A Monoclonal Antibody Labelling the Keratinocyte Membrane: a Marker of Epidermal Differentiation

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A murine hybridoma secreting an IgM monoclonal antibody (KL_3) was produced by cell fusion of mouse myeloma cells with spleen cells from mice immunized with human epidermal keratins. On normal human epidermis KL_3 stained the intercellular spaces from the stratum germinatum to the stratum granulosum with a fluorescence intensity increasing from the basal layer to the upper layers. Basal cells were not stained on the side facing the basement membrane. About 90% of free keratinocytes isolated after trypsinization were labelled by KL_3 in a punctate staining. Immunoelectron microscopy allowed us to show that the antigen recognized by KL_3 was exclusively localized on the keratinocyte membrane especially in the desmosomal plaques. KL_3 reactivity was not modified by pre-incubation of skin sections with lectins showing a selective intercellular labelling of upper layers of epidermis or pemphigus antisera, nor by adsorption of the antibody on NP_{40} soluble proteins of the epidermis. Though KL_3 reactivity was completely abolished after adsorption of purified keratins, no immunological reactivity of KL_3 was detected with epidermal keratin polypeptides blotted on nitrocellulose paper. In psoriatic epidemis and epidermal tumors KL_3 reactivity was drastically modified. These results suggest that KL_3 recognized a keratinocyte membrane antigen implied in the epidermal differentiation process. *Key words: Differentiation antigen.* (Received May 15, 1984.)

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In the epidermis, basal cells, differentiating keratinocytes and stratum corneum are attached to each other by an undefined "glue substance" which fills the epidermal intercellular spaces. The cell surfaces and the intercellular spaces constitute the glycocalyx (1) which is important in the maitenance of a functionally normal epidermis. During the differentiation process of epidermis, keratinocyte cell membrane antigens undergo major changes. At the level of the stratum granulosum and stratum corneum there is a thickening of cell membranes, narrowing of intercellular spaces and lysis of desmosomes. A complex set of components rich in glycoproteins and glycolipids such as receptors for hormones, antibodies, lectins, blood group and HLA antigens, "pemphigus" antigen(s), desmosomal intercellular material or "desmoglea"... is involved in this process (2). All these antigens play an important role in many aspects of keratinocyte physiology: cell recognition, cell morphology, cell differentiation ... In this study we describe the properties of a monoclonal antibody (KL_3) reacting with the keratinocyte membrane which therefore seems useful for keratinocyte differentiation studies of normal and abnormal human epidermis.

Abbreviations: DAB = Diaminobenzidine, DMEM = Dulbecco's minimum essential medium, FCS = Fetal calf serum, NP-40 = Nonidet P-40, SDS PAGE = Sodium dodecyl sulfate polyacrylamide gel electrophoresis, TCA = Trichloroacetic acid.

MATERIAL AND METHODS

I. *Preparation of monoclonal antibody* (MCA)

Balb/c mice were immunized by injection of 50 μg of purified epidermal keratins according to a technique already described for the preparation of monoclonal antikeratin antibody KL_1 (3). Briefly, spleen cells were fused with SP_2 myeloma cells according to the technique of Köhler & Milstein (4). The supernatants of the different colonies were screened by immunofluorescence on skin sections. The culture corresponding to KL_3 was subcloned. The secreted antibody was found to belong to the IgM class by imunodiffusion. KL_3 antibodies were produced either in culture or by induction of ascites in Balb/c mice.

II. *Sources of substrates for immunofluorescence (IF) studies*

Epidermal cell suspensions. Normal human skin obtained from plastic surgery was trypsinized (5) and the epidermal cell suspensions in Hanks medium with 15% FCS were used for IF studies.

Tissue sections (4 μm). Snap-frozen biopsies of *normal tissues*: human skin, monkey (cynomolgus) skin, rabbit and mouse lips and tongues were used in the study of KL_3 rectivity pattern.

Pathologic human skin biopsies. Four samples of well-developped, non-treated, statiionary psoriatic plaques, five basal cell carcinomas (BCC), three squamous cell carcinomas (SCC), five plantar and five hand warts were included in the IF study. The excised tissues were immediately divided into two parts: one-snap-frozen in liquid nitrogen for IF examination, another, fixed in Bouin medium, for routine histopathology.

