HBD: A new tool to enhance human skin self-defence against micro-organisms

Ingrid Pernet[(1)], Corinne Reymermier[(2)], Anne Guezennec[(3)], Jacqueline Viac[(1)], Jean-Eric Branca[(2)], Joëlle Guesnet[(3)], Eric Perrier[(2)].

[(1)]INSERM U346, E. Herriot Hospital, Lyon, France; [(2)]COLETICA, Lyon, France; [(3)]YSL Beauté, Paris, France

Keywords: beta-defensin; human; keratinocytes; anti-microbial peptides; cell-culture.

Summary

Normal human skin, constantly challenged by environmental micro-organisms, has an innate ability to fight invading microbes through antimicrobial peptides. These peptides, described in both plant and animal kingdoms are able to inactivate a broad spectrum of micro-organisms.

Mammalian defensins constitute one of the most common antimicrobial peptide family. Among the three human beta-defensins hBD1, hBD2 and hBD3 produced in epithelia, only hBD2 and hBD3 are inducible and additionally have been described as expressed by differentiated keratinocytes at site of inflammation and infection.

The aims of these studies were to define a cell culture model in which the basal production of hBD could be detected and up-regulated in order to enhance skin auto-protection against micro-organisms.

A specific Polymerase Chain Reaction method have been developed for hBD2 and hBD3 mRNA detection in non-differentiated monolayer keratinocytes cell culture.

We have been able to demonstrate that *in vitro*, hBD2 and hBD3 expression in normal human keratinocytes could be detected and enhanced by TNF-alpha and IFN-gamma, in hypercalcic culture conditions. This research opened the possibility of the development of cosmetic active compounds, able to induce the expression of skin natural antibiotic peptides responsible about microflora ecology of the skin.

Introduction

Normal human skin is constantly exposed to injury and challenged by environmental microorganisms, which do not produce evidence of clinical infection. This suggests that skin has an innate ability to fight invading microbes [1, 2]. Antimicrobial peptides are evolutionarily conserved elements, of this innate immune system [3]. More than 500 of these peptides have been described in both plant and animal kingdoms. They are small cationic molecules characterized by an amphipathic structure [4]. They are able to inactivate a broad spectrum of microorganisms, like bacteria, fungi and viruses.

Mammalian defensins constitute one of the most common antimicrobial peptide family. They are divided into two subgroups of α? and β–defensins which differ from one another by the spacing and the connectivity of their six cysteine residues [5]. Human α-defensins are found in neutrophile granules and in the Paneth cells of the small intestine, whereas β–defensins have been localised to surface epithelial cells [6]. The three β–defensins so far identified, human β–defensins hBD1, hBD2 and hBD3, are produced in various epithelia [7-11]. Whereas hBD1 is constitutively expressed in normal human skin, hBD2 and hBD3 are induced predominantly by contact with microbial products such as endotoxin or proinflammatory cytokines and are highly expressed by differentiated keratinocytes at site of inflammation and infection [7-12].

HBD2 is highly effective in killing gram-negative bacteria and has only a bacteriostatic activity against gram-positive bacteria; hBD3 reveals a broader spectrum than hBD2, as it exhibits antimicrobial activity against several gram-positive and gram-negative bacteria [12, 13].

However, overexpression of β–defensin has been reported, until now, to be associated in vivo with activation of keratinocytes and release of epidermal cytokines [14,15], in particular with the release of Macrophage Inflammatory Protein-3α (MIP3α/CCL20), a member of the CC-chemokine family.

MIP3α is also described in differentiated keratinocytes of psoriatic skin and in epithelial cells activated by proinflammatory cytokines [16, 17].

MIP3α as well as β–defensins bind to, and activate, the chemokine receptor CCR6 which is expressed by immature dendritic cells and memory T cells and promote adaptive immune responses by recruiting dendritic and T cells to the site of microbial invasion [18].

The findings that defensins also act as chemokines and the in vivo coexpression of hBD2 and

MIP3α suggest important and complementary role in both innate and adaptative immune response against microbial invasion.

