ϵ -(γ -Glutamyl)lysine Cross-link in the Terminal Differentiation of Mammalian Epidermis

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Purified horny cell membrane of the epidermis of newborn rat was prepared by discontinuous sucrose density gradient ultracentrifugation after 0.1 N sodium hydroxide treatment. ϵ -(γ -Glutamyl)-lysine cross-link was identified mainly in the horny cell membranes of the epidermis of newborn rat, cow snout, and human stratum corneum. When, [14 C]-lysine was incubated with minced epidermis of newborn rat, [14 C]-lysine labeled ϵ -(γ -glutamyl)lysine cross-link was identified in purified horny cell membrane. Results indicate that the protein newly synthesized in the granular layer might play a role in the formation of thickened cell membrane in the late stage of the epidermal differentiation.

During epidermal differentiation, the cell membranes are thickened about twice as thick as the plasma membranes of nonkeratinized cells^{1~4}) and become highly resistant to the action of keratinolytic reagents.^{4,5}) Chemical studies have shown that the thickened membrane of the cornified cell is the most resistant component of the protective system and have suggested the presence of an unknown cross-link.^{4,6}) In connection with the biochemical insolubilization of protein, ϵ -(γ -glutamyl)lysine cross-link has been reported in an ever-increasing number of biological materials which are insoluble proteins or stabilized tissues such as fibrin polymer, wool, guinea-pig hair and porcupine quill (for a recent review see ref. 7).

Earlier studies in this laboratory indicated the presence of ϵ -(γ -glutamyl)lysine cross-link in the 0.5 N KOH insoluble fraction in the epidermis of newborn rat.^{8,9}) This fraction corresponds to the horny cell membrane reported by Matoltsy.¹⁰) In addition, the incorporation of [14 C]-lysine labeled soluble epidermal protein into the keratin fraction was demonstrated in the presence of epidermal transglutaminase. Recently the presence of ϵ -(γ -glutamyl)lysine in the human stratum corneum¹¹) and horny envelope of human callus¹²) has been reported.

While numerous studies have been reported

on structural transformation of cell membrane during the epidermal differentiation, little is known about the biochemical bases except such as the papers mentioned above. This paper reports the localization of ϵ -(γ -glutamyl)lysine cross-link in the horny cell membrane of the epidermis of newborn rats which was purified by sucrose density gradient centrifugation, cow snout and human stratum corneum, and also reports the formation of ϵ -(γ -glutamyl)lysine cross-link in the late stages of epidermal differentiation.

MATERIALS AND METHODS

Materials. Carboxypeptidase A (bovine pancreas, Type II) and prolidase were purchased from Sigma Chemical Co., St. Louis, Mo. Pronase P was products of Kaken Chemicals, Tokyo. Aminopeptidase M (Lörm and Haas) was obtained from Peptides Center, Osaka. Synthetic ϵ -(γ -glutamyl)lysine was obtained from Cyclo Chemical Corp., Los Angeles, Calif. L-[4,5- 3 H]-lysine and L-[U- 14 C]lysine were purchased from The Radiochemical Centre, Amersham.

Epidermis and human stratum corneum. The epidermis of newborn rats (2~3 days or less than 24 hr old) were separated from the dermis by incubating the skin in 0.24 M NH_4Cl , pH 9.5, for 15 min at 0°C, or in 0.01 % trypsin in Earle's isotonic solution for 90 min at 0°C with constant slow stirring.¹³) Human stratum corneum was sliced from the sole of healthy human foot with a razor blade and the epidermis of cow snout

was removed in thin slices with a razor blade.

Preparation of horny cell membranes. The epidermis or stratum corneum were suspended in 0.1 N sodium hydroxide solution and horny cell membranes were prepared according to Matoltsy and Matoltsy¹⁰⁾ except that the suspension of minced or homogenized tissue was stirred with a teflon coated magnetic bar in a beaker. When it was necessary, the horny cell membrane from the epidermis of the newborn rat (2~3 days old) was suspended in 0.1 N sodium hydroxide-0.2% Brij 35 solution and submitted to discontinuous sucrose density gradient ultracentrifugation.¹⁴⁾ The preparations of horny cell membrane were checked by phase-contrast microscopy and electron microscopy, and stored after lyophilization. The horny cell membrane of cow snout used in preliminary experiments was a gift from Dr. A. G. Matoltsy and subsequently prepared from cow snout epidermis.

0.1 N sodium hydroxide soluble protein and prekeratin. The 0.1 N sodium hydroxide soluble fractions of the epidermis or stratum corneum were adjusted to pH 4.5 and the sediments were lyophilized. Prekeratin¹⁵⁾ of cow snout was a gift from Dr. A. G. Matoltsy.

