Neuroimmunological Activities of Keratinocytes

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Abstract
The ability of keratinocytes to produce catecholamines indicates that the skin reacts to different types of environmental stress by secreting hormones that regulate vascular reactivity locally and blood flow through the microvascular circulation.

Several recent investigations have shown that epithelial cells actively take part in the control of homeostasis of the skin organism.This concept is essentially based on three observations:

- Keratinocytes are able to synthesize cytokines and, thus, interfere with immunological processes.[1]
- Keratinocytes express receptors for catecholamines (CA), which are any of a group of amines derived from catechol that have important physiological effects as neurotransmitters and hormones and include epinephrine, norephinephrine and dopamine.[2]
- Keratinocytes locally synthesize CA hormones.[3]

These hormones, as well as the cytokines, contribute to the interconnection of the three systems that control homeostasis: the nervous system, the immune system and the endocrine system.

This article reviews the published literature describing the secretion and use of CAs by keratinocytes and the involvement of these cells in the immune response. In addition, this article reports our own results on a study analyzing the capability of keratinocytes to produce CAs.We also report our findings on the biological mechanism involved in the modulation of the interaction between keratinocytes and the neuroendocrine system.

Keratinocytes and Catecholamines: A Literature Review

Keratinocytes as controllers of stress hormones: When keratinocytes are exposed to various pathogens, they are stimulated to several acts:

- They secrete pro-inflammatory cytokines.
- They express adhesion molecules on their surfaces that increase and/or regulate the blood supply to the inflamed skin.[4]
- They establish interactions with other cells present in the skin (mast-cells, lymphocytes T, γ, δ, etc.) which participate more or less directly in the inflammatory process.

On the grounds of these observations, Barker et al.[5] formulated the hypothesis that keratinocytes are involved in inflammation.

It has also been observed that the mitotic activity of keratinocytes is influenced by the serum concentration of epinephrine[6] and that the addition of adrenergic agonists to keratinocyte cultures increases the cyclic adenylic acid (cAMP) concentration in the cytoplasm.[7]

Many reports have focused on the effects of treating keratinocytes with adrenergic agonists in vitro and in vivo and have led to discordant conclusions regarding the activity of these compounds.

Results have shown that modulation of cytoplasmic concentrations of cAMP induces opposite effects that likely depend on the parameters studied.[8,9]

These observations raise questions regarding the pathways and mechanisms by which β-adrenergic stimulation influences keratinocyte physiology. Fairly convincing evidence obtained from studies on human and animal keratinocytes shows that these cells display specific receptors for compounds with β-adrenergic activity.[10] Gazith et al. (1983) clearly proved that the density of β-adrenergic receptors expressed by human keratinocytes is higher than that of other cell compartments normally controlled by CAs.[11]

It has also been shown that in response to the specific binding of an agonist these receptors induce an enzy-

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matic cascade that leads to the synthesis of cAMP.[8]

More recently, adrenergic receptors have also been detected on Langerhans cells. It is well known that these cells play an important role in antigen presentation to the immune system and that they are connected to epidermic nerves.[12]

The observation that they display adrenergic receptors provides additional proof of the interconnection between the immune system and the nervous system and also suggests that stress hormones can modulate the build-up of an immune response in the skin and vice versa.

Keratinocytes as synthesizers of CAs: Another putative role for keratinocytes in the control of stress hormone activity derives from the observation that they are able to synthesize CAs. Like hepatocytes and neuronal cells, keratinocytes display a sophisticated array of enzymes that allows them to synthesize CAs from phenylalanine and L-tyrosine.[13]

It has also been shown that the biosynthesis of CAs and the expression of their receptors on the keratinocyte membrane are closely correlated in less differentiated cells. Moreover, the adrenergic stimulation of keratinocytes by epinephrine determines an increase in cytoplasmic Ca2+ and cAMP which is followed by the induction of the gene encoding the enzyme tyrosine-hydroxylase and the increase of the corresponding messenger RNA.[14] Thus, poorly differentiated keratinocytes produce catecholamines which control the calcium homeostasis and the transformation of tyrosine into melanin (in melanocytes) or into catecholamine (in keratinocytes) either by autocrine and/or paracrine actions. This synthesis is inversely proportional to the differentiation and occurs at the end of the maturation pathway.[13-15]

The relationships between the ability of keratinocytes to synthesize CAs and their immunological activities are yet to be determined.