It has been recently demonstrated that β–defensins are expressed and can be up-regulated in differentiated human reconstructed epidermis [19]. Moreover, it was recently reported that an essential amino acid L-isoleucine induces epithelial β–defensin expression [20].

These data confirmed the interest to look for substances which could improve skin protection by stimulating the production of hBD2 and/or hBD3 but without inducing inflammatory cytokines.

For this purpose, we have developed an in vitro model to screen molecules for their ability to stimulate β–defensin expression without inducing inflammatory cytokines. First of all, we have defined a monolayer normal human keratinocyte culture model in which the basal production of hBD could be detected and up-regulated by reference molecules. Then, in order to be able to screen a large number of molecules, we have adapted this model to obtain a keratinocyte culture model in 96-well plates, in specific calcium conditions. Semi-quantitative RT-PCR and quantitative Real Time PCR (Q-PCR), adapted to 96-wells plates in vitro models, have then allowed the measure of defensins hBD2 and hBD3 mRNA expression, while measuring in the same time the levels of MIP3α expressed in cell culture media.

We report here that, in vitro, hBD2 and hBD3 expression in monolayers of human normal keratinocytes and in 96-wells plate's culture model, could be enhanced by TNFα and IFNγ, in hypercalcic culture conditions, and that this stimulation is correlated with an enhanced secretion of the chemokine MIP3α .

Nevertheless, it remains important to investigate whether the stimulation of β–defensins in epidermal keratinocytes is, or not, always followed by the release of MIP3α or other proinflammatory cytokines such as IL1α or IL8.

Materials and Methods

Human keratinocyte isolation and cultures

Normal human keratinocytes (NHK) derived from neonatal foreskins (n=12) were used in this study. Freshly isolated cells were cultured in serum-free medium K-SFM (Invitrogen, Cergy-Pontoise, France) supplemented with 1% penicillin/streptomycin (Invitrogen, Cergy-Pontoise, France) and epidermal growth factor (EGF) and bovine pituitary extract (BPE). Cells were grown and amplified

at 37°C and 5% CO_2 in air.

NHK were seeded in 6-well plates in K-SFM at three different calcium chloride concentrations: 0.09mM (basal concentration of K-SFM), 0.8mM and 1.7mM (concentrations obtained by addition of a solution of calcium chloride ($CaCl_2$, Sigma, L'Isle d'Abeau, France) in sterile water).

Cell treatments:

After 4 days, cells were washed and fresh medium added, containing neither EGF nor BPE, at the three previous concentrations of $CaCl_2$, and cultured either in absence (control) or in presence of 4 rising concentrations of Tumour Necrosis Factor alpha (TNF-α): 1, 10, 100 and 500 ng/ml (Abcys, Paris, France; 1ng = 20 U). All conditions were performed in triplicate.

Cells cultured at 1.7mM of $CaCl_2$, were also treated with 3 rising concentrations of Interferon gamma (IFN-γ): 1, 10, 100 and 500 ng/ml (Sigma, L'Isle d'Abeau, France).

After 16h, supernatants were collected, centrifuged at 10,000g for 10 min and stored at -70°C. Dishes were then rinsed with PBS, cells were trypsinated, counted and stored as dry pellets at -80°C.

Total RNA extraction and semi-quantitative Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA were extracted using the High Pure™ RNA Isolation Kit (Roche Diagnostics, Meylan, France) or using the SV 96 Total RNA Isolation System (Promega, Meylan, France) for the 96-wells plates conditions, according to the manufacturer's instructions. Total RNA integrity and purity were verified on a 2% agarose gels, and quantities were evaluated photometrically.

