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Elemental Analysis Mirrors Epidermal Differentiation

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Using a scanning nuclear microprobe, the distribution of elements and trace elements of skin cross sections of normal skin, non-lesional psoriatic skin and in dry atopic skin have been mapped. In non-lesional psoriatic skin and in dry atopic skin the epidermal Ca-gradient is higher than that of normal skin. In addition, abnormally high Fe and Zn levels were recorded in the stratum granulosum and corneum regions in the pathological skin. It is suggested that these findings correlate to an increased cell turnover in the basal cell layer of the psoriatic and atopic skins. The ratio of Ca/Zn in stratum corneum of paralesional psoriatic skin is approximately 8 : 1 compared to 12 : 1 in normal skin and 15 : 1 in atopic skin. This suggests that the differentiation process in paralesional psoriatic skin may actually be an example of disturbed programmed cell death. ***Key words: apoptosis; calcium; trace elements; human skin; particle probe analysis; PIXE.***

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Trace elements such as calcium (Ca), zinc (Zn) and iron (Fe) have been shown to be involved in the regulation of cell turnover, cell metabolism and in the case of Ca and Zn in the final extinction process often expressed in the process of apoptosis (1, 5). We have assessed the levels of these trace elements in normal and pathological skin in order to contribute to the elucidation of their physiological roles in the chosen conditions, dry atopic skin and clinically normal skin from psoriatics compared with skin from non-afflicted individuals. In this process we have tentatively interpreted our data in light of the present-day knowledge of apoptosis, which shares a number of characteristics with the normal cornification process that is the final part of epidermal differentiation.

The epidermis represents a special kind of tissue in that the transformation of the keratinocytes into the corneocytes of the stratum corneum represents an advanced metamorphosis of the basal cell progeny. During the process of differentiation the keratinocytes not only produce the intracellular fibrous protein keratin, synthesize proteins that will form the corneocyte envelope, they also produce enzymes active at different differentiation stages and some possibly responsible for the shedding of the stratum disjunctum cells (cf. 10). In addition, a number of lipids that will constitute the chemical barrier will be produced during the differentiation process. As the transformed cells of the stratum granulosum move into the corneocyte compartment, i.e. the stratum corneum, there is a complete dissolution of the nuclear material that has already been compacted in the stratum granulosum layer. Obviously, such a process shares a number of characteristics with the apoptosis process, as evidenced in other cellular systems (cf. 1, 8, 12, 16, 18).

Studies focusing on epidermal apoptosis have centred mainly around immunological and biochemical markers rather than ion markers. It is therefore interesting to note that in the development of epidermal tissue cultures a Ca^{2+} content of the culture medium exceeding 0.1 mM Ca^{2+} was necessary for the production of a complete epidermis with a stratum corneum (17). A lower Ca^{2+} content results in an incomplete cornification of the stratum corneum cells. In the wake of our studies of the elemental distributions over skin cross sections using particle probes, X-ray microanalysis (XRMA) in the scanning transmission microscope, and proton-induced X-ray emission (PIXE) analysis, the study of pathological skin has revealed interesting data on trace element levels, e.g., Ca, Fe and Zn, in normal and pathologically changed epidermal tissues. Here we summarize some recent findings that may have a direct bearing on the programmed cell death in the human epidermis.

METHODS AND MATERIAL

Skin biopsies were obtained from five patients (29–50 years) with a clinically verified diagnosis of atopic dermatitis according to the criteria of Hanifin-Rajka (9). All patients had dry, non-eczematous skin on the back where biopsies were taken under local anaesthesia (lidocain/adrenalin) with a 4 mm punch. This skin had not been topically treated with any ointment 1 week before biopsy. Normal skin biopsies for control were taken from a corresponding area of eight healthy volunteers in matching age range with no records of skin disorders or family history of atopy. In a corresponding manner, skin biopsies were immediately obtained from the lower back in six psoriatics at least 3 cm from lesions or lesional rests.

The biopsies were immediately quench-frozen in liquid nitrogen and stored in a deep freezer at –20°C. Sectioning was performed at –20°C in a cryostat (AMES, LAB-TEK) with a nominal section thickness of 16 μm. The sections were transferred to specimen support rings covered with Kimfol® foil, which gives virtually no contribution to the particle induced X-ray spectrum. The complete specimen support sandwich was stored in sealed vessels with drying material to prevent water uptake until analysis. The particle probe technique, including sampling of specimens and processing for analysis, has been presented in detail elsewere (7, 15).

