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N^1N^8-Bis(γ-glutamyl)spermidine cross-linking in epidermal-cell envelopes

Comparison of cross-link levels in normal and psoriatic cell envelopes

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N^1N^8-Bis(γ-glutamyl)spermidine was found in exhaustive proteolytic digests of isolated cell envelopes from human epidermis at levels comparable with those of ε-(γ-glutamyl)lysine. Significantly higher than normal amounts of these compounds, particularly the bis(γ-glutamyl)polyamine, were observed in envelopes from afflicted areas (scales) of psoriatic patients. These findings support the notions that N^1N^8-bis(γ-glutamyl)spermidine, like ε-(γ-glutamyl)lysine, functions in cell envelopes as an enzyme-generated protein cross-link and stabilizing force and that individuals with the chronic, recurrent skin disease, psoriasis, exhibit in involved epidermis abnormal cell-envelope-protein cross-linking.

INTRODUCTION

Epidermal cells undergo terminal differentiation culminating in the establishment of the stratum corneum. This outermost layer of the epidermis, which consists of dead and desquamating cells, is capable of retaining water and confers coherence, plasticity and resistance to the integument. The key enzymic role played by transglutaminase(s) in epidermal-cell terminal differentiation is well documented. These enzymes catalyse the production of ε-(γ-glutamyl)lysine cross-links (Fig. 1*a*) between several cellular proteins to form highly insoluble structures, called cell envelopes [1,2] (initially termed marginal bands [3]), that contribute to the protective function of the skin [4,5].

Although it is well documented that high cellular levels of spermidine and spermine generally parallel cell proliferation, little is known about the specific biological functions of these ubiquitous polycations (for reviews, see [6] and [7]). Post-translational modifications in which structural elements of the polyamines are incorporated into proteins have been described [8,9]. In this regard all of the polyamine associated with a highly insoluble protein fraction of cultured epidermal cells during terminal differentiation was found to occur in γ-glutamylamine linkage [10]. This observation, together with the knowledge that transglutaminases can catalyse cross-linking of model protein molecules through bis(γ-glutamyl)polyamine bonds (Fig. 1*b*) [11], suggested a role of enzyme-catalysed polyamine incorporation in cell-envelope formation. Reported here is the finding of polyamine cross-links in cell envelopes at levels indicative of their significant contribution to envelope structure stabilization.

Psoriasis is a chronic hereditary recurrent papulosquamous dermatosis. Because disorder of epidermal differentiation and elevated cutaneous levels of polyamines [12–14] are both associated with psoriasis, this disease was chosen as a model to study potential abnormalities of cell-envelope cross-linking. The results of this study are also reported here.

MATERIALS AND METHODS

Samples of whole skin were obtained from individuals of different ages, sexes and races who displayed no clinical symptoms of psoriasis. Skin was removed from various body locations (legs, feet or breasts) during surgery or immediately *post mortem*. Epidermal callus was removed from the soles of normal volunteers of different ages, sexes, and races and from the soles of psoriatic patients by scraping with a scalpel. Psoriatic patients who showed no visible clinical involvement of their soles were selected, because callus was chosen as the source of clinically uninvolved epidermis from these patients. Patients of different ages, sexes, and races were recruited at the Dermatology Clinic, National Cancer Institute, National Institutes of Health, Bethesda, MD, U.S.A. The disease, ascertained by clinical examination, was confirmed by biopsy of involved skin. None of the patients had been under treatment for psoriasis or any other disorder during a period of at least 6 weeks preceding sample collection. Scales of clinically involved skin were collected by gently stroking with a smooth dermal curette. All samples were stored frozen in liquid N_2 until processed.

Separation of the epidermis from the dermis of skin samples collected during surgery was carried out by their treatment with the enzyme Dispase as outlined previously [15]. A single sample of cell envelopes was isolated from a pool of epidermal sheets obtained by this means. Samples of epidermis from skin obtained *post mortem* were prepared free of dermis by treatment with 0.24 M-NH_4Cl at pH 9.5 as described [16]. Cell envelopes were isolated from epidermis, callus and psoriatic scales by gentle overnight stirring at room temperature in 0.025 M-Tris/HCl buffer, pH 9, containing 8 M-urea, 2 mM-2-mercaptoethanol and 0.2% SDS, followed by centrifugation for 20 min at 750 *g*. The insoluble crude envelopes were freed of remaining soluble impurities by washing three times for 2 h with the same buffer mixture at room temperature and three times for 5 min with this mixture at 100 °C. Buffer salts and detergent were removed by multiple extractions with water, two washes with ethanol, and one wash with aq. 5% formic acid. Upon microscopic examination, each envelope preparation displayed the features characteristic of the isolated form of this epidermal component. Examination of each of the preparations by SDS/PAGE after

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disruption in a sonicator (Heat Systems) revealed an absence of protein bands on the running gel and thus provided evidence for satisfactory isolation.