Semiquantitative RT-PCR was performed using Superscript™ One-Step RT-PCR with Platinum Taq (Invitrogen, Cergy-Pontoise, France). HBD2 primers were designed as 5'-CCA GCC ATC AGC CAT GAG GGT-3' (forward) and 5'-GGA GCC CTT TCT GAA TCC GCA-3' (reverse) [7]. HBD3 primers were designed as 5'-AGC CTA GCA GCT ATG AGG ATC-3' (forward) and 5'-CTT CGG CAG CAT TTT CGG CCA-3' (reverse) [12]. Amplification using these primers resulted respectively in a 255 bp and 206 bp fragments. The Reverse-Transcription-PCR was carried out in a Tetrad (MJ Resarch, Strasbourg, France) using 50 ng of total RNA. RT-PCR conditions for hBD2 and hBD3 were: synthesis of first strand cDNA at 48°C for 30 min, followed by denaturation at 94°C for 2 min,

then 32 cycles of amplification (94°C for 30 s, 60°C for 30 s, 68°C for 30 s) and a final extension step at 72°C for 10 min. The number of cycles and the quantity of total RNA were previously determined by different assays, the optimal semi-quantitative conditions falling in the linear range of the reaction when the PCR signals intensity was not saturated. For each experiments, internal controls lacking template or reverse transcription step (reaction mix pre-heated to denature reverse transcriptase) were performed and verified to be negative. In parallel, amplification of an house-keeping gene, β-actine, was performed on each RNA sample in order to ensure that differences between the time points for hBD2 and hBD3 mRNA levels are due to the regulation of the defensins. β–actine primers were designed as 5′-GTG GGG CGC CCC AGG CAC CA-3′ (forward) and 5′-CTC CTT AAT GTC ACG CAC GAT TTC-3′ (reverse) [12] and were predicted to amplify a 540 bp fragment. RT-PCR was carried out in the same conditions as HBD2 and hBD3 (but with 30 cycles instead of 32). Reaction products were mixed and visualized by electrophoresis on 2% precast agarose gels (E-gels, Invitrogen, Cergy-Pontoise, France). Quantification of each band was achieved using a digital camera used on gels and an image analysis software (Phoeretix 1D).

Cytokines levels in culture supernatants

MIP-3α levels were measured by enzyme-linked immunosorbent assay (ELISA, R&D systems, Abingdon, UK) using 96-well microliter plates in accordance with the manufacturer's instructions. Supernatants were not diluted. The levels of MIP-3α were calculated using standard curves obtained with human recombinant (hr)MIP3–α (from 0.47 $pg.ml^{-1}$ to 500 $pg.ml^{-1}$).

Data Analysis

The results were expressed as the mean ± S.D. in $pg.ml^{-1}.(RNA\ concentration)^{-1}$ and differences assessed using a one way ANOVA analysis and Benferroni tests.

Results

Calcium induced a co-ordinated up-regulation of β–defensin hBD2 mRNA and MIP3α secretion from TNFα-activated NHK

The switch in $CaCl_2$ concentration from 0.09 mM to 1.7 mM induced little morphological changes in keratinocytes. Cells appeared small and tight, but there was no stratification even after 4 days. Cell

morphology was not modified upon TNFα or IFNγ.

When we examined the effect of calcium on the basal level of β-defensins of NHK, we observed that a switch in $CaCl_2$ concentration from 0.09 mM (basal concentration in K-SFM medium) to 1.7 mM for four days, was efficient to improve the detection of hBD2 and hBD3 mRNA in keratinocyte cultures from different skin donors with some inter-individual variation (Fig 1). As a rule, we found more hBD2 than hBD3 transcripts in unstimulated NHK.

The addition of TNFα (1 to 500 ng/ml) for 16h, to NHK cultured in different calcium concentrations (0.09, 0.8 and 1.7 mM) induced an up-regulation of mRNA expression of hBD2 but not hBD3 and the signal was increased in a dose-dependent manner (Fig 2). It is important to note that, in such conditions and in a hypocalcic medium (0.09mM), basal or stimulated levels of hBD2 and hBD3 transcripts were not detected. This confirmed that calcium was essential to trigger the stimulation of defensins by TNFα. Moreover, TNFα strongly intensified hBD2 mRNA but was a poor enhancer of hBD3 mRNA.

As far as the secretion of MIP3α, evaluated by ELISA as described above, we could notice that in basal conditions (0.09mM $CaCl_2$), NHK secreted very low amounts of MIP3α. TNFα and $CaCl_2$ (Fig 3) were significantly increasing the levels of this molecule in a dose-dependent manner.