Informed consent was obtained from all probands. Permission to conduct the investigation was given by the Ethics Committee of the Karolinska Institute and the University of Uppsala, respectively.

Statistics

A multivariate statistical method was applied to analyse the covariation of trace elements with the three main strata of the epidermis as related to distributions in normal skin, atopic dry skin and paralesional psoriatic skin. The method makes it possible to find the number of true factors that control elemental composition in the samples and to find the composition of each factor expressed in the detected elements, e.g., which elements and trace element co-variates with a particular stratum in the normal skin and if this co-variation is also true for a diseased

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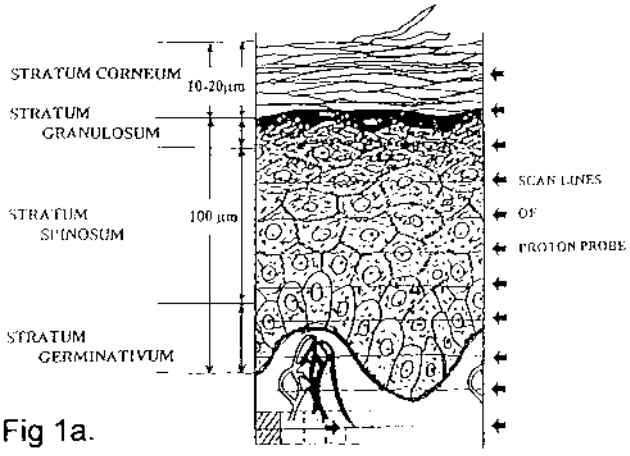

Fig 1a.

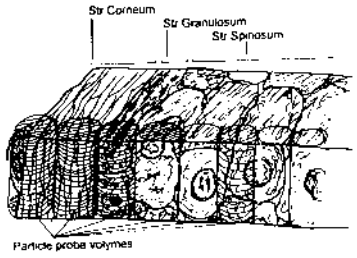

Fig 1b.

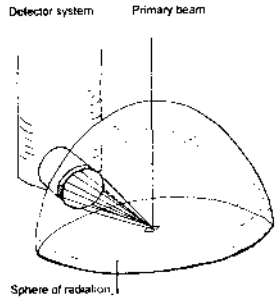

Fig1c.

Fig. 1. (*a*) Schematic representation of how the nuclear scanning probe retrieves information from a selected area of the epidermis. (*b*) Schematic representation of how the resolution of the nuclear scanning probe is influenced by probe size and overlapping cells in the tissue. (*c*) Schematic representation of the effective angle of the detector at PIXE analysis.

skin. The actual analysis is done in eight dimensions, which eventually are projected down to two or three dimensions to be comprehensible (13).

RESULTS

General comments on the effect of the spatial resolution of the present Lund nuclear microprobe

The mass distribution profile peaks in the stratum corneum. Because of the comparatively large probe size compared to the width of the stratum corneum, the mass peak, as well as the elemental and trace elemental distribution curves, will show tapering wings (Figs. 1 and 2). The comparatively low spatial resolution of PIXE, achieved with a 5 µm diameter proton probe (Fig. 1b), will therefore not allow detection of gradients *within* the stratum corneum. The low spatial resolution also has bearings on the elemental data distributions, e.g. the probe area will overlap into the topmost stratum granulosum or the space outside the stratum corneum (Fig. 1b). Our previous experiences with the electron microprobe in the scanning transmission electron microscope (XRMA: probe size <0.5 µm) and data from the work of Warner (6, 19, 20) suggest that the present values of phosphorus (P), chlorine (Cl), potassium (K) and calcium (Ca) may therefore be too high.

Characteristics of normal skin

The Ca gradient has a bimodal form with a relatively high level in the basal region and a high level in the stratum granulosum/corneum junction, subsequently dropping conspicuously to very low levels. Sulphur (S) follows the mass in principle, whereas P, Cl and K decline from high values in the basal region to low values in the stratum corneum region. Iron (Fe) has its peak value in the basal region and declines towards the stratum corneum and zinc (Zn) has its peak value in the stratum spinosum layer (Table I).