Acid hydrolysis was performed in 6 M-HCl for 18 h at 110 °C. Proteolytic digestion was conducted at 37 °C with the use of the enzymes Pronase, aminopeptidase M and carboxypeptidases A and B as outlined in [8]. At the completion of this procedure, digestion was continued for an additional 8 h period after addition of carboxypeptidase Y at a level of 0.01 mg/initial mg of envelope [17]. Amino acids were determined with the use of an automated amino acid analyser using ninhydrin detection. Analyses for polyamines were carried out on acid hydrolysates of cell envelopes by means of an automated ion-exchange procedure employing the three-buffer system for elution and *o*-phthalaldehyde for detection [8]. For determination of the γ-glutamylamines, the first step was conducted as outlined [18], using the same ion-exchange procedure employed for polyamine analyses, with the exceptions that elution was with the five-buffer system [8] and effluent was collected direct from the column in fractions without mixing with the fluorimetric reagent. The fractions containing bis(γ-glutamyl)putrescine were combined, as were those containing ε-(γ-glutamyl)lysine, those containing both *N*-(γ-glutamyl)putrescine and N^1N^8-bis(γ-glutamyl)spermidine, and those containing both N^1- and N^8-(γ-glutamyl)spermidines. The components of the individual pools were derivatized with *o*-pthalaldehyde and the γ-glutamylamine derivatives were separated and quantified by reverse-phase h.p.l.c. [18].

The quantities of γ-glutamylpolyamines in the pooled fractions from ion-exchange chromatography were verified by measurement of polyamines produced upon acid hydrolysis. The use of the enzyme γ-glutamylamine cyclotransferase [19] provided a means of confirming the identity and, further, a third method for verifying the amounts, of γ-glutamylamines. This enzyme, which is specific for release of amines from γ-glutamylamines, was employed, as outlined in [19], to analyse pooled fractions from the ion-exchange chromatography of random samples of envelope digests.

The envelope cross-link data were analysed with the use of the Wilcoxon rank sum test for unpaired differences and the Wilcoxon signed rank test for paired differences. A Bonferroni correction factor of 3 was applied in each case. The values are expressed as means ± S.D.

RESULTS

In order to determine directly the type and quantity of covalent binding between protein chains in cell-envelope preparations, it was necessary to isolate simple compounds that contain the bonds connecting the chains in order to define their structures and to measure their amounts. Characterization and quantification of the isopeptide ε-(γ-glutamyl)lysine was the means employed by Rice & Green [20] to determine one type of covalent bonding in cell envelopes. Because all cross-linking bonds produced by the catalytic action of transglutaminases are in the form of amides of the γ-carboxy group of protein-bound glutamic acid (Fig. 1) and because these γ-amides are degraded by chemical treatments to essentially the same degree as are peptide bonds, special means are needed to preserve cell-envelope cross-links during disassembly of the envelope protein chains which they connect. The method employed here was to cleave the peptide bonds by exhaustive digestion with proteolytic enzymes and to determine the cross-links by identification and measurement of the γ-glutamylamine residues which are resistant to proteolysis. This proteolytic-digestion methodology is routinely employed for liberation of ε-(γ-glutamyl)lysine from protein