It is noteworthy that the lower dose of TNFα (1 ng/ml) was already efficient to promote both an up-regulation of hBD2 mRNA expression and MIP3α secretion.

IFNγ strongly up-regulated hBD3 but not hBD2 mRNA in NHK cultured in high calcium concentration (1.7 mM)

As TNFα was a poor enhancer of hBD3 mRNA, we tested the effect of IFNγ, a potent pro-inflammatory cytokine recently reported to stimulate hBD3 in HaCaT cell line [13]. Its effect was evaluated under the optimal condition obtained for TNFα, *i.e.* 1.7mM $CaCl_2$. IFNγ induced in NHK, a strong and dose-dependent up-regulation of hBD3 mRNA whereas the effect on hBD2 mRNA which was also dose-dependent remained very slight (Fig 4).

96-wells plates Cell culture model

As we showed in the first part, keratinocytes cultured in hypercalcic conditions (1.7mM) allowed an

easier detection of β-defensin signal after mRNA amplification. In 96-wells culture plates, we were able to extract 80µl of a solution of almost 10ng.µl^{-1} of total RNA per well. In chosen RT-PCR conditions (50ng of initial total RNA), non treated cells showed no β-defensin expression but high-intensity bands appeared when cells were treated with positive control *i.e.* TNFα 100ng.ml^{-1} for hBD2 and IFNγ 100ng.ml^{-1} for hBD3.

Discussion

Our results clearly demonstrated that calcium triggers the up-regulation of β-defensin mRNA and MIP3α secretion in activated NHK. Calcium is a physiological regulator of keratinocyte maturation and a gradient of both intracellular and extracellular Ca^{2+} occurs, *in vivo*, in human epidermis and modulating extracellular Ca^{2+} is a frequently used method to induce maturation of culture human keratinocytes. Our striking observation was the significant and dose-dependent induction of hBD2 mRNA in stimulated keratinocytes under moderate and high Ca^{2+} levels and the coordinated increase of MIP3α secretion in cell supernatants. Unlike hBD2, hBD3 transcripts were poorly up-regulated by TNFα in keratinocyte cultures. This may explain the discrepant results recently reported [12,13] and suggests that in keratinocytes, different signalling pathways and specific inducibility are involved in hBD2 and hBD3 regulation. This was confirmed by the preferential up-regulation of hBD3 by IFNγ, as recently reported in HaCaT cells [13].

We reported here that a high calcium concentration (1.7 mM) alone applied for four days in the culture medium, may be sufficient to induce β-defensins mRNA and to enhance MIP3α secretion in keratinocyte monolayers.

In vivo, the normal human skin is inhabited by a population of commensal bacteria that are regarded as beneficial to the host. Recent data suggest that commensals provide protection by chronically stimulating epithelial surfaces to express antimicrobial peptides at levels that kill pathogen microbes [21]. In epidermis, only highly differentiated keratinocytes *i.e.* corneocytes are prone to exhibit an antibacterial activity, together with sweat which contains at least the antimicrobial protein dermicidin [22]. It seems likely that hBD2 in cooperation with MIP3α is also involved in the maintenance of Langerhans cells in normal epidermis and that both β-defensins and

MIP3α contribute to the immunosurveillance of the skin barrier function. The fact that it is possible, in vitro, to induce β-defensins and MIP3α in activated keratinocyte monolayers derived from foreskins, matured under appropriate calcium levels, provides a simple tool to study β-defensins and associated cytokines expression under various keratinocytes stimulation. The use of 96-wells plate format suited at the same time for cell cultures, RNA extraction, RNA quantification and RT-PCR and Q-PCR reactions allow the possibility to test in a short time, libraries of molecules. The use of positive controls, TNFα and IFNγ, chosen on the basis of our reported results is a reliable mean to valid the efficiency of each experiment.

Qualitative RT-PCR allows in the first step of a screening to detect rapidly, potentially interested molecules. Nevertheless, this method is not quantitative and results must be analysed cautiously. Then, a quantitative method would have to be developed to confirm interesting selected molecules.