Characteristics of paralesional psoriatic skin

The mass distribution in paralesional psoriatic skin rises from low values in the basal layer to very high levels in the stratum corneum (Table I). The latter values are even up to 10-fold as high as those of normal skin. S has a bimodal distribution and is about 3-fold as high in the basal layer compared to normal skin. The peak value of S is found in the stratum corneum. Although higher than in normal skin, Cl and K distribute as in normal skin, whereas P actually has a peak value in the stratum granulosum/corneum. Fe is almost 4-fold as high in paralesional psoriatic skin and about three times higher in stratum corneum compared with normal skin. Zn has a peak in stratum corneum and is 4–5 times higher than in normal skin.

Characteristics of dry atopic skin

All elements are higher than normal skin (Table I). The most conspicuous feature is the increasing Zn, which is even higher than in the paralesional psoriatic skin. It is notable that the Zn is high in the stratum corneum in contrast to normal skin.

DISCUSSION

The differentiation process of the normal human epidermis has the corneocyte as end product. This is an extremely flattened, polygonal cell with a diameter to height ratio around 100:1. Corneocytes characteristically are devoid of a normal cytosol, contain no cell organelles and, in addition, normally lack even fragments from a nucleus. The lipid membrane of the keratinocytes of the viable epidermis is exchanged for a protein envelope in the corneocyte, rendering the cell highly resistant to detergents and enzymes. The overlapping corneocytes are firmly joined by special desmosomes providing a three-dimensional scaffold that mechanically protects the intercellular