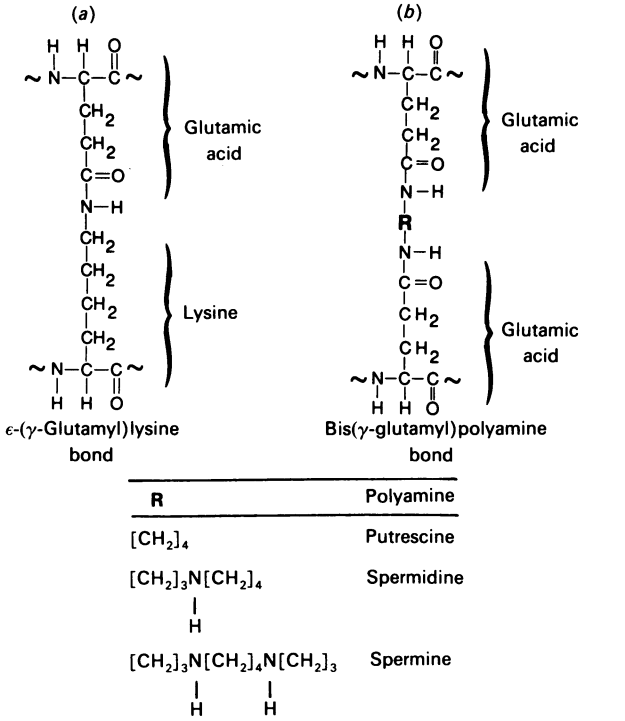

Fig. 1. Structures of the covalent cross-links in human epidermal-cell envelopes

preparations [21] and has been shown to be applicable also for release of γ-glutamylpolyamines [8].

When proteolytic digests of cell-envelope preparations from body epidermis, callus and psoriatic scales were examined for γ-glutamylpolyamines, bis(γ-glutamyl)spermidine was found at levels comparable with those of ε-(γ-glutamyl)lysine (Fig. 2; cf. *b* and *a*). Much smaller amounts of bis(γ-glutamyl)putrescine (< 0.6 per 1000 amino acid residues) were seen in digests of envelopes from psoriatic scales (results not shown). Digests of envelopes from epidermis and callus were found to contain only traces, if any, of bis(γ-glutamyl)putrescine. None of the digests contained bis(γ-glutamyl)spermine at detectable levels.

In addition to the bis(γ-glutamyl)polyamines, small amounts of mono(γ-glutamyl)spermidines (< 0.2 per 1000 amino acid residues) and traces of mono(γ-glutamyl)putrescine were seen in digests from psoriatic scale envelopes. Only mono(γ-glutamyl)spermidines were seen in some digests of envelopes from callus, and only at trace levels. These mono(γ-glutamyl)polyamines are in all probability cross-link precursors, and thus were generally detectable only in samples high in polyamine cross-links.

A comparison of the amounts of bis(γ-glutamyl)spermidine cross-linking in representative samples of cell envelopes as measured by the three procedures outlined in the Materials and methods section showed close agreement in the three values obtained for each of the samples. For example, with each of five samples from callus of normal individuals the values determined by the three methods differed by less than 18%; likewise the three values obtained with each of four samples from scales of psoriatic patients agreed within 8%.

When cell envelopes from callus and from psoriatic scales were hydrolysed with acid and the polyamine contents of the hydrolysates measured, good correspondence was observed in each case between the spermidine released by acid (Fig. 2*c*) and the spermidine found in cross-link form (Fig. 2*b*). This close agreement indicates that essentially all of the spermidine

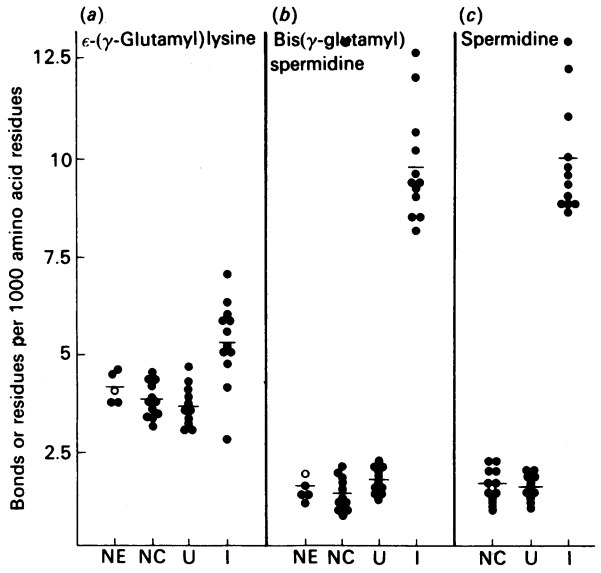

Fig. 2. Cross-link levels (*a* and *b*) and spermidine content (*c*) of cell envelopes isolated from specimens of epidermis, epidermal callus and psoriatic scales

NE, samples of envelopes from epidermis prepared from skin collected *post mortem* from normal individuals (●) and the single sample of envelopes from pooled epidermis prepared from skin obtained during surgery from normal individuals (○); NC, envelopes from callus of normal individuals; U, envelopes from uninvolved areas (callus) of psoriatic patients; I, envelopes from involved areas (scales) of psoriatic patients. The group means are denoted by the horizontal lines.