Delipidation. The samples were extracted with chloroform-methanol mixture (2:1, v/v)¹⁶⁾ and/or chloroform-methanol-water (1:2:0.8 v/v/v).¹⁷⁾

Proteolytic digestion of the membrane protein. After delipidation, the membranes were suspended by degassing in 0.1 M Tris-acetate, pH 8.0, containing 5 mM calcium chloride at a concentration of approximately 0.5% (w/v). Pronase P was added to give an enzyme to substrate ratio of about 1:100 and allowed to be digested at 37°C with stirring by magnetic stirrer. A small amount of thymol was added as a bacteriostatic agent. The mixture was incubated for 5 days with four more addition of pronase P at 24 hr intervals. During the incubation, after 2 days, the mixture was made 0.5 M in cobaltous chloride which is required for the aminopeptidase activity contained in pronase P. The digestion was continued further for 48 hr with two additions of carboxypeptidase A (1:100) applied at 24 hr intervals. Then the mixture was made to 5 mM manganese chloride and prolidase (1:100) (activated with manganese ion by preincubation) was added. After 24 hr digestion, aminopeptidase M (1:200) was added and incubation was continued for final 24 hr. The supernatant solution of the digest was subjected to gel chromatography. A similarly incubated mixture of the proteolytic enzymes used for digestion without substrate was incubated as a control.

Gel chromatography. The soluble fraction of the digest of samples was applied to a column of Bio-Gel P-2 (2×91 cm=300 ml) equilibrated with 0.1 N acetic

acid and eluted with the same solution. The column had been calibrated previously with a standard mixture of amino acids and ϵ -(γ -glutamyl)lysine. The isopeptide fraction almost free from neutral and acidic amino acids was analyzed by an amino acid analyzer.

Identification and estimation of ϵ -(γ -glutamyl)lysine. The analysis was carried out essentially according to the method of Lorand *et al.*¹⁸⁾ on a Hitachi KLA-3B amino acid analyzer with a custom 2612 resin column (0.9×52 cm) equilibrated in 0.2 N citrate buffer, pH 3.31, at a flow rate of 60 ml/hr. A modified accelerated procedure with a 0.2 N sodium citrate buffer, pH 3.57, (K. Sugawara, unpublished) was also applied in the final stage of this experiment. In this method, the peak of ϵ -(γ -glutamyl)lysine appeared at about 114 min between those of methionine (101 min) and isoleucine (135 min). The details will be published elsewhere.

Structural characterization of the cross-link. After isolation of the ϵ -(γ -glutamyl)lysine from the horny cell membrane of newborn rat epidermis by a preparative run on the amino acid analyzer, the impurities arising from the citrate buffer were removed by gel filtration on a Bio-Gel P-2 column. To confirm the characteristic structure of the isopeptide, dansylation¹⁹⁾ was carried out, followed by paper electrophoresis at pH 3.7²⁰⁾ to resolve α -DNS-lysine and ϵ -DNS-lysine. After hydrolysis at 110°C for 22 hr with 6 N HCl, amino acid composition of these samples was analyzed by a Hitachi amino acid analyzer.

Incorporation of radioactive lysine into the ϵ -(γ -glutamyl)lysine in vitro. The epidermis of newborn rat was separated from the dermis by trypsin treatment. The minced epidermis, 6 g wet weight, was shaken at 37°C with L-[U-¹⁴C]lysine or L-[4,5-³H]lysine in 10 ml of Earl's solution containing 300 units/ml penicillin G and 50 μ g/ml of streptomycin. The horny cell membrane was prepared from the labeled tissue and submitted to the proteolytic digestion. The soluble fraction of the digest was fractionated by gel chromatography on a column of Bio-Gel P-2. The elution profile of ϵ -(γ -glutamyl)lysine was the same as that of lysine. The radioactive fractions containing lysine and ϵ -(γ -glutamyl)lysine were collected and fractionated by paper electrophoresis at pH 3.7²⁰⁾ to resolve lysine and ϵ -(γ -glutamyl)lysine. After lysine and ϵ -(γ -glutamyl)lysine were eluted from the paper with 50 mM acetic acid, the cpm was estimated by a liquid scintillation counter in toluene-NT scintillator.²¹⁾

Phase-contrast and electron microscopy. A phase-contrast microscope (Olympus) was used for the examination of the horny cell membranes. Electron microscopic examination of the membrane was kindly performed by Mr. M. Shirai of the Laboratory of Applied Microbiology, Ibaraki University.