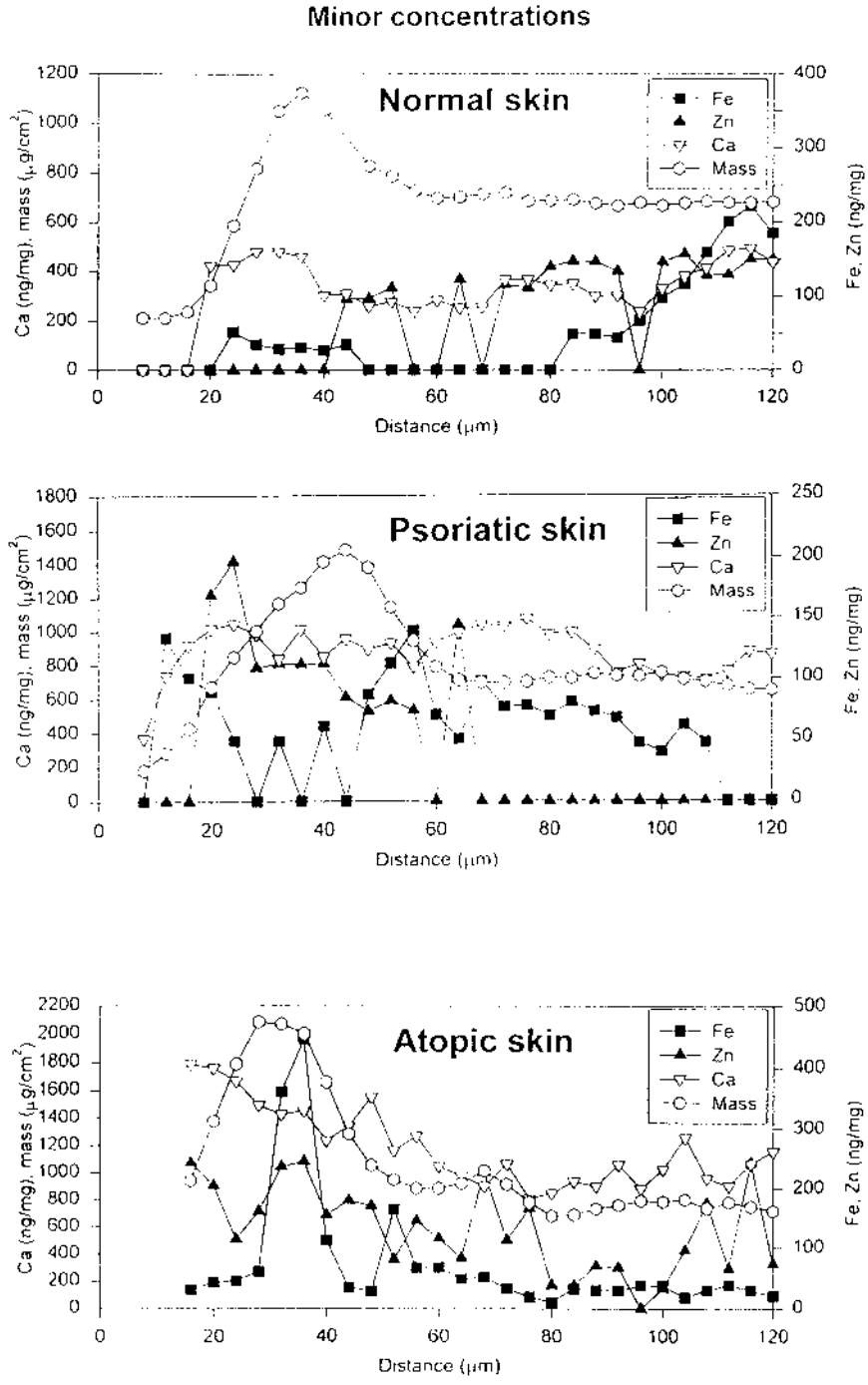

Fig. 2. Examples of cross-section distribution of trace elements Ca, Fe and Zn as related to mass distribution in normal skin (*top panel*), in paralesional psoriatic skin (*middle panel*), and in atopic skin (*lower panel*). Note that the values on the vertical axes are different for the three types of skin.

stacks of lipid bilayers that constitute the chemical barrier (3). These features result in a rigid structure that, due to the internal "fiber reinforcement" of keratin fibrils, prevents the corneocyte from swelling in the horizontal dimension and thus the integrity of the skin surface is preserved when the body is immersed in water (14). It is also interesting to note that the transition from the granular cells that carry highly condensed nuclei and a lipid envelope to the corneocyte is a very rapid process, and there are no reports of transition cells sharing the characteristics of both strata at either light microscopic or electron microscopic resolution.

Taking the above facts within the context of programmed cell death, or apoptosis, a number of conspicuous facts emerge. Apoptotic bodies formed from fragments of a cell undergoing apoptosis have been shown to hold highly cross-linked protein envelopes that are resistant to detergents or chaotropic agents (4). The insolubility is due to formation of ε(γ-glutamyl)lysine isopeptide bonds. These bonds are the characteristic products

Table I. *Elemental and trace elemental distributions over skin cross sections (ng/mg). Data given in mean ± SD*

Element	Normal skin			Paralesional psoriatic skin			Atopic skin		
	Basal	Spinous	Corneum	Basal	Spinous	Corneum	Basal	Spinous	Corneum
P	4882 ± 3207	7651 ± 3617	5362 ± 3460	5647 ± 4700	9623 ± 6478	7376 ± 4315	10820 ± 3635	14170 ± 5434	11600 ± 8597
S	7296 ± 1609	8073 ± 1282	7512 ± 1344	9573 ± 3275	10400 ± 3146	11400 ± 3263	11020 ± 22524	12530 ± 4553	14110 ± 5773
Cl	10930 ± 2671	7725 ± 1889	6313 ± 1425	20120 ± 10090	20160 ± 11410	13280 ± 6856	15010 ± 4961	13660 ± 6412	12350 ± 7258
K	8791 ± 2236	9276 ± 2726	8147 ± 2289	12580 ± 5227	13370 ± 5055	10060 ± 4942	12970 ± 5719	14060 ± 5125	9346 ± 5328
Ca	320.3 ± 150	231.7 ± 158.4	350.1 ± 199.4	688.9 ± 397.5	631.9 ± 336.7	826.7 ± 270.3	3523 ± 1622	3645 ± 1873	3118 ± 1281
Fe	105.5 ± 84.93	36 ± 46.82	16.6 ± 26.27	141.2 ± 86.92	95.26 ± 84.77	36.42 ± 45.36	169.8 ± 105.6	151.6 ± 110.2	81.76 ± 41.89
Zn	23.12 ± 46.76	46.67 ± 65.82	27.74 ± 46.77	20.68 ± 65.45	53.4 ± 90.73	106.2 ± 74.11	168.3 ± 137.7	165.7 ± 146.3	220.4 ± 122.1

of Ca^{2+} dependent transglutaminases. The apoptotic process is also very fast, cells having been reported to disintegrate and disappear completely within 4 h (2). Ca^{2+} plays a crucial role in many reports on apoptosis (5) and increased intracellular levels of Ca^{2+} may elicit series of secondary intracellular events such as activation of endonuclease, transglutaminase as well as morphological changes.