III. *Immunofluorescence (IF) tests*

Screening of culture fluids. Frozen sections (4 μm) of normal human epidermis (foreskin) were fixed in acetone (−20°C), rinsed in PBS and incubated either with the supernatant of the culture fluids or the ascitic fluids for 30 min. After washing in PBS (30 min) sections were incubated with a 1/20 goat anti mouse FITC conjugate (Meloy) for 30 min. After another washing in PBS, the slides were mounted with polyvinyl alcohol medium. Control reactions included frozen sections: 1) treated with the conjugate alone and 2) incubated either with culture medium or another unrelated ascitic fluid without activity.

KL_3 reactivity pattern. The study of KL_3 reactivity pattern on normal and pathologica tissues was performed as described above. Ascitic fluid at 1:50 dilution was used.

Reciprocal blocking immunofluorescence test. A specific inhibition of the KL_3 reactivity by lectins and pemphigus antisera was tested on unfixed frozenskin sections.

Lectins (HPA, PNA, SBA, SJA; see Table I) and pemphigus antisera (titer 1280 at 10^{-1} dilution) were preincubated for 1 h at 37°C on skin sections. After washing, incubation in KL_3 (10^{-2} dilution) was performed for 30 min. Sections were then stained by a goat anti-mouse FITC conjugate as indicated above.

Blocking IF tests were also performed by preincubation with KL_3 followed by FITC lectins or pemphigus antiserum (stained by a goat anti human IgG, FITC, Inst. Pasteur, 1% 70 dilution).

Lectins and FITC lectins were kindly provided by CIRD-Sophia Antipolis, France.

IV. *Immunoelectron microscopy*

Indirect immunoperoxidase(IPO) reactions using KL_3 were performed on frozen skin sections and on epidermal cell suspensions:

12 μm-thick sections of snap-frozen normal human skin biopsy wre fixed with 3% paraformaldehyde (in PBS) for 15 min, washed 15 min in PBS and incubated with KL_3 (1:20) at 37°C for 45 min. The murine antibody fixed on the skin sections was recognized by rabbit anti-mouse IgM (mu chain specific) Zymed peroxidase conjugate (1:50) during 45 min incubation at 37°C and the immune complexes were post-fixed with 2% glutaraldehyde in cacodylate buffer for 20 min. The peroxidase activity was then revealed by DAB. After washing n Tris-HCl buffer (pH 7.6) for 15 min, the sections were post-fixed again, with 1% osmium tetroxide for 10 min, washed and dehydrated in ethanol and embedded in epoxy medium. Some of ultrathin sections were counterstained with lead citrate and uranyl acetate.

The epidermal cell suspensions were kept at +4°C, in a Hanks medium enriched with 15% FCS for 4 h, then fixed with 3% paraformaldehyde at +4°C for 2 h and washed overnight in PBS at +4°C. IPO staining with KL_3 as well as post-fixation and dehydratation inclusion techniques were the same as for skin section samples.

A heat-inactivated normal mouse serum was used for the control experiments.

V. *Immunological reactivity of KL_3 with epidermal keratins and NP40 soluble proteins from epidermis*

Adsorption of KL_3 on epidermal keratins. Epidermal keratins were prepared as previously described and precipitated with TCA at 4°C for 1 h. The resulting pellet was washed with methanol ether (50 : 50) mixture, then in PBS. 1 ml of KL_3 previously diluted with PBS was added to 10 mg of epidermal keratins and incubated at 37°C for 1 h, then overnight at 4°C. After centrifugation the supernatant obtained was used as absorbed MCA and tested in immunofluorescence.

For control, a pemphigus antiserum at 1 : 50 dilution was adsorbed on epidermal keratins and used in parallel in IF studies.

Adsorption of KL_3 on NP_{40} soluble proteins. After dermo-epidermal separation by heat treatment (6) the NP_{40} soluble proteins of epidermis were extracted according to the technique described by Brisk & Snider (7). The extract protein content was determined by the Bradford method (8). 5 mg of protein were precipited with TCA, then washed three times in PBS. Immunoadsorption was performed as described above.