RESULTS

Isolation of horny cell membrane by sucrose density gradient ultracentrifugation

The horny cell membrane fraction of the epidermis of newborn rat was purified by discontinuous sucrose density gradient as described in the method. The major portion of the membrane was present at the interface d 1.0/ d 1.6 and sometimes also obtained at the interface d 1.16/ d 1.18 and d 1.18/ d 1.20. The impurities including hair were sedimented at the bottom under the layer of $d=1.20$ of the tube, when the membranes were prepared from the epidermis of more than 3 days old rat. A trace amount of impurities was found in the preparation from the epidermis of 2~3 days old rat, while no impurities were detected from the epidermis of newborn rats less than 24 hr old. About 30~60 mg of the horny cell membrane was obtained from 1 g of the epidermis of newborn rat based on dry weight.

Identification of ϵ -(γ -glutamyl)lysine cross-links

When the proteolytic digests of the horny cell membranes were fractionated on Bio-Gel P-2, ϵ -(γ -glutamyl)lysine and basic amino acids were separated from neutral and acidic amino acids (Fig. 1). The peptide fractions were collected and applied on an amino acid analyzer using pH 3.31 buffer. A peak (about 270 min) was located between the peaks of leucine (about 243 min) and tyrosine (about 314 min) as determined with a standard amino acid mixture. When authentic ϵ -(γ -glutamyl)lysine was added to the proteolytic digest of the samples and analyzed, only the peak eluting at 270 min was increased and the extent of augmentation was as expected. Further confirmation was made by isolating the ϵ -(γ -glutamyl)lysine from a large scale run. The peptide fraction was collected directly from the amino acid analyzer column. The material was desalted on a Bio-Gel P-2 column, and subjected to several methods of structural identification. Amino acid analysis showed only glutamic acid and lysine, and the ratio of glutamic acid to lysine was approximately 1. Acid hydrolysate of the

FIG. 1. Representative Gel Filtration Profile of the Proteolytic Digest of Horny Cell Membrane.

The supernatant of the digest was applied to a column of Bio-Gel P-2 (2.1 $\times$ 91 cm) to separate the ϵ -(γ -glutamyl)lysine from neutral and acidic amino acids. The column was equilibrated and eluted with 0.1 N acetic acid. Peak A contains ϵ -(γ -glutamyl)lysine and basic amino acids free from neutral (peak B) and acidic (peak C) amino acids. $\circ$ --- $\circ$, authentic ϵ -(γ -glutamyl)lysine (Cyclo Chemicals); $\square$ — $\square$, proteolytic digest of horny cell membrane.

dansyl derivative of the peptide was analyzed by high voltage paper electrophoresis and thin layer chromatography, and dansyl-glutamic acid and α -dansyl-lysine were detected. These results support the original assignment of the dipeptide as ϵ -(γ -glutamyl)lysine.

Estimation of ϵ -(γ -glutamyl)lysine in the horny cell membranes

Proteolytic digests of the membranes were fractionated on a column of Bio-Gel P-2 and the peptide fractions were submitted to an amino acid analyzer for the estimation of ϵ -(γ -glutamyl)lysine. The results are summarized in Table I. On the other hand, the amount of ϵ -(γ -glutamyl)lysine cross-link in the 0.1 N sodium hydroxide soluble fraction was very low as compared with the value of the membranes (Table II). No ϵ -(γ -glutamyl) lysine cross-link was detected in the digest of prekeratin from cow snout.

TABLE I. ϵ -(γ -GLUTAMYL)LYSINE IN HORNY MEMBRANES OF THE EPIDERMIS

Source	nmol	Molecular weight of protein
	mg Protein ^a	ϵ -(γ -Glutamyl)-lysine
Newborn rat (2~3 days old)	31	32,200
Cow snout	21	47,600
Human stratum Corneum	42	23,800

^a The amount of digested protein was calculated from the decrease in the dry weight of insoluble materials during the proteolytic digestion.

TABLE II. ϵ -(γ -GLUTAMYL)LYSINE IN 0.1 N SODIUM HYDROXIDE SOLUBLE EPIDERMAL PROTEIN AND PREKERATIN

Source	nmol
	mg Protein ^a
Newborn rat (2~3 days old)	0.47
Cow Snout	0.63
Human Stratum Corneum	1.05
Prekeratin (Cow Snout)	0

^a About 50 mg of samples were used and almost completely solubilized except sample from newborn rat (digestibility was 83%). The value of protein of newborn rat was determined in the same way as noted in Table I.

The incorporation of labeled lysine into the ϵ -(γ -glutamyl)lysine cross-links *in vitro*

The epidermis of the newborn rat (less than 24 hr old) was incubated with L-[4,5-³H]-lysine for 2 hr and the epidermis of newborn rat (2~3 days old) was incubated with L-[U-¹⁴C]-lysine for 2 hr, respectively. Then, the horny cell membranes were isolated and digested exhaustively with the proteases as described in the method. The digest was chromatographed on a Bio-Gel P-2 column. A major portion of the radioactivity of the digest was recovered in the fractions of free lysine and ϵ -(γ -glutamyl)lysine (Fig. 2, peak A). The peptide fractions containing free lysine were concentrated and separated from lysine by high voltage paper electrophoresis. The radioactivity was detected only in the spots of ϵ -(γ -glutamyl)lysine and lysine. Then the radioactivity of the cross-linked lysine and the free lysine were estimated.