McCall & Cohen (11) have shown that endonuclease(s) responsible for the DNA fragmentation of the granular cells act in patterns that are seen in other cases of programmed cell death; 5mM Ca^{2+} in TMS suspension of either isolated granular or basal cells elicited fragmentation of the DNA, demonstrating that even basal cells contained endonucleases and that the keratinocytes of epidermis are destined to programmed cell death although under normal conditions the basal cells are protected from enzyme activation.

A unique property of PIXE analysis is that primary data are simultaneously collected, i.e. *all* elements within a particular pixel are simultaneously recorded. This makes it possible to study changes in ratios of elements of physiological significance, e.g., Ca/Zn, and increases the significance of *the interpretation of elemental data in a physiological context*.

An inherent property related to the fact that the metabolic changes in a particular cell fluctuate with time is the fact that quantitative analysis of elements in a population of cells will generally show conspicuous variability in the absolute amounts of elements. In addition, the smaller the volume of analysis, the greater the variation expected in the obtained data, and this will be reflected in the standard variation in small samples. The epidermis, which is a dynamic, differentiating tissue will consequently yield data of great variability even when the analysis refers just to a single stratum. Standard deviations will therefore be large in small populations and the interpretation of data uncertain. The multivariate statistical analysis that we have performed in this investigation highlights the feasibility of this analysis technique and allows a biological interpretation not provided by the one-dimensional statistical analysis.

The variability in elemental distributions is well demonstrated by the cluster plot (Fig. 3a), where the mean values for each stratum in each specimen are plotted in a cluster diagram for the stratum corneum. Two of the psoriasis cases (P1 and P6) appear in the region where the normal skin resides. Hence it appears that there is a great variability in the expression of the disease even in the normal skin.

The corresponding plot for stratum spinosum (Fig. 3b) reveals that a number of psoriatics share the properties of normal skin. In contrast, a considerable number of atopics appear well outside the normal population, a fact which may indicate the immaturity of the atopic spinous layer.

The basal region shows great overlaps of psoriatics over normals. The atopics are sequestered outside these populations, although with a small overlap. This may be an expression of an increased cellular activity in the germinative pool to compensate for the functional impairment of the barrier (16). An increased cell turnover may also explain the high levels of Fe in the dry atopic skin compared to normal skin.

When looking closer at our nuclear microprobe results we find that the ratio of Ca/Zn in stratum granulosum/corneum of paralesional psoriatic skin is approximately 8 : 1. This should be compared to a Ca/Zn ratio of 12 : 1 in normal skin and 15 : 1 in atopic skin, i.e. in the latter cases a clear dominance of Ca in the stratum granulosum/corneum region. It