Table 1. Amino acid composition of human epidermal-cell envelopes

The envelopes from epidermis of normal individuals were from those samples of skin obtained *post mortem*. Tryptophan and cysteine were not determined. All values are means ± S.D. for samples from four or five individuals. Abbreviation: N.D., not detected.

		Composition (residues/1000 residues)			
Amino acid	Source of envelopes...	Epidermis of normal individuals	Callus of normal individuals	Callus of psoriatic patients	Scales of psoriatic patients
Asx		54±4	50±5	50±6	83±6
Thr		32±3	37±6	29±5	21±5
Ser		137±9	160±9	160±7	18±5
Glx		166±4	170±10	175±8	240±20
Pro		96±3	82±5	97±4	122±12
Gly		231±5	210±12	171±20	73±9
Ala		28±2	38±5	44±6	64±3
Val		59±2	56±5	69±3	60±5
Met		2±1	7±2	N.D.	N.D.
Ile		21±2	20±1	17±7	22±6
Leu		31±3	33±2	37±1	40±2
Tyr		19±2	16±2	19±3	N.D.
Phe		7±2	10±1	14±3	15±1
Lys		56±3	60±6	63±4	185±6
His		20±2	20±2	24±3	24±5
Arg		41±2	32±2	34±4	34±7

associated with envelopes is in the form of polyamine cross-links and is further evidence for good quantification of bis(γ-glutamyl)spermidine. In addition to spermidine, hydrolysates of all samples were also found to contain small amounts of putrescine (levels < 10% of those of spermidine). The amounts of spermine found in acid hydrolysates of the tissues from which envelopes were prepared, i.e. 0.12±0.09 nmol per mg wet weight of callus of normal individuals ($n = 12$) and 1.08±0.58 nmol per mg wet weight of psoriatic scales ($n = 12$), were within the ranges reported for free spermine [12–14]. However, no spermine was found in hydrolysates of any of the envelope preparations. This observation is consistent with the failure to detect spermine cross-linking in these preparations.

Little variation in amino acid composition occurred among samples of envelopes prepared from epidermis of normal individuals (Table 1). This was also the case among samples in each of the other groups. Striking differences, however, were apparent between the composition of envelopes from psoriatic scales and that of envelopes from body epidermis of normal individuals or those of envelopes from callus of either normal subjects or psoriatic patients. Envelopes from epidermis and those from callus of normal subjects and psoriatic patients were found to be of similar amino acid composition.

Comparison of the values for cross-link content of cell envelopes based upon the amino acid composition of the envelopes and reported as number of cross-links per 1000 amino acid residues (Figs. 2*a* and 2*b*) shows that the level of the ε-(γ-glutamyl)lysine cross-link (5.35±1.10) and that of the bis(γ-glutamyl)spermidine cross-link (9.84±1.40) in envelopes from psoriatic scales are significantly higher than those (4.16±0.38 and 1.66±0.22 respectively) in envelopes from body epidermis of normal individuals [2α (two-sided significance probability in Wilcoxon tests) < 0.015 and $2\alpha < 0.006$ respectively], than those (3.85±0.45 and 1.41±0.56 respectively) in envelopes from callus of normal individuals ($2\alpha < 0.006$ and $2\alpha < 0.003$ respectively) and than those (3.75±0.48 and 1.83±0.31 respectively) in envelopes from callus of psoriatic patients ($2\alpha < 0.024$ and $2\alpha < 0.006$ respectively. No significant differences in cross-link levels were seen between envelopes from epidermis of normal subjects, those from callus of normal subjects and those from callus of psoriatic patients.

The mean level of ε(γ-glutamyl)lysine cross-link in envelopes from epidermis of normal individuals and those from callus of normals and psoriatic patients were calculated to range between 36.8 and 40 nmol per mg of protein, which corresponds to between 25 and 27 kDa of protein/cross-link; that in envelopes from psoriatic scales was calculated as 47.4 nmol/mg of protein or 21 kDa of protein/cross-link. The mean levels of bis-(γ-glutamyl)spermidine cross-link ranged between 14.1 and 17.9 nmol/mg of protein, corresponding to 55.9–70.9 kDa of protein/cross-link, in envelopes from epidermis and callus of normal subjects and callus of psoriatic patients; that in envelopes from psoriatic scales was calculated as 87.2 nmol/mg of protein or 11.5 kDa of protein/cross-link.

