation was done by the method of adding cell proliferation reagent WST-1 (tetrazolium salt; Roche Molecular Biochemicals, Mannheim, Germany) [9, 10]. The absorbance of the samples was measured against a background control as a blank using a scanning multiwell spectrophotometer (ELISA reader, Shimadzu, Dual-wavelength Flying-spot Scanner CS-9000, Tokyo, Japan). The wavelength for measuring the absorbance of the formazan product was 450 nm and the reference wavelength was 690 nm. All experiments were performed in four cultures.

Results

Effects of protein coatings on cell attachment

Primary keratinocytes were seeded at a density of 4×10^4 cm^{-2} on collagen, gelatin and hydrolysate, and cells grown on these substrates were compared with cells cultured on plastic as a control.

The percentage of cell adhesive rate at different times is shown in Table I and Fig. 1 is their adhesive rate curves. Cells grown on collagen showed a significant increase in rate of attachment compared with that of cells grown on plastic within 16 h (Fig. 2A–C), but rates of attachment to gelatin (Fig. 2D–F) and hydrolysate (Fig. 2G–I) did not differ significantly from that of cells cultured on plastic alone (Fig. 2J–L). Results of preliminary experiments on cell attachment to collagen, gelatin and collagen hydrolysate coatings over a period of 20 h showed that maximum attachment was achieved within 16 h, and the rate of attachment at 20 h was similar to that at 16 h in all experiments. Twenty hours, a duration that is sufficient for cells to attach but not to proliferate, was therefore used as the culture time in subsequent experiments. After 16 h, 61.4% (±2.6% SEM) of the cells had adhered to collagen, but only 40.9% (±3.2% SEM) of the cells had adhered to gelatin and only 38.2% (± 2.4% SEM) of the cells had adhered to collagen hydrolysate, rates of attachment similar to that of the plastic control (36.0 ± 2.5% SEM).

Table I Percentage of cell adhesive rate (±SEM)*

	8 h	12 h	16 h	20 h
Hydrolysate	12.5 ± 2.2	31.8 ± 3.2	38.2 ± 2.4	36.0 ± 3.4
Gelatin	13.6 ± 2.5	34.1 ± 2.8	40.9 ± 3.2	40.5 ± 3.3
Collagen	22.7 ± 3.1	52.3 ± 2.3	61.4 ± 2.6	59.8 ± 2.9
Control	11.4 ± 2.4	26.0 ± 3.0	36.0 ± 2.5	34.0 ± 3.2

*Mean of four experiments shown.

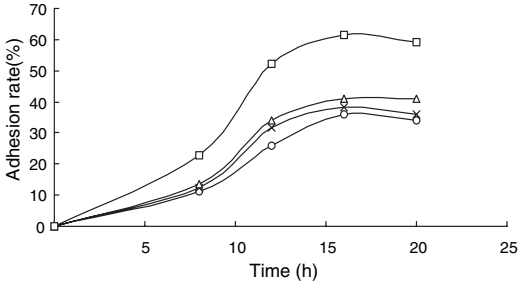

Figure 1 Adhesive rate curves of keratinocytes cultured on various substrata for 20 h. Cells were seeded at 4×10^4 cm^{-2} on collagen (□), gelatin (△), hydrolysate (×) and plastic (○). Data represent the means of four cultures.

Effect of protein coatings on cell proliferation

In order to determine whether differences in protein coatings had any sustained effect on keratinocyte proliferation, cells were seeded at various densities (0.875, 1.75 and 3.5×10^4 per well) in 96-well microplates coated with collagen, gelatin and hydrolysate. The results were compared with the proliferation of cells on plastic. Figure 3 shows the cell proliferation rates over periods of 6 days in which cells were seeded at high density (Fig. 3A), medium density (Fig. 3B) and low density (Fig. 3C). Cells seeded at high density showed a maximum rate of proliferation after 2 days of culture, whereas cells seeded at medium density and those seeded at low density showed maximum rates of proliferation after 3 and 4 days of culture, respectively. As can be seen in Fig. 3, cells grown on collagen showed the greatest increase in