Conclusions

Human β-defensins which play an important antimicrobial role in host defense against cutaneous pathogens have been described to be expressed by differentiated keratinocytes, in vivo, in normal epidermis, or in epidermal organotypic culture [15,19]. As we demonstrated here that keratinocyte monolayers cultured in high calcium concentrations allowed to detect mRNA of both hBD2 and hBD3, this prompt us to establish a culture model for the screening of molecules able to stimulate epidermal defensins aimed at improving the protection of sensitive skin against environmental pathogens. Whereas overexpression of β-defensin has been reported, until now, to be associated in vivo with activation of keratinocytes and release of epidermal inflammatory cytokines, we will need to insure that β-defensin stimulating products screened in our new model will not stimulate inflammatory cytokines usuallly associated with these antimicrobial peptides induction.

References

1. Huttner, K.M. and Bevins, C.L. Antimicrobial peptides as mediators of epithelial host defense. *Pediatr. Res.* **45,** 785-794 (1999).
2. Gallo, R.L. and Huttner, K.M. Antimicrobial peptides: an emerging concept in cutaneous biology. *J. Invest. Dermatol.* **111**, 739-745 (1998).
3. Ganz, T. Defensins and host defense. *Science* **286,** 420-421 (1999).
4. Martin, E., Ganz, T. and Lehrer, R.I. Defensins and other endogenous peptide antibiotics of vertebrates. *J. Leukoc. Biol.* **58,** 128-136 (1995).
5. Ganz, T. and Weiss, J. Antimicrobial peptides of phagocytes and epithelia. *Semin. Hematol.* **34,** 343-354 (1997).
6. Diamond, G. and Bevins, C.L. Beta-defensins: endogenous antibiotics of the innate host response. *Clin. Immunol. Immunopathol.* **88**, 221-225 (1998).
7. Harder, J., Bartels, J., Christophers, E. and Schröder, J.M. A peptide antibiotic from human skin. *Nature* **387**, 861 (1997).
8. Zhao, C.Q., Wang, I. and Lehrer, R.I. Widespread expression of beta-defensive HBD-1 in human secretory glands and epithelial cells. *FEBS Lett.* **396,** 319-322 (1996).
9. Valore, E.V., Park, C.H., Quayle, A.J., Wiles, K.R., McGray, P.B. and Ganz, T. Human beta-defensin-1: an antimicrobial peptide of urogenital tissues. *J. Clin. Invest.* **101,** 1633-1642 (1998).
10. Bals, R., Wang, X., Wu, Z., Freeman, T., Bafna, V., Zasloff, M. and Wilson, J.M. Human beta-defensine 2 is a salt-sensitive peptide antibiotic expressed in human lung. *J. Clin. Invest.* **102,** 874-880 (1998).
11. Hiratsuka, T., Nakazato, M., Date, Y., Ashitani, J., Minematsu, T., Chino, N. and Matsukura, S. Identification of human beta-defensin-2 in respiratory tract and plasma and its increase in bacterial pneumonia. *Biochem. Biophys. Res. Commun.* **249,** 943-947 (1998).
12. Harder, J., Bartels, J., Christophers, E. and Schröder, J.M. Isolation and characterization of human β-defensin-3, a novel human inducible peptide antibiotic. *J. Biol. Chem.* **276**, 5707-5713 (2001).
13. Garcia, J.R.C., Jaumann, F., Schulz, S., Krause, A., Rodriguez-Rimenez, J., Forssman, U., Adermann, K., Klüver, E., Vogelmeier, C., Becker, D., Hedrich, R., Forsmann, W.G. and Bals,

R. Identification of a novel, multifunctional β-defensin (human β-defensin 3) with specific antimicrobial activity. *Cell. Tissue. Res.* **306,** 257-264 (2001).