DISCUSSION

For a number of years the ε-(γ-glutamyl)lysine bond was imputed to be the covalent linkage that connects certain of the proteins of keratinocytes to produce the cell envelope [20]. The insolubility and chemical resistance of the envelope was attributed largely to cross-linking through this bond. The present report provides evidence for the occurrence of an additional covalent cross-link, N^1N^8-bis(γ-glutamyl)spermidine, in the epidermal-cell envelope. The significantly high normal level of this linkage, its frequency as compared with that of ε-(γ-glutamyl)lysine, and the pronounced aberrations in the levels of the two cross-links in envelopes from affected areas of patients with psoriasis are consistent with important and similar functions for both.

That the bis(γ-glutamyl)spermidine cross-link, like the ε-(γ-glutamyl)lysine cross-link, is a product of transglutaminase

action seems most certain. Despite their probably common enzymic origin, however, these two linkages differ significantly in several respects. First, the more extended chemical structure of the bis(γ-glutamyl)spermidine bond allows greater distance between cross-linked protein chains and, consequently, may provide chain attachments at positions not suitable for cross-linking through ε-(γ-glutamyl)lysine bonds. Secondly, because the specificity of transglutaminase is limited to primary amines [22], the secondary amino group of the spermidine moiety of the bis(γ-glutamyl)spermidine linkage remains unconjugated and thus may contribute to the overall charge in the vicinity of this cross-link. Thirdly, for ε-(γ-glutamyl)lysine cross-linking, both the carboxy contributor, a carboxamide groups of a glutamine residue, and the amine contributor, the ε-amino group of a lysine residue, must be located in macromolecules. For bis(γ-glutamyl)spermidine cross-linking, however, since the amine portion of the bond is contributed by the low-molecular-mass biological cation spermidine, the macromolecules need only have carboxamide groups available to the cross-linking enzymes in order to enter into cross-link formation. Thus the conformations of the envelope precursor proteins and their non-covalent interactions at the time of cross-link production may, to a large degree, regulate the type of cross-linking. One would expect that the conformations and interactions required for ε-(γ-glutamyl)lysine bond formation would not be the ones most suitable for bis(γ-glutamyl)polyamine bond formation. This seems a reasonable expectation in the light of the pronounced differences in molecular structure between the two cross-links.

The failure to detect γ-glutamylspermine cross-linking in any of the envelope samples examined here is in accordance with an earlier observation that no γ-glutamylspermine derivatives are produced in mouse epidermal cells under various culture conditions [10]. The evidence against participation of spermine in cross-link formation in epidermal cells is in contrast with an earlier finding that this polyamine is effectively incorporated *in vitro* into derivatized casein by transglutaminases with the resultant formation of N^1N^{12}-bis(γ-glutamyl)spermine cross-links between casein molecules [11]. Consistent with both observations is preliminary evidence that the ubiquitous cellular enzyme polyamine oxidase is capable of catalysing the degradation of both γ-glutamylspermine and peptide-bound forms of this polyamine conjugate to produce free spermidine and free or peptide-bound γ-glutamyl-3-aminopropionaldehyde respectively (S. Beninati & J. E. Folk, unpublished work). It is therefore possible that, even though spermine may be incorporated into certain epidermal-cell proteins during terminal differentiation, no conjugates of spermine accumulate and no spermine cross-linking occurs because of the efficient polyamine oxidase-catalysed breakdown of γ-glutamylspermine conjugates.

The significance of elevated levels of cutaneous polyamines reported in psoriasis has been questioned [23]. Because the polyamines were measured in full-thickness biopsies of normal and psoriatic skin [12–14], it was pointed out [23] that the variations may simply result from ratio changes in the epidermis and dermis, the polyamine contents of which differ significantly. Alternatively, these variations may be due to the parakeratotic nature of psoriatic epidermis, the nuclei of which could contribute substantially to the polyamine content. Variations in epidermal-cell-envelope cross-linking (Fig. 2), as well as in amino acid composition (Table 1), on the other hand, must be construed as strong evidence for an abnormality in an essential tissue component produced during cellular differentiation. Indeed, it has been suggested that epidermal-cell envelopes are composed of those proteins available to the cross-linking enzymes at the moment that they become activated during terminal differentiation and, therefore, that the composition of the envelopes is not strictly determined [24]. Thus comparison of the peptides produced by CNBr cleavage of envelopes from normal epidermis with those from psoriatic lesions revealed striking differences [25]. In accordance with this finding are the amino acid compositional and cross-linking differences reported here.

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