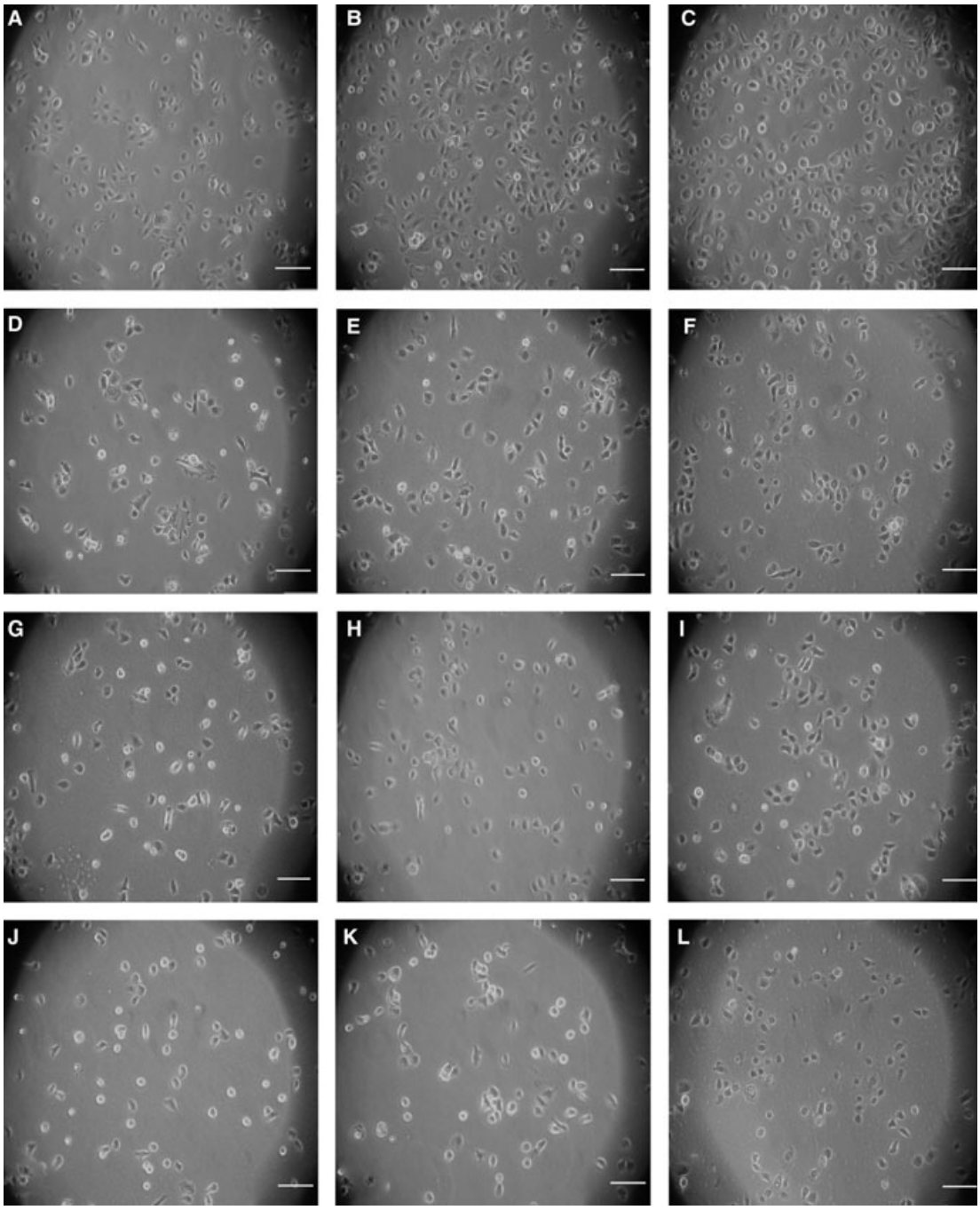

Figure 2 Photomicrographs of keratinocytes cultured on various substrata for 8 h (2A, 2D, 2G and 2J), 12 hours (2B, 2E, 2H and 2K) and 16 hours (2C, 2F, 2I and 2L). Cells were seeded at $4 \times 10^4/cm^2$ on collagen (2A, 2B and 2C), gelatin (2D, 2E and 2F), hydrolysate (2G, 2H and 2I) and plastic (2J, 2K and 2L). Bars: 100 μm.

proliferation at any seeding density. The proliferation rates of cells grown on gelatin and on hydrolysate were higher than the proliferation rate of cells grown on plastic, but the differences were not significant.