To our knowledge, no studies of these two aspects of stress hormone biology and their correlations currently exist. However, a great deal of data suggest, though indirectly, that cytokines and CAs can indeed interact. It has been shown that:

- Interferon α is able to modulate both CA synthesis and the expression of the corresponding receptors on circulating lymphocytes in vitro and in vivo.

- Interleukins IL1, IL6, IL2 can modulate the synthesis of various stress hormones by interacting with specific receptors in the pituitary gland and/or in the diencephalon.[16]
- Interferons can directly interact with β-adrenergic receptors.[17-19]

These data suggest the following hypothesis: if different compounds that act on the same cellular target are produced in a given microenvironment by the same type of cells, then their activity will be influenced by the corresponding concentrations and affinities to the receptors.

This becomes especially relevant when analyzing the skin response to damage from various causes, such as ultraviolet light (UV), one of the most harmful factors affecting the physiological balance of keratinocytes and causing cutaneous neoplasia.[20]

The cytotoxic activity of UV light is tightly linked to its ability to induce keratinocytes to synthesize immunosuppressive cytokines and other suppressive factors.[21] Similarly, various inflammatory or allergic stimuli determine the synthesis and the secretion of cytokines in the epidermis.

Through the interaction with CA receptors these cytokines induce the expression of the genes that regulate CA synthesis.

Reactive oxygen toxic species (ROTS) that accumulate in the skin as a consequence of the same processes that induce cytokine synthesis[22] might also contribute to this hypothetical process. The presence of molecular oxygen indeed promotes the transformation of L-tyrosine into melanin and/or CAs.[13] Therefore, the pathway can be delineated as follows:

1. An irritating stimulus that strikes the skin either directly or indirectly activates the adrenergic receptors of keratinocytes and simultaneously induces oxydative radicals.
2. Induction of the genes encoding the enzymes that regulate the production of CAs whose activity is enhanced by the presence of molecular oxygen.
3. Cytokines must be present and they must be able to act as modulating factors in the same microenvironment.

The link to the nervous, immune and endocrine systems: The aim of our

studies during the last three years has been to verify these hypotheses. To date, we have confirmed that keratinocytes actively take part in various interactions among the major systems that control the physiological homeostasis. These systems are the immune system, the endocrine system and the nervous system.

In particular, it has been possible to show that keratinocytes are able to secrete cytokines, to express the corresponding receptors and/or to attract and entrap activated immunocompetent cells within the same microenvironment. Therefore, we designed an experimental approach for evaluating the possibility that even CAs participate in the neuroimmunoendocrine interactions within the skin.

When formulating this working hypothesis we took into account that CAs are able to interfere with mitogenesis and with the synthesis of cAMP by keratinocytes. We have also taken into consideration recent observations concerning the production and the secretion of CAs by activated lymphocytes.

In this context, research by Vevy Europe addresses the synthesis of peptides that modulate the formation of CAs at the local level (skin anticytostressors[a]).

The connections described above, which link the neuroendocrine system to the immune system, may be seen as a triangle in which bi-directional stimuli link the cells of the three tissues in order to adapt the biological responsiveness of each of them to incidents that affect one of them.[23,24] The role of the skin, and especially the keratinocytes, in this linkage has been reported in the literature.

- In vitro, keratinocytes have been shown to produce a cytokine cascade that is either able to attract cells of the immune system within its microenvironment or to modulate its local functional capacities.[25] Moreover, keratinocytes express membrane-bound proteins that interact in an autocrine fashion with the cytokines they produce themselves and that interact by a paracrine mechanism with those secreted by the attracted immunocompetent cells.[26]
- Keratinocytes express hormone receptors and have the fine biological machinery to produce local hormonal substances.[27]
- Both the skin and the immune system are provided with direct sympathetic innervation to the hypothalamic nuclei.