Immunoblot techniques. (*a*) Epidermal keratin polypeptides separated by gel electrophoresis were transferred to nitrocellulose sheets according to the technique described by Towbin et al. (9). The electrophoretic blots were soaked in 5% BSA in saline (0.9% NaCl, 10 mM Tris-HCl pH 7.4) for 1 h at 40°C and 24 h at 4°C. They were incubated either with KL_1 MCA or KL_3 MCA diluted in 3% BSA in saline. The sheets were washed with 6 changes of buffer and incubated for 2 h at room temperature with anti-mouse IgG or IgM peroxidase-conjugated rabbit F(ab')2 (Zymed) (10). The immune reaction was revealed with DAB.

(*b*) NP_{40} soluble proteins: After separation of the NP_{40} soluble proteins on 5–15% gradient slab gel electrophoresis, immunoblotting procedure was performed as previously described.

RESULTS

From several colonies showing an IF staining on skin sections, one gave rise to a stable monoclonal line (KL_3) which was found to produce antibodies labeling epithelial intercellular spaces. By immunodiffusion analysis KL_3 was found to be an IgM.

Immunofluorescence analysis

On frozen sections of normal human epidermis KL_3 labelled the intercellular spaces from the stratum germinatum to the stratum granulosum with a fluorescence intensity increasing from the basal to the upper layers. Basal cells were not stained on the side facing the basement membrane and showed a weak intercellular staining. There was no staining in the stratum corneum (Fig. 1 *a*). Positive labelling of outer root sheath of hair follicles and of excretory sebaceous duct epithelium was observed, while structures corresponding to eccrine and apocrine sweat glands remained negative. No staining of dermis or subcutaneous tissue was found. To determine the species specificity of KL_3, animal tissues were tested: monkey epidermis, rabbit and mice lips and tongues. A slight KL_3 binding was detected with these tissues limited to the malpighian layer. Only monkey epidermis was stained as human epidermis.

Table I. *Lectins used in the reciprocal blocking immunofluorescence tests*

		Sugar specificity
HPA	Helix pomatia AGG	*N*-acetyl—D-galactosamine
PNA	Peanut AGG	D-galactose
SBA	Soybean AGG	D-galactose; *N*-acetyl—D-galactosamine
SJA	Sophora japonica	D-galactose; *N*-acetyl—D-galactosamine

These lectins showed an intercellular staining of the stratum spinosum and the stratum granulosum; no fluorescence was seen in the basal membrane or the basal cell layer and the intercellular fluorescence increased from the lower malpighian layer to the upper stratum granulosum (12).

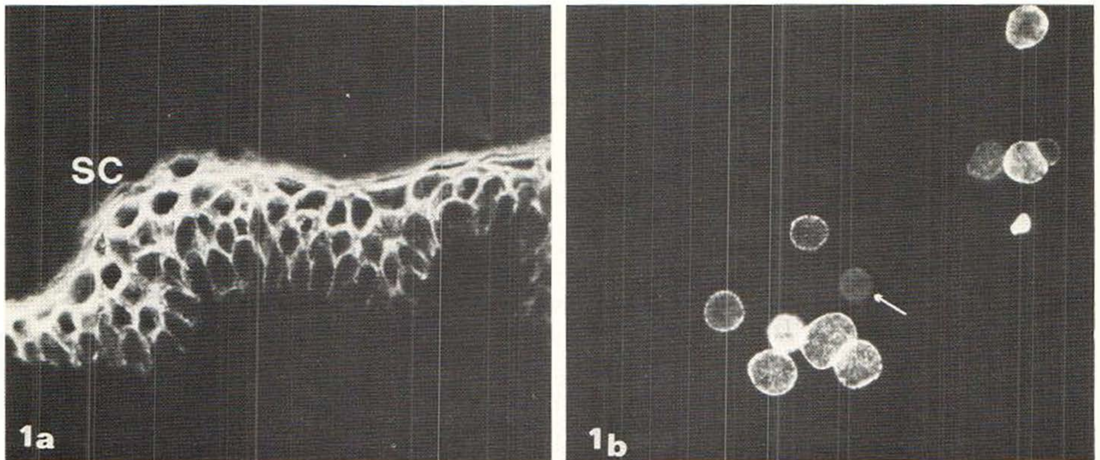


Fig. 1. (*a*) Indirect immunofluorescent staining patterns of normal human epidermis by KL_3 (×500). Note the positive gradient of fluorescence from the basal layer to the stratum granulosum. The stratum corneum (SC) remains negative. (*b*) Epidermal cell suspension labelled by KL_3 (×500). Note the fine patchy fluorescent pattern on the keratinocyte membrane. Arrows denote a negative cell.

