

## NEUROIMMUNOLOGICAL ACTIVITIES OF KERATINOCYTES

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### SUMMARY

The skin constitutes a complex structure that regulates the interaction between the environment within and without the body. In order to perform this function the skin is connected to the main homeostatic systems which allow the human body to modify its biological behaviour based on the stimulus of environmental events. The above mentioned connections, which link the neuroendocrine system to the immune system, may be seen as a triangle in which bi-directional stimuli link the cells of three tissues in order to adapt the biological responsiveness of each of them to incidents that affects one of them (23-24).

The scientific bases of the above reported concepts are the following:

1. 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 functional capacities in loco (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 immune competent cells (26).
2. Keratinocytes express hormone receptors and have the fine biological machinery to produce hormonal substances in loco (29).
3. Both the skin, as well as the immune system, are provided with direct sympathetic innervation that connects these structures directly with the hypothalamic nuclei.

These anatomic connections suggest that the skin and the immune and neuroendocrine systems possess the tools for an efficient cross-talk and reciprocal adjustment (27-28).

While the data concerning cytokine and hormone networks are based on experimental evidence, the ones concerning the sympato-immune-cutaneous network require experimental work to be fully delineated. Recently, we demonstrated that human peripheral blood lymphocytes (PBL) are able to produce catecholamines (CA), (30). Moreover, preliminary observations strongly suggest that inhibiting catecholamine synthesis by PBL affects their in vitro proliferative responsiveness to antigenic stimulation (Table 5).

The aims of this study were:

- a) to analyze the capability of keratinocytes to produce catecholamines.

To investigate the biological mechanism involved in the modulation of the interaction between keratinocytes and the neuroendocrine system.

### INTRODUCTION

Several recent investigations have shown that epithelial cells actively take part in the control of homeostasis of the organism.

This concept is essentially based on three observations:

1. keratinocytes are able to synthesize cytokines and thus interfere with immunological processes (1);
2. keratinocytes express receptors for catecholamines (2);
3. keratinocytes locally synthesize catecholamine 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 short review will mainly focus on the secretion and the use of catecholamines by keratinocytes, and will also include highlights regarding the involvement of these cells in the immune response.

When keratinocytes are exposed to various pathogens they are stimulated to:

1. secrete pro-inflammatory cytokines;
2. express adhesion molecules on their surfaces that increase and/or regulate the blood supply to the inflamed skin (5);
3. establish interactions with other cells present in the skin (mast-cells, lymphocytes T,  $\gamma$ ,  $\delta$  etc.) which participate more or less directly in the inflammatory process.

On the grounds of these observations Barker et al. (4) 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 cAMP concentration in the cytoplasm (7).

Many reports have focussed 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  $\beta$ -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  $\beta$ -adrenergic activity (10). Gazith et al. (1983) clearly proved that the density of  $\beta$ -adrenergic receptors expressed by human keratinocytes is higher than that of other cell compartments normally controlled by catecholamines (11).

It has also been shown that in response to the specific binding of an agonist these receptors induce an enzymatic cascade that leads to the synthesis of cAMP (8).

More recently (12) 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*.

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

It has also been shown that the biosynthesis of catecholamines 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  $Ca^{2+}$  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 catecholamines 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 catecholamines can indeed interact. It has been shown that:

1. interferon  $\alpha$  is able to modulate both catecholamine synthesis and the expression of the corresponding receptors on circulating lymphocytes *in vitro* and *in vivo*;
2. 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);
3. interferons can directly interact with  $\beta$ -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 micro-environment by the same type of cells then their activity will be influenced by the corre-

sponding concentrations and affinities to the receptors.

This becomes especially relevant when analysing the skin response to damage of various origin, 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 catecholamine receptors these cytokines induce the expression of the genes that regulate catecholamine synthesis.

ROTS (Reactive Oxygen Toxic Species) 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 catecholamines (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 catecholamines whose activity is enhanced by the presence of molecular oxygen;
3. presence of cytokines that act as modulating factors in the same micro-environment.