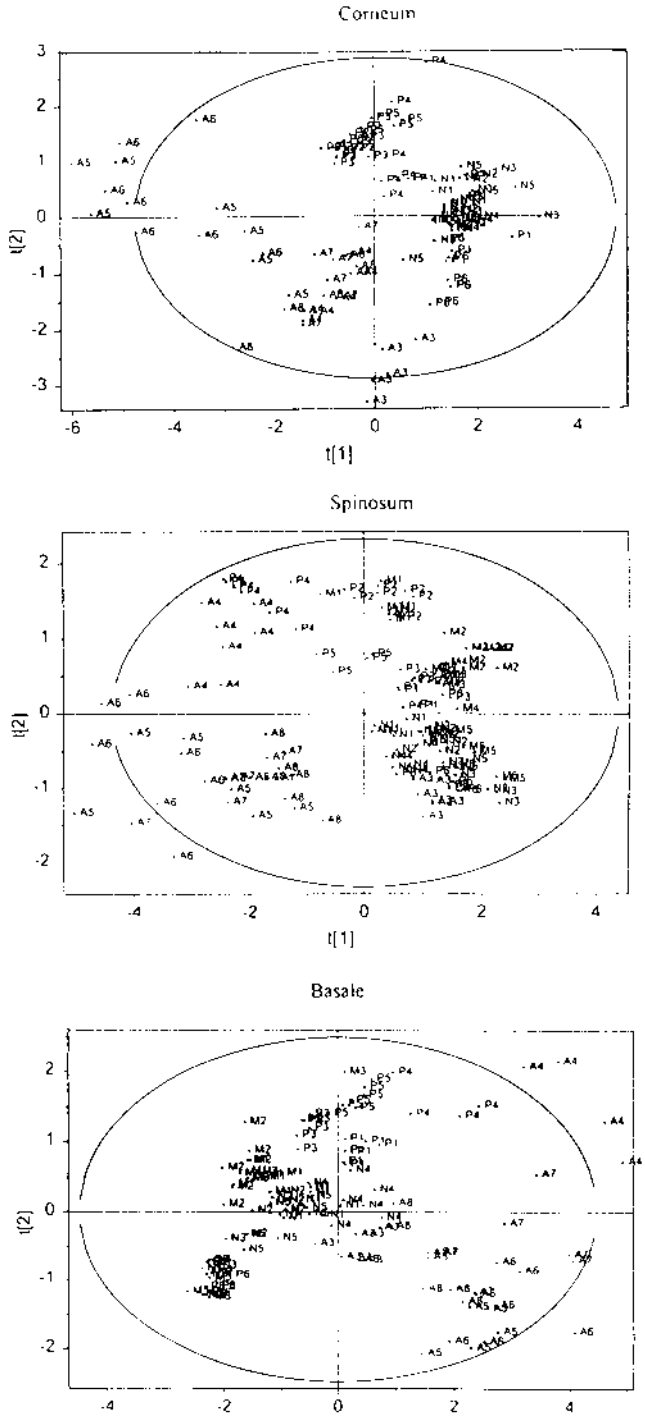

Fig. 3. Diagrams of cluster analysis of data from stratum corneum (*top panel*), stratum spinosum (*middle panel*) and stratum basale (*bottom panel*) obtained by multivariate statistical analysis. The process of principal component modelling corresponds geometrically to fitting a plane to the data (represented as points in a multidimensional space) with the variables (e.g. the strata and the skin type, respectively) as axes. The t_1, t_2, plots show how the samples are distributed on this projection plane. Thus this type of fitting procedure helps find the maximized correlations between a particular stratum and a skin type. N: normal skin; A: dry atopic skin; P: paralesional psoriatic skin; numbers refers to individuals.

can be speculated that the comparatively high Zn inhibits the final differentiation (apoptotic) process in the paralesional psoriatic skin. Thus this may actually be an example of disturbed programmed cell death.

The Ca gradients over normal skin cross sections that we have previously reported (cf. 6, 7) are in harmony with these findings. However, we still lack an understanding of the processes that provide the increased Ca^{2+} gradient in paralesional psoriatic skin and in dry atopic skin, since the particle probe analysis provides us with *total* Ca, ignoring the fact that some of the Ca may be bound to intracellular stores (bound to proteins and lipids) and part exists in ionized form. Thus, there appears to be an influx of Ca^{2+} ions into the topmost strata of the epidermis, but the creation of this gradient remains to be elucidated.

CONCLUSION

In conclusion, we may state that particle probe analysis in combination with multivariate statistical analysis can provide new insights into the physiology of the epidermal differentiation process. The simultaneous recording of elements within a given specimen volume makes it possible to apply a multivariate statistical method for the analysis of covariation of a number of biologically interesting variables such as trace elements versus epidermal strata, and skin type. Further, PIXE makes it possible to assess ratios of physiologically important elements, in our particular case, Ca and Zn. Such ratios are expected to mirror the pathophysiology of skin disorder, e.g., the conflicting effects of Ca^{2+} and Zn^{2+} to the final stages of epidermal differentiation.