14. Ali, R.S., Falconer, A., Ikram, M., Bisset, C.E., Cerio, R. and Quinn, A.G. Expression of the peptide antibiotics human β-defensin-1 and human β-defensin-2 in normal human skin. *J. Invest. Dermatol.* **117**, 106-111 (2001).
15. Liu, A.Y., Destoumieux, D., Wong, A.V., Park, C.H., Valore, E.V., Liu, L. and Ganz, T. Human beta-defensin-2 production in keratinocytes is regulated by interleukin-1, bacteria and the state of differentiation. *J. Invest. Dermatol.* **118,** 275-281 (2002).
16. Charbonnier, A.S., Kohrgruber, N., Kriehuber, E., Stingl, G., Rot, A. and Maurer, D. Macrophage inflammatory protein-3alpha is involved in the constitutive trafficking of epidermal Langerhans cells. *J. Exp. Med.* **190,** 1755-1768 (1999).
17. Dieu-Nosjean, M.C., Massacrier, C., Homey, B., Vanbervliet, B., Pin, J.J., Vicari, A., Lebecque, S., Dezutter-Dambuyant, C., Schmitt, D., Zlotnik, A. and Caux, C. Macrophage inflammatory protein 3alpha is expressed at inflamed epithelial surfaces and is the most potent chemokine known in attracting Langerhans cell precursors. *J. Exp. Med.* **192,** 705-712 (2000).
18. Tohyama, M., Shirakara, Y., Yamasaki, K., Sayama, K. and Hashimoto, K. Differentiated keratinocytes are responsible for TNF-α regulated production of macrophage inflammatory protein 3a/CCL20, a potent chemokine for Langerhans cells. *J. Dermatol. Sci.* **27,** 130-139 (2000).
19. Chadebech P, Goidin D, Jacquet C, Viac J, Schmitt D, Staquet MJ. The β-defensin hBD2 is expressed in differenciated human reconstructed epidermis at mRNA and peptide levels and is up-regulated in response to bacterial LPS. Submitted.
20. Fehlbaum P, Rao M, Zasloff M, Anderson GM. An essential amino acid induces epithelial β-defensin expression. *Proc. Natl. Acad. Sci. USA* **97**, 12723-12728 (2000)
21. Boman HG. Innate immunity and the normal microflora. *Immunol. Rev.* **173**, 5-16 (2000)
22. Schitteck B, Hipfel R, Sauer B et al Dermicidin, a novel human antibiotic peptide secreted by sweat glands. Innate immunity and the normal microflora. *Nature Immunol.* **2**, 1133-1137 (2001).

Figures:

Fig 1: Comparative hBD2 and hBD3 basal expression in normal human keratinocytes derived from 12 donors (A to L) and cultured either in low or high calcium concentrations (0.09 and 1.7 mM). Note some inter-individual variations and a β-defensin mRNA expression increase in $CaCl_2$ 1.7 mM. 10µl of semi-quantitative RT-PCR products were loaded on a 2% agarose gel stained with Ethidium Bromide.

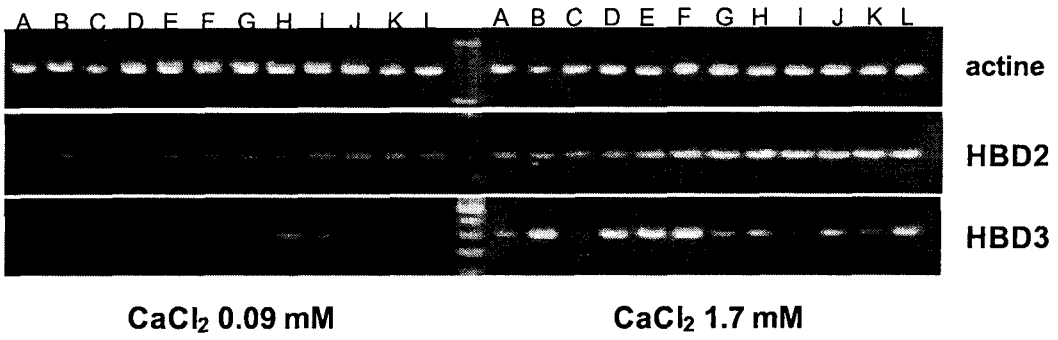

Fig 2: Differential effect of TNF-α (1 to 500 ng/ml) on hBD2 and hBD3 mRNA expression in normal human keratinocytes cultured under 3 different calcium concentrations. In order to improve the reliability of the ratio hBD/actine, RT-PCR products of hBD2, hBD3 and actine were loaded in the same well (3µl actine + 6 µl hBD2 + 6 µl hBD3) on a 2% precast agarose gel. In such conditions, basal level of hBD could not be detected.