These anatomic connections suggest that the skin and the immunoneuroendocrine systems possess the tools for an efficient cross-talk and reciprocal adjustment.[28,29]

While the data concerning cytokine and hormone networks are based on experimental evidence, the ones concerning the sympato-immuno-cutaneous network require experimental work to be fully delineated. Recently, we demonstrated that human peripheral blood lymphocytes (PBL) are able to produce CAs.[30] Moreover, as we will show later in this article, preliminary observations strongly suggest that inhibiting CA synthesis by PBL affects their in vitro proliferative responsiveness to antigenic stimulation.

In our laboratory we undertook a study that had two aims:

1. To analyze the capability of keratinocytes to produce CAs.
2. To investigate the biological mechanism involved in the modulation of the interaction between keratinocytes and the neuroendocrine system.

Materials and Methods

Reagents: The reagents and mitogens we used and their sources are listed in Table 1. Water was purified by Milli-RO 12[+] and Milli-Q filters[b] in series.

Cell preparation: Heparinized peripheral blood samples four identical (repeated) samples, 150-180 mL each) were obtained from 12 healthy volunteers (age 28-39 years, 8 males and 4 females). Samples were diluted 1:1 with Hank's balanced salt solution[c], stratified 5:3 on Ficoll-Hypaque[d] gradient and centrifuged (400 g for 40 min at +4°C). The floating ring containing peripheral blood light-density mononuclear cells (PBMC) was collected and washed three times with the salt solution at 400 g for 10 min. Cells were then resuspended at $1x10^6$/mL in RPMI (Rosweli Park Memorial Institute) 1640 medium[e] containing 10% (v/v) heat-inactivated fetal calf serum[e], 200 mM glutamine and penicillin/streptomycin (complete medium).

Mononuclear adherent cells were identified by two consecutive PBMC incubations, first in plastic flasks at 37°C for 1 h in complete medium and then, lastly, overnight in the same conditions. Nonadherent cells (95-99% T and B lymphocytes) were then resuspended at a final concentration of $2x10^6$ (cell cultures in microwell plates) to $5x10^6$ (CA secretion studies) cells/mL in complete medium. The lympho-

[a] *Anticytostressor is trade marked and patented by Vevy Europe S.p.A., Genova, Italy.*

[b] *Supplied by Millipore Corp, Milford, Massachusetts, USA*
[c] *HBSS, Difco, Detroit, Michigan, USA*
[d] *Eurobio, Paris, France*
[e] *Gibco, Grand Island, New York, USA*

Table 1. Reagents and Sources

Abbreviation	Material*	Source	Comment
	Reagents		
	L-tyrosine	a	
	L- Dopa	a	
DHBA	dihydroxybenzylamine	a	internal standard of catecholamine assay
	dopamine		
	α-methyl-p-L-tyrosine	a	inhibitor of tyrosine-hydroxylase - EC 1.14.16.2
	benserazide	a	inhibitor of L-dopa-decarboxylase - EC 4.1.1.28
	5-butylpicolinic acid	a	fusaric acid, inhibitor of dopamine-α-hydroxylase - EC 1.14.17.1
DMSO	dimethylsulfoxide	a	
	sodium metabisulfite	a	
	Mitogens		
PHA	phytohemagglutinin	a	
IL-2	interleukin 2	b	
MoAbCD3		c	monoclonal antibody against CD3
	^{3}H-thymidine	d	specific activity 2 μCi/mmol

* Sources
a = Purchased from Sigma Chemical Company, St. Louis, Missouri, USA
b = Roche, Milan, Italy
c = internal production
d = Amersham, Aylesbury, UK

cytes-T and lymphocytes-B underwent experimental procedures, which are described below.

Cell lines: PC 12 cells, a clonal line of rat adrenal pheochromocytoma, were grown at 37°C in a humidified atmosphere of 95% air, 5% CO_2 in disposable flasks in RPMI 1640 medium, Hepes modified supplemented with fetal bovine serum, horse serum and 200 mM L-glutamine.