Discussion

It is known that the epidermis in mammals (including humans) is a regenerating tissue composed of four stratified cell layers, the stratum

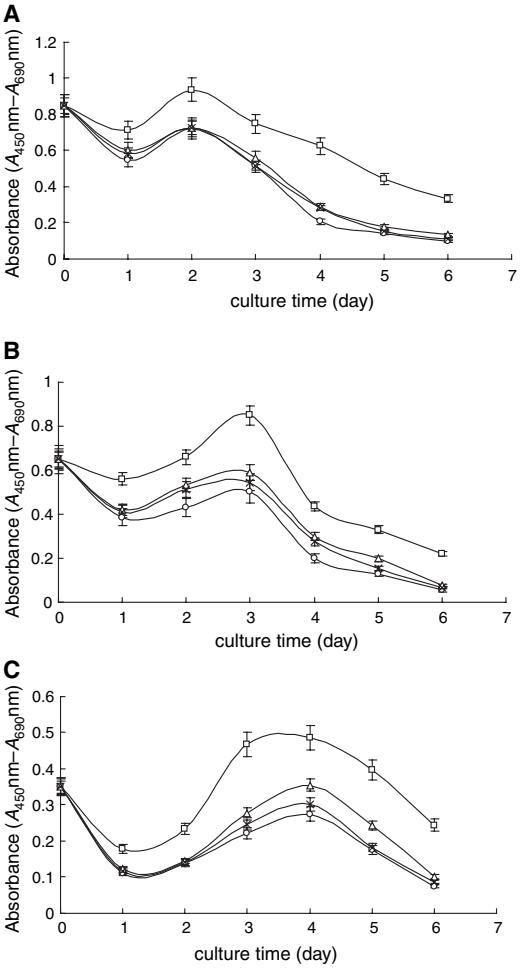

Figure 3 Proliferation curves of keratinocytes cultured on various substrata for 6 days. Cells were seeded at 3.5×10^4 per well (3A), 1.75×10^4 per well (3B) and 0.875×10^4 per well (3C) on collagen (□), gelatin (△), hydrolysate (×) and plastic (○). Data are expressed as the means ± SD of four independent measurements.

germinativum (basal cell layer), stratum spinosum (spinous cell layer, including a suprabasal cell layer immediately above the basal cell layer), stratum granulosum (granular cell layer), and stratum corneum (cornified layer) in order of increasing degree of cell differentiation. The basal cells adhere to a basement membrane zone (BMZ) that separates them from the dermis. Differentiation of the epidermis is accompanied by decreased basal cell-substratum adhesion to the BMZ as the cells move into the suprabasal and spinous cell layers. The cells of the epidermis thus arise at the basal layer as active cells that are capable of division and they are discarded at the surface as dead horny scales. Between 6 and 14 g of these dead scales is discarded each day in humans [11]. For this reason, a major function of the skin is the production of keratinocytes. Basal keratinocyte cultures serve as models for skin regeneration, and cell–substratum interactions play a major role in regulating the morphogenesis of the epidermis. The result of Nagelschmidt and Struck studying on the penetration of collagen as cosmetics was that collagen did not penetrate the dermis but was absorbed by the epidermis[5], indicating that collagen, gelatin and collagen hydrolysate could have effects on keratinized cells. However, the significantly higher rates of adhesion and proliferation of keratinacytes cultured on collagen than those of keratinacytes cultured on gelatin and collagen hydrolysate found in our experiments indicate that collagen is a more effective cosmetic material than is gelatin or collagen hydrolysate.

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