Epidermal cell suspensions displayed a fine patchy fluorescent pattern with KL_3 suggesting a non-uniform distribution of the antigen on the keratinocyte membrane. About 90% of the cells were found positive, among them 10 to 15% being only slightly positive (Fig. 1 *b*).

Immunoelectron microscopy

The immunoperoxidase technique applied in electron microscopy allowed us to precise either on skin sections or epidermal cell suspensions, that the positive reaction was localized on keratinocyte membrane, especially in the desmosomal regions (Fig. 2*a*, *b*). In cell suspensions, melanocytes and Langerhans cells recognized after routine counterstaining by their cytoplasmic characteristics, were found negative with KL_3.

Comparison of KL_3 receptor with those for lectins and pemphigus antisera

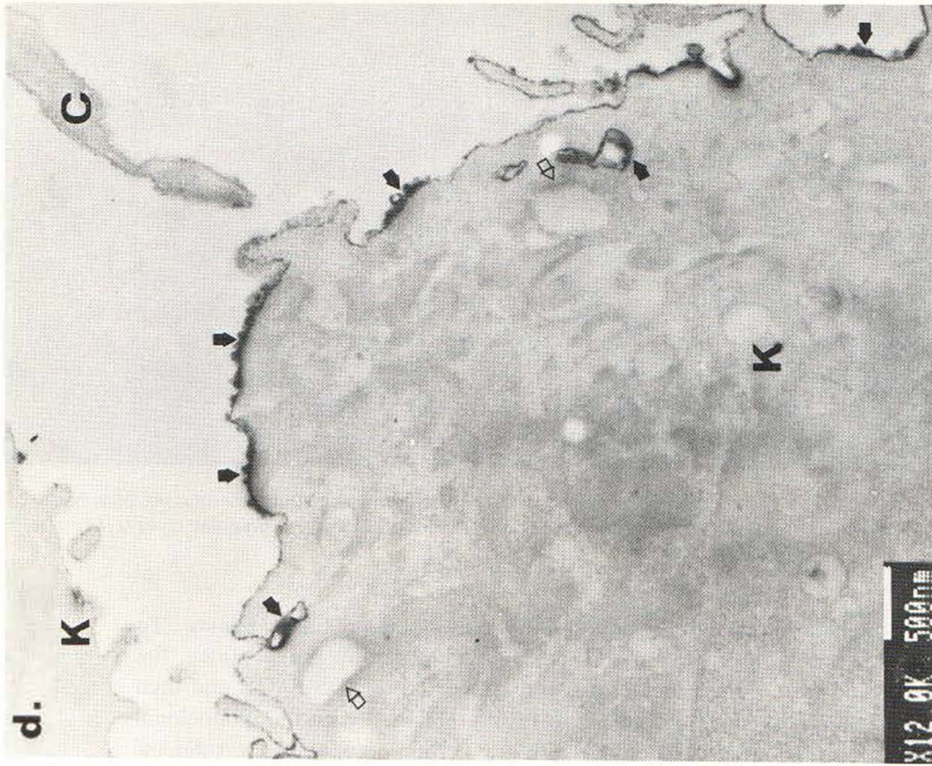
The pretreatment of skin sections with lectins PNA, HPA, SJA, SBA (Table I) and pemphigus antisera did not block the KL_3 reactivity.

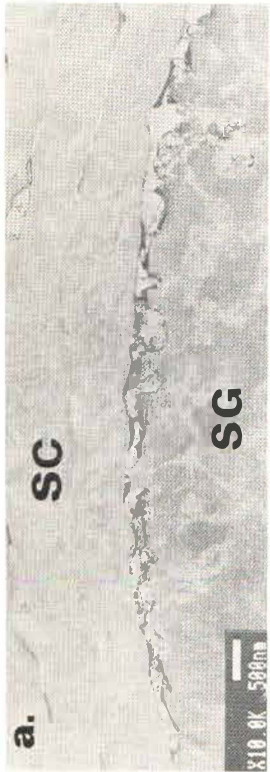
Conversely, the pretreatment of skin sections with KL_3 did not block the reactivity of any FITC lectin.