The details of distributions within the stratum granulosum and stratum corneum respectively remain to be scrutinized when a higher resolution nuclear probe becomes available. We therefore suggest that future studies with biochemical, morphological and immunological methods should, when possible, be combined with high resolution particle probe analysis to reveal the important physiological aspects of the process of programmed cell death in normal and pathological epidermis.

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REFERENCES

1. Barr PJ, Tomei LD. Apoptosis and its role in human disease. Biotechnology 1994; 12: 487–493.
2. Bursch W, Paffe S, Putz B, Barthel G, Schulte-Hermann R. Determination of the length of the histological stages of apoptosis in normal liver and altered hepatic foci of rats. Carcinogenesis 1990; 11: 847–853.
3. Fartasch M, Bassuskas ID, Diepgen TL. Structural relationship between epidermal lipid lamellae, lamellar bodies and desmosomes in humans epidermis: an ultrastructural study. Br J Dermatol 1993; 128: 1–9.

4. Fesus L, Thomazy V, Autuori F, Ceru MP, Tarcsa E, Piacentini M. Apoptotic hepatozytes become insoluble in detergents and chaotropic agents as a result of transglutaminase action. FEBS Lett 1989; 245: 150–154.
5. Fesus L, Davies PJA, Piacentini M. Apoptosis: molecular mechanisms in programmed cell death. J Cell Biol 1991; 56: 170–177.
6. Forslind B, Roomans GM, Carlsson L-E, Malmqvist KG, Akselsson KR. Elemental analysis on freeze-dried sections of human skin: studies by electron microprobe and particle induced X-ray emission analysis. Scanning Electron Microsc 1984; 2: 755–759.
7. Forslind B. X-ray microanalysis of the integument. In: Ingram P, Shelburne JD, Roggli VL. Microprobe analysis in medicine. New York, London: Hemisphere Publishing Corp. 1989: 207–218.
8. Haake AR, Polakowska RR. Cell death by apoptosis in epidermal biology. J Invest Dermatol 1993; 101: 107–112.
9. Hanifin JM, Rajka G. Diagnostic features of atopic dermatitis. Acta Derm Venereol (Stockh) Suppl 1980; 92: 44–47.
10. Lundström A, Egelrud T. Cell shedding from human plantar skin in vitro: evidence that two different types of protein structures are degraded by a chymotrypsin-like enzyme. J Invest Dermatol 1990; 282: 234–237.
11. McCall CA, Cohen JJ. Programmed cell death in terminally differentiating keratinocytes: role of endogenous endonuclease. J Invest Dermatol 1991; 97: 111–114.
12. Miracco C, Spina D, Santopietro R, Sforza V, Leoncini L, Pacenti L, et al. Apoptotic index: discriminant feature for the differentiation of cutaneous diffuse malignant follicular center cells lymphomas from lymphoid hyperplasia. J Invest Dermatol 1993; 100: 699–704.
13. Morrison DF. Multivariate statistical methods. Singapore: McGraw-Hill, 1998.
14. Norlén L, Emilson A, Forslind B. Stratum corneum swelling. Biophysical and computer assisted quantitative assessments. Arch Dermatol Res 1997; 289: 506–513.
15. Pallon J, Knox J, Forslind B, Werner-Linde Y, Pinheiro T. Applications in medicine using the new Lund microprobe. Nucl Instr Meth Phys Res 1992; B 77: 287–293.
16. Polakowska RR, Piacentini M, Barlett R, Goldsmith LA, Haake AR. Apoptosis in human skin development: morphogenesis, perderm, and stem cells. Dev Dyn 1994; 199: 176–188.
17. Ponec M, Kempenaar J. Calcium induced modulation of lipid synthesis in cultured human epidermal keratinocytes. Invest Dermatol 1985; 84: 452.
18. Sayama K, Yonehara S, Watanabe Y, Miki Y. Expression of FAS antigen on keratinocytes in vivo and induction of apoptosis in cultured keratinocytes. J Invest Dermatol 1994; 103: 330–334.
19. Warner RR, Myers MC, Taylor DA. Electron probe analysis of human skin. Element concentration profiles. Invest Dermatol 1988; 90: 78–85.
20. Warner RR, Bush RD, Ruebusch NA. Corneocytes undergo systematic changes in element concentrations across the human inner stratum corneum. J Invest Dermatol 1995; 104: 530–536.