FIG. 2. Gel Filtration Profile of the Proteolytic Digest of L-[4,5-³H]lysine Labeled Horny Cell Membrane.

The epidermis of less than 24 hr old newborn rat was labeled with L-[4,5-³H]lysine, *in vitro*, and the horny cell membrane was digested and chromatographed. The other conditions were similar to those described in Fig. 1. ○—○, radioactivity, cpm; ●—●, ninhydrin color reaction, absorbance at 570 nm.

TABLE III. INCORPORATION OF LABELED LYSINE INTO THE HORNY CELL MEMBRANE OF NEWBORN RAT

Epidermis	Incubation (hr)	dpm		(B) %
		Lysine (A)	ϵ -(γ -Glutamyl)-lysine (B)	
Less than 24 hr old rat ^a	2	79980	11270	12.4
2~3 days old rat ^b	2	2850	630	18.1
	5	3400	1800	34.6

^a Labeled with L-[4,5-³H]-lysine.

^b Labeled with L-[¹⁴C]-lysine.

The results are shown in Table III. About 18% of the total lysine recovered was present in the cross-linked form when the epidermis of 2~3 days old newborn rat was incubated for 2 hr. After 5 hr incubation, the value of cross-linked lysine increased to about 35%. The value of less than 24 hr old newborn rat, which was incubated for 2 hr, was 12.4%.

DISCUSSION

The present experiments show the presence and the localization of ϵ -(γ -glutamyl)lysine cross-links in the horny cell membranes of the epidermis of newborn rat, cow snout and human sole. The amounts of ϵ -(γ -glutamyl)lysine cross-link in horny cell membranes of these mammalian epidermis were 31, 21, and 42 mol per megagram of protein for newborn rat, cow snout, and human sole, respectively (Table I). The values of this range are less than those of clotted seminal protein of guinea pig²²⁾ (370 mol per megagram of protein), which is one of the most cross-linked proteins ever described, citrulline containing medullary protein of hair²³⁾ (115~200 mol per megagram), and cornified cell envelope of human plantar callus¹²⁾ (130 mol per megagram). However, the values obtained in the present experiments are much higher than those of human plasma coagulated fibrin^{24,25)} (6.6~8.8 mol per megagram) and plasma membrane of mouse L cell²⁶⁾ (1.7 mol per megagram). Compared with these levels of cross-link, the values, 21~42 mol per megagram of protein seem enough to account for the extreme insolubility of horny cell membranes of the mammalian epidermis. Presumably, a moderate degree of cross-link will be needed for providing stratum corneum of the epidermis with flexibility and elasticity against physical attacks.

A low level of the cross-link was detected in the 0.1 N sodium hydroxide soluble fractions in three samples (Table II). Since the cross-link was not detected in 8 M urea-DTT soluble fraction of the epidermis,^{8,9)} it can be presumed that the cross-linked protein was released from the horny cell membrane with 0.1 N sodium hydroxide. This suggests that the cross-linked protein in this fraction is bound to the membrane by alkali labile binding such as ester or ester-like bonds. Further studies are required to elucidate the properties and the role of the alkali labile cross-linked protein.

The mammalian epidermis is a continuously differentiating tissue, in which the daughter

cells, produced by mitosis in the inner basal layer, migrate toward the outer surface through a series of morphologically and biochemically distinct states to the ultimate cornification. In the final stages of this differentiation, the membranes of the cells thicken and cytoplasmic components disintegrate except for the fibrils which consolidate into keratin, and the membrane resists the action of chemical attack such as keratinolytic agents. Matoltsy suggested that these membranes were stabilized by unknown highly resistant bonds.^{4,6)} Furthermore, it was postulated that the characteristic insolubility of the stratum corneum is mainly due to the cell membrane,²⁷⁾ since mechanical disruption of the cells after swelling in urea allowed the release of protein.

When [¹⁴C]-lysine was incubated with minced epidermis for 2 hr, the ratio of radioactivity of ϵ -(γ -glutamyl)lysine to free lysine in the horny cell membrane was 1:4.5. However, after incubation for 5 hr, the proportion of the cross-linked form of [¹⁴C]-lysine increased to 1:1.9. The epidermis used, consisted mainly of horny cell layer and granular cells,¹³⁾ where terminal cytodifferentiation would be taking place. These facts demonstrate that the protein synthesized in granular cells participates extensively in the cross-linking and plays an important role in the terminal differentiation of the epidermis by contributing to the formation of ϵ -(γ -glutamyl)lysine cross-links.

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