The national collection of type culture (NCTC) cells are a continuous cellular line of human keratinocytes. They were suspended in MEM EAGLE medium supplemented with fetal bovine serum, nonessential aminoacids, vitamins and L-glutamine 200 mM.

A peptide for inhibiting CA synthesis: We used the ACS2 peptide[f] obtained by synthesis to inhibit CA synthesis.

Cell cultures: Peripheral lymphocytes were cultured in 24-well plates at 37°C in a 5% CO_2 atmosphere up to 120 h with phytohemagglutinin (PHA), interleukin 2 (IL-2) or monoclonal antibody against CD3 (MoAbCD3). Incubation times were 24, 48 and 72 h for PHA or IL-2, and 72, 96 and 120 h for MoAbCD3. Mitogen concentrations were 10 μg/mL for PHA or MoAbCD3 and 10^2 U/mL for IL-2.

CA pathway inhibitors (αMPT, BENS and FUS) were added at the beginning of the culture period at the concentrations shown in Table 2. L-dopa was employed at final in-well 10^{-7} M and L-tyrosine was employed at final in-well $5x10^{-5}$ M.

DNA synthesis: The proliferative capacity of cultured peripheral lymphocytes was assessed by incorporating ^{3}H-thymidine. Thus, cultures were pulsed with 10 μCurie ^{3}H-thymidine for the last 8 h of culture. Cultures were stopped at 24, 48, 72, 96 or 120 h after the beginning. They were harvested by an automated device and radioactivity was measured by a β counter. The results are expressed as mean count per minute (cpm) of quintuplicate experiments. At the end of each culture period CA production was also evaluated.

CA determination: At the end of the incubation periods, aliquots of 10^7 cells were centrifuged at 650 g, 15°C for 5 min (supernatant assay) or disrupted by ultrasound waves[g] (cell CA content). Following that, 2 mL were then assayed.

CAs were separated by HPLC with electrochemical detection following the alumina-batch extraction procedure described elsewhere.[31] Intra- and inter-assay coefficients of variation were less than 9%. Detection limits were 94, 6 and 13 femtomole for L-dopa, nor-epinephrine (NE) and dopamine (DA), respectively. The recovery rates were 78, 86 and 84% for L-dopa, NE and DA, respectively.

Statistical methods: Standard statistical methods were employed to analyze the results. We used ANOVA followed by Bartlett's statistics and by Student-Newman-Keuls or by Bonferroni multiple comparison test. We also used an analytical software package.[h]

[f] *Vevy Europe S.p.A., Genova, Italy*

[g] *Model 250 Branson Sonic Power Co., Danbury, Connecticut, USA*
[h] *SPSS 6.1, SPSS Inc., Chicago, Illinois, USA*

Table 2. CA pathway inhibitors and their concentrations

Abbreviation	Inhibitor	Concentration
αMPT	α-methyl-p-L-tyrosine	10^{-5} M
BENS	benserazide	$5x10^{-6}$ M
FUS	fusaric acid in 35% DMSO	$5x10^{-4}$ M

Table 3. Intracellular catecholamine levels in selected cell types

	NE*	DA**
Cell type and incubation conditions	(x/mL)	(x/mL)
PC12 alone	42	1334
PC12 + L-dopa 10^{-7} M	46	3098
PC12 + L-tyrosine $5x10^{-5}$ M	136	8747
NCTC alone	15.41	24.52
NCTC + L-dopa 10^{-7} M	36.66	?
NCTC + L-tyrosine $5x10^{-5}$ M	48.37	?
PBMC alone	0.227	0.171
PBMC + L-dopa 10^{-6} M	0.364	n.d.***
PBMC + L-dopa 10^{-7} M	0.291	n.d.
PBMC + L-tyrosine $5x10^{-5}$ M	0.2	18.29

*NE: nor-epinephrine; **DA: dopamine; ***n.d.: not detectable
PC12 = rat lymphocytes (adrenal pheochromocytoma cells)
NCTC = human keratinocytes
PBMC = human peripheral blood light-density mononuclear cells

Table 4. Intracellular catecholamine levels in PC12 and NCTC after incubation with the anti-cytostressine peptide (Peptide ACS2)

Cell type and incubation conditions (x/mL)	(x/mL)	
PC12 alone	152	1838
PC12 + Peptide ACS2	117	1233
NCTC alone	46.69	?
NCTC + Peptide ACS2	36.71	?