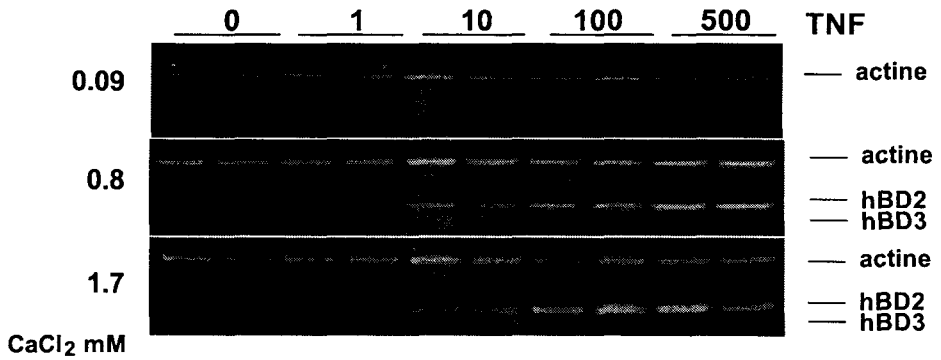

Fig 3: Effect of TNF-α treatment on MIP3-α secretion by normal human keratinocytes cultured in 3 different calcium concentrations. Cells were treated with TNFα: □ 1ng/ml, ■ 10 ng/ml, ■ 100 ng/ml, ■ 500 ng/ml towards a non-treated control ■ . The results were expressed as the mean ± SD in pg/ml/10^6 cells. *p<0.05; **p<0.01;*** p<0.001.

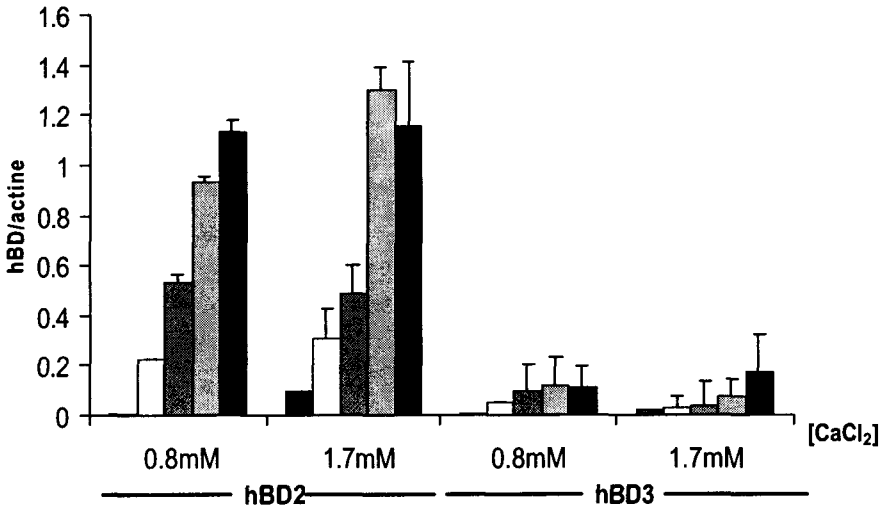

Fig 4: Effect of TNF-α treatment on MIP3-α secretion by normal human keratinocytes cultured in 3 different calcium concentrations. Cells were treated with TNFα: □ 1ng/ml, ■ 10 ng/ml, ■ 100 ng/ml, ■ 500 ng/ml towards a non-treated control ■. The results were expressed as the mean ± SD in pg/ml/10^6 cells. *p<0.05; **p<0.01;*** p<0.001.