The aim of our studies over 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 i.e. 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 to entrap activated immunocompetent cells within the same micro-environment. Therefore we designed our experimental approach in order to evaluate the possibility that even catecholamines participate in the neuro-immuno-endocrine interactions within the skin. When formulating this working hypothesis we took into account that catecholamines are able to interfere with mitogenesis and with the synthesis of cAMP by keratinocytes. We have also taken into consideration recent ob-

servations concerning the production and the secretion of catecholamines by activated lymphocytes.

In this context, research by Vevy Europe addresses the synthesis of peptides that modulate the formation of catecholamines at the local level (skin *anticytostressors*<sup>1</sup>).

## MATERIALS AND METHODS

### Reagents

The following reagents were purchased from Sigma Chemical Co. St.Louis, MO: L-tyrosine, L- Dopa, dihydroxybenzylamine (DHBA, internal standard of CA assay), dopamine,  $\alpha$ -methyl-p-L-tyrosine (inhibitor of tyrosine-hydroxylase - EC 1.14.16.2), benzerazide (inhibitor of L-dopa-decarboxylase - EC 4.1.1.28), 5-butylicolic acid (fusaric acid, inhibitor of dopamine- $\alpha$ -hydroxylase - EC 1.14.17.1), dimethylsulfoxide (DMSO) and sodium metabisulfite. Water was purified by Milli-RO 12<sup>+</sup> (Millipore Corp. Milford, MA) and Milli-Q (Millipore Corp.) filters in series.

The mitogens we employed were: phytohemagglutinin (PHA) (Sigma Chemical Co., St.Louis, MO), interleukin 2 (IL-2) (Roche, Milan, Italy) and monoclonal Ab against CD3 (MoAbCD3) (internal production). <sup>3</sup>H-thymidine (specific activity 2  $\mu$ Ci/mmol) was purchased from Amersham, Aylesbury, UK).

### Cell Preparation

Heparinized peripheral blood samples (four repeated samples, 150 - 180 mL each) were obtained from 12 healthy volunteers (age 28-39 yrs, 8 males and 4 females). Samples were diluted 1:1 with Hank's balanced salt solution (HBSS, Difco, Detroit, MI, USA), stratified 5:3 on Ficoll-Hypaque (Eurobio, Paris, France) 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 HBSS at 400 g for 10 min. Cells were then resuspended at  $1 \times 10^6$ /mL in RPMI 1640 medium (Gibco, Grand Island, NY, USA) containing 10% (v/v) heat inactivated fetal calf serum (Gibco), 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 hour in complete medium and then, lastly, overnight in the same conditions. Nonadherent cells (95 to 99% T and B lymphocytes) were then resuspended at a final concentration of  $2 \times 10^6$

<sup>1</sup>Anticytostressor: Trade Mark and Patent of Vevy Europe S.p.A.

(cell cultures in microwell plates) to  $5 \times 10^6$  (CA secretion studies) cells/mL in complete medium. The lymphocytes underwent experimental procedures.

#### *Cell lines*

PC 12 cells, a clonal line of rat adrenal pheochromocytoma, are grown at 37°C in a humidified atmosphere of 95% air 55% CO<sub>2</sub> in disposable flasks in RPMI 1640 medium, Hepes modified supplemented with fetal bovine serum, horse serum and L-glutamine 200 mM.

NCTC cells are a continuous cellular line of human keratinocytes. They were suspended in MEM EAGLE medium supplemented with fetal bovine serum, non essential aminoacids, vitamins and L-glutamine 200 mM.

#### *CA synthesis inhibiting peptides*

The ACS2 peptide, obtained by synthesis (Vevy Europe S.p.A., Genova, Italy), was used.

#### *Cell cultures*

Peripheral lymphocytes were cultured in 24-well plates at 37°C in a 5% CO<sub>2</sub> atmosphere up to 120 h with PHA, IL-2, or MoAbCD3. Incubation times were: 24h, 48h, 72h for PHA or IL-2, and 72h, 96h and 120h for MoAbCD3. Lastly, mitogen concentrations were: PHA 10 µg/mL, IL-2 10<sup>2</sup> U/mL and MoAbCD3 10 µg/mL.