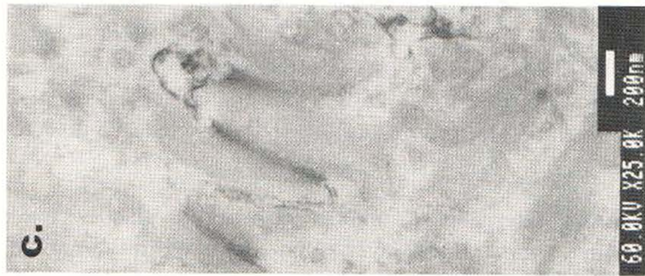
Immunological reactivity of KL_3 with NP_{40} soluble proteins

The adsorption of KL_3 on epidermal keratins (i.e. the antigen used for immunization), completely abolished the reactivity of the MCA on skin sections. Control adsorption of

Fig. 2. Electron microscopic identification of KL_3 MCA binding structures by IPo staining (no counterstaining). (*a*, *b*, *c*) On skin sections: (*a*) SC = stratum corneum; no staining. SG = stratum granulosum; the most superficial epidermal layer still stained. (*b*) SS = stratum spinosum; note keratinocyte membrane staining including desmosomes (▶▶). (*c*) Detail of SS desmosome staining. (*d*) On epidermal cell suspension: C = corneocyte; no staining. K = keratinocyte; cell membrane staining with predominance at desmosome region (→) (note tonofilament bundles in contact with the membrane) some internalized desmosomes are not stained (⇒) (presumably not at contact with the cell surface at time of fixation). On the other keratinocyte only traces of membrane staining.


d.
C
K
K
X12.0K 500nm


a.
SC
SG
X10.0K 500nm


c.
60.0KV X25.0K 200nm

b.
SS

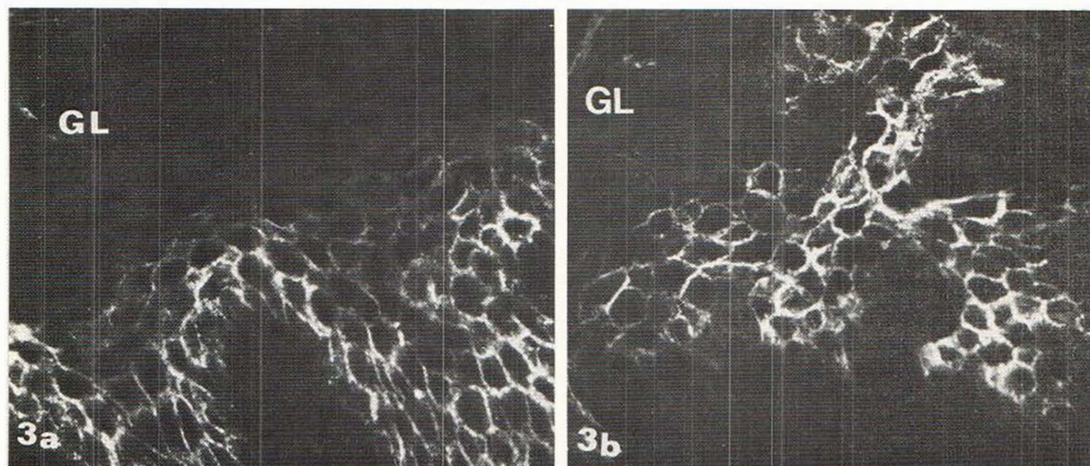


Fig. 3. KL_3 reactivity pattern (*a*) psoriatic epidermis (×500). (*b*) epidermis above tumor masses of a basal cell carcinoma (×500). A discontinuous and irregular staining is observed with a negative granular layer (*GL*).

pemphigus antibody on keratins did not modify the typical intercellular staining. To determine a possible binding specificity of KL_3 to individual keratin polypeptides, the keratin proteins purified from normal epidermis were subjected to SDS-PAGE and then transferred to nitrocellulose sheets. In contrast to results obtained with the monoclonal antikeratin antibody KL_1 (3), no KL_3 reactivity with any keratin polypeptide was detected.

The adsorption of KL_3 on NP_{40} soluble proteins of epidermis did not modify the reactivity pattern of the MCA. SDS-PAGE of NP_{40} soluble proteins showed numerous proteins but keratins were essentially absent. No immunological reactivity of KL_3 with any of these proteins transferred to nitrocellulose sheets could be detected.