*NE: nor-epinephrine; **DA: dopamine
PC12 = rat lymphocytes (adrenal pheochromocytoma cells)
NCTC = human keratinocytes

Table 5. Effects of inhibitors of catecholamine synthesis on the lymphocyte proliferation activity induced by PHA or interleukin 2

Cell type and incubation conditions	PHA	Interleukin 2
PBMC alone	4815 cpm	2167 cpm
PBMC + αMPT	3257	1891
PBMC + BENS	681	139
PBMC + FUS	62	106

PBMC = human peripheral blood light-density mononuclear cells

Results

CA production by keratinocytes: In order to assess whether keratinocytes produce CAs, human NCTC cells were tested both in basic conditions and following incubation with L-dopa or L-tyrosine. The results reported in Table 3 show that the amount of NE increased significantly in the cells exposed to L-dopa or to L-tyrosine, thus indicating that those cells, as well as the lymphocytes used as controls (Table 3), possess the ability to synthesize CA. The amount of NE from PRMC with and without L-dopa and L-tyrosine is also shown.

In order to analyze the possible inhibitory activity of the ACS2 peptide, the experiment was repeated, but this time the cells were exposed to the CA activating synthesis stimuli plus the ACS2 peptide. The results reported in Table 4 show that in three different experiments – PC12/NE, PC12/DA and NCTC/NE – the presence of the ACS2 Peptide inhibits 50% of the CA synthesis by both lymphocytes and keratinocytes.

In order to analyze this result better, the experiment was repeated using the cell line PC12 as target cells. This line is derived from a pheochromocytoma, which is considered a gold standard for CA production.

The results reported in Table 4 clearly show that the ACS2 Peptide exerts its inhibitory capacity even in an experimental setting.

CA synthesis and cellular biology: In order to assess whether CA synthesis is related to the biological activity of CA-secreting cells, we performed a series of experiments aimed at analyzing the mitogen responsiveness of human lymphocytes to mitogenic stimuli in the presence of CA synthesis-inhibiting factors. The results of these experiments (Table 5) indicate that inhibition of CA synthesis is related to the incapacity of human peripheral blood lymphocytes to proliferate in response to mitogenic stimuli.

Discussion

The data show that different cell lines are able to synthesize CA. In particular, it is of interest that the skin epithelial cells are included among the cells that possess the machinery to produce CA. Such a finding suggests that the skin reacts to different types of environmental stress by secreting hormones that regulate vascular reactivity locally and the blood flow through the microvascular circulation.

Moreover, our data indicate that immunocompetent cells are equipped with the same fine device and that the inhibi-

tion of CA synthesis by lymphocytes affects the in vitro responsiveness to mitogenic stimuli.[30] Since the current literature does not include data concerning this observation, it is truly difficult to understand the real pathophysiological implications of these findings.

Nonetheless, the similarity between keratinocytes and lymphocytes may indicate that epithelial cells play an active role in the immune defense of mucosal and skin surfaces, either through their physical role or by secreting soluble factors aimed at adapting the responsiveness of cells located in the local microenvironment to the needs triggered by internal or external factors.

Whether CA synthesis represents one of these mechanisms is not known, despite the fact that the correlation between lymphocyte responsiveness and CA synthesis strongly suggests that this is the case. Based on these conclusions we can postulate that the modulation of CA production at the skin level could prove to be beneficial when the skin is exposed to factors that pathologically stimulate its biological reactivity.

The finding that a peptide (ACS2) can inhibit the CA synthesis in vitro indicates that the pharmaceutical industry could provide tools to modulate cell responsiveness to stressors and possibly reduce their harmful effects.

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