CA pathway inhibitors were added at the beginning of the culture period. Concentrations were:  $\alpha$ -methyl-p-L-tyrosine 10<sup>-5</sup> M, ( $\alpha$ MPT) benserazide  $5 \times 10^{-6}$  M (BENS) and fusaric acid  $5 \times 10^{-4}$  M in 35% DMSO (FUS). L-dopa was employed at final in-well 10<sup>-7</sup> M and L-tyrosine was employed at final in-well  $5 \times 10^{-5}$  M.

#### *DNA synthesis*

The proliferative capacity of cultured peripheral lymphocytes was assessed by incorporating <sup>3</sup>H-thymidine. Thus, cultures were pulsed with 10 µCi <sup>3</sup>H-thymidine for the last 8 h of culture. Cultures were stopped at 24, 48, 72, 96, or 120h after the beginning, they were harvested by an automated device and radioactivity was measured by a  $\beta$  counter. The results are expressed as mean c.p.m. of quintuplicate experiments. At the end of each culture period CA production was also evaluated.

#### *Catecholamine determination*

At the end of the incubation periods, aliquots of 10<sup>7</sup> cells were centrifuged at 650 g, 15°C for 5 min (supernatant assay) or disrupted by ultrasound waves (Mod. 250 Branson Sonic Power Co., Danbury, CT) (cell CA content). 2 mL were then assayed.

CA were separated by HPLC with electrochemical detection following alumina-batch extraction, as reported (31). Intra- and inter-assay coefficients of variation are < 9%. Detection limits are: L-dopa 94 fmol, nor-epinephrine (NE) 6 fmol, dopamine (DA) 13 fmol. The recovery rates are: L-dopa 78%, NE 86%, DA 84%.

#### *Statistical methods*

Standard statistical methods were employed to analyze the results. ANOVA followed by Bartlett's statistics and by Student-Newman-Keuls or by Bonferroni multiple comparison test was utilized. The SPSS 6.1 software package (SPSS Inc., Chicago, IL) was used for analysis.

## CONCLUSIONS

#### *CA production by Keratinocytes*

In order to assess whether keratinocytes produce catecholamines, NCTC cells were tested both in basic conditions and following incubation with L-dopa or L-tyrosine. The results reported in Table .2 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, possess the ability to synthesize CA.

In order to analyze the possible inhibitory activity of the ACS2 Peptide the above reported experiment was repeated, but this time the cells were exposed to the CA activating synthesis stimuli plus the ACS2 Peptide. The results plotted in Table 4 show that in three different experiments 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 plotted 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 are plotted in Table 5, and they indicate that inhibition of CA synthesis is related to the incapacity of human PBL 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 inhibition 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 defence 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 micro environment 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 pharmaceuticals industry could provide tools to modulate cell responsiveness to stressors and possibly reduce their harmful effects.

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**Table 1. Intracellular catecholamine levels in PC12 cells.**

	NE* (x/ml)	DA** (x/ml)
PC12 alone	42	1334
PC12 + L-dopa $10^{-7}$ M	46	3098
PC12 + L-tyrosine $5 \times 10^{-5}$ M	136	8747

\*NE: nor-epinephrine; \*\*DA: dopamine

**Table 2. Intracellular catecholamine levels in NCTC cells.**

	NE* (x/ml)	DA** (x/ml)
NCTC alone	15.41	24.52
NCTC + L-dopa $10^{-7}$ M	36.66	?
NCTC + L-tyrosine $5 \times 10^{-5}$ M	48.37	?

\*NE: nor-epinephrine; \*\*DA: dopamine

**Table 3. Intracellular catecholamine levels in PBMC.**

	NE* (x/ml)	DA** (x/ml)
PBMC alone	0.227	0.171
PBMC + L-dopa $10^{-6}$ M	0.364	n.d.***
PBMC + $10^{-7}$ M	0.291	n.d.
PBMC + L-tyrosine $5 \times 10^{-5}$ M	0.2	18.29

\*NE: nor-epinephrine; \*\*DA: dopamine; \*\*\*n.d.: not detectable

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

	NE* (x/ml)	DA** (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

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

	PHA	Interleukin 2
PBMC alone	4815*	2167
PBMC + $\alpha$ MPT	3257	1891
PBMC + BENS	681	139
PBMC + FUS	62	106

\*: cpm.