KL_3 reactivity on pathological human skin samples

Intensity of epidermal intercellular staining on psoriatic plaque samples was clearly diminished especially in the granular layer when compared to the normal and its degree varied from biopsy to biopsy (Fig. 3*a*). In tumor masses of BCC and SCC, KL_3 gave a negative or faintly positive staining. The epidermis above the tumors showed a discontinuous and irregular staining (Fig. 3*b*). In warts, the reactivity intensity was also reduced and its pattern altered. The loss of reactivity was more evident in plantar warts than in hand warts. In most cases, the granular layer of epidermal warts was negative whereas malpighian layers showed a faint and irregular staining.

DISCUSSION

By immunizing mice with human epidermal keratins we have obtained a MCA of unexpected specificity since KL_3 recognizes an antigen present exclusively on keratinocyte membrane.

The reactivity pattern of KL_3 on normal epidermis showed a positive gradient of fluorescence which may represent an enhanced availability of the antigen due to a gradual modification in the steric configuration of the keratinocyte membrane receptor during the differentiation process.

The antigen recognized by KL_3 was trypsin resistant since epidermal cell suspensions

were labelled by the antibody. The punctate staining pattern of keratinocyte membrane was confirmed by immunoelectronmicroscopy which allowed us to show that the antigen was preferentially associated with desmosomal plaques.

As various lectins show characteristic patterns of staining on epidermis which have been related to the development of an increasingly complex series of glycoconjugates during keratinocyte maturation (11) we studied the blocking effect of lectins showing a selective intercellular labelling of upper layers of epidermis (12) on KL_3 binding. No blocking effect was observed either with these lectins or with pemphigus antisera indicating that they do not recognize the same structure as KL_3. The results were partially confirmed by the fact that, in addition, KL_3 reactivity was not modified after adsorption on NP_{40} soluble antigens.

Though KL_3 reactivity was completely abolished after adsorption of the antibody on the antigen used for immunization (i.e. the human epidermal keratins) no immunological reactivity was detected with any keratin polypeptide band blotted on nitrocellulose paper.

So it is probable that small amounts of cross-linked molecules such as "keratolinin" (15) or a precursor of envelope protein as contaminating impurities present in immunizing antigen may be responsible for the described KL_3 specific reactivity. Such an hypothesis needs further investigations.

Recently, glycokeratins have been described in the epithelial cell line ME 180 (potential extracellular components of the intracellular cytoskeleton) (13) and a MCA (HK_1) against hair keratin unexpectedly stained cell membranes of human and mouse epidermal keratinocytes, predominantly in desmosomal areas as shown by electron microscopy (15). Our results showed that KL_3 recognized a highly insoluble keratinocyte membrane antigen preferentially concentrated at desmosome regions but not desmosome specific. The lack of KL_3 reactivity in immunoblotting might be explained by the fact that some monoclonals did not react with denature antigens.

Epidermal cell membranes undergo changes of their surface during differentiation, proliferation and malignant transformation (16). Similarly, changes of KL_3 staining patterns were observed on various lesions in which disorders of keratinization and cell differentiation are known to occur.

The dermal masses of BCC and SCC tumors showed the most striking alterations since no KL_3 staining was observed. More interestingly the alterations of keratinocyte antigenicity of morphologically normal epidermis, just above the tumor masses were observed.

In psoriasis, no important abnormality was reported in keratinocyte membrane antigens using β_2 microglobulin, Con A or pemphigus antisera (17) whereas modifications of other lectin binding sites have been described (17). The decreased KL_3 reactivity confirmed dysfunction of surface membrane receptors in this disease.

In warts, the disturbance of KL_3 reactivity might be correlated with the intensity of the viral cytopathic effect and the human papilloma virus type.

In conclusion, KL_3 antibody defines a keratinocyte membrane antigen, insoluble in non-ionic detergent, trypsin resistant, greatly modified in lesions with disorders of keratinization and cell differentiation. Though the biochemical nature of this antigen remains to be clarified, it is potentially implicated in the epidermal differentiation process and thus KL_3 can be considered as a useful and sensitive probe to explore the complex phenomenon of cell adhesion and its relevance in epidermal cell pathology.

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