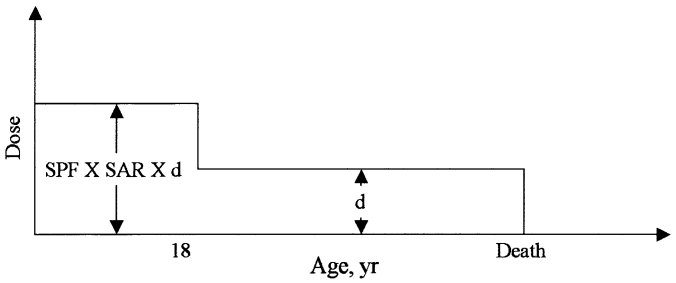

Figure 1
Age pattern of ultraviolet-B exposure. SPF indicates sun protective factor; SAR, sun affinity ratio; and d, dose affiliations

ple that is truly representative of the Danish population, Thieden's findings are likely to be more applicable to 21st-century Denmark, a quite northern country (latitude 55°) blessed with far more generous vacations than those who worked in not quite tropical Boston (latitude 42°) in the 1980s.

Robert S. Stern,
Department of Dermatology,
Beth Israel Deaconess Medical Center,
Harvard Medical School, Boston,
Massachusetts, USA.

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Address correspondence to: Robert S. Stern, Department of Dermatology, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, Massachusetts, USA. Emails: rthornto@bidmc.harvard.edu, rstern@caregroup.harvard.edu, sjanus@bidmc.harvard.edu

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Vitamin D Induces the Antimicrobial Protein hCAP18 in Human Skin

To the Editor:

Cathelicidins are a class of mammalian antimicrobial peptides expressed in leukocytes and at epithelial surfaces (Zanetti, 2004). Human cathelicidin antimicrobial protein hCAP18 is encoded by *CAMP* (Ensembl Gene ID ENS G00000164047) on chromosomal location 3p21 and is the sole cathelicidin protein in humans. Recent studies have shown that cathelicidins, in addition to being antimicrobial, are multifunctional proteins with receptor-mediated effects on eukaryotic cells and activity in chemotaxis, angiogenesis, and wound healing (Zaiou *et al*, 2003; Zanetti, 2004). In the skin, there is low constitutive expression of hCAP18 in the basal layer of keratinocytes but rapid upregulation upon inflammation and injury (Frohm Nilsson *et al*, 1999; Dorschner *et al*, 2001; Heilborn *et al*, 2003).

Molecular mechanisms controlling the expression of *CAMP* are still poorly understood. We have investigated whether its expression could be influenced by agents that affect the proliferation and differentiation of skin keratinocytes. Human neonatal epidermal keratinocytes (Cascade Biologics, Portland, Oregon) were cultured in EpiLife serum-free keratinocyte growth medium (Cascade Biologics) containing growth supplements and a calcium concentration of 0.06 mM. At 60% confluency, the agents assayed were added to the medium and cells were harvested after 24 h. RNA was extracted and reverse transcribed by standard methods, and the expression was quantified by Real-Time RT-PCR on an ABI Prism 7700 (Applied Biosystems, Foster City, California) using 5 ng of cDNA according to standard protocols. Sequences were 5′-GTCACCAGAGGATTGTGACTTCAA-3′ and 5′-TTGAGGGTCACTGTCCCCATA-3′ for the primers, and 6-FAM–5′-CCGCTTCACCAGCCCGTCCTT-3′–BHQ1 for the fluorigenic probe.

An upregulation of *CAMP* of about one order of magnitude was achieved by treatment with 100 nM MC903/calcipotriol, a vitamin D analog applied for psoriasis treatment (Kragballe, 1995). Calcium is known to regulate major functions of the epidermis including terminal differentiation. Pretreatment of cells by 1.5 mM calcium for 48 h increased the expression by about 1.5-fold, and was synergistic to the effects of MC903 (Fig 1*a*). Based on these findings, we assayed the effect of vitamin D and its metabolites (all from Fluka, Buchs, Switzerland). Both biologically active forms of vitamin D_3, i.e., $1,25(OH)_2D_3$ and $25(OH)D_3$, stimulated *CAMP* expression at the same magnitude as MC903. The corresponding vitamin D_2 analogs were slightly less efficient. All compounds were active down to levels of 10 nM (shown for $1,25(OH)_2D_3$). The precursor of vitamin D biosynthesis, 7-dehydrocholesterol (7-DHC), was ineffective. Western blot analysis confirmed that the elevated transcription was reflected on the protein level (Fig 1*b*).

An *in silico* analysis revealed two putative vitamin D responsive elements (VDRE) of the DR3 type, and one putative heterodimer site of the DR5 type, within 1 kb upstream of the transcription start (Table I). This region was subcloned

into a luciferase construct (pGL2-Basic) (Promega, Madison, Wisconsin) and its promoter activity assayed by transfection into the human keratinocyte line HaCaT (Boukamp *et al*, 1988). Treatment of the HaCaT cells with $1,25(OH)_2D_3$ resulted in a 3-fold stimulation of the luciferase activity. By combining 5′-deletions in the promoter, and 10-bp deletions or point mutations, the crucial element was located in VDRE1 at −494/−480, the deletion or mutation of which removed 90% of the stimulatory effect (Fig 1*c*). In all mutation constructs, a small responsivity (15% of the vitamin D stimulatory response) to $1,25(OH)_2D_3$ remained, indicating that minor effects outside VDRE1 contributed to the overall effect. While this manuscript was being finalized, Wang *et al* (2004) reported the stimulation of antimicrobial peptide expression by $1,25(OH)_2D_3$, the responsive element described being identical with VDRE1.

To assay the stimulation of *CAMP* expression *in vivo*, we applied MC903/calcipotriol to the skin of 4 healthy individuals. Twenty-five micrograms calcipotriol in 0.5 g ointment (Daivonex, LEO Pharma, Malmö, Sweden) was applied to a test area of 2 × 2.5 cm localized on the lower abdomen. Vaseline (ACO, Stockholm, Sweden) served as control. Punch biopsies were obtained after 24 h and frozen instantly. All participants gave their written informed consent. The study was approved by the Regional Committee of Ethics and was conducted according to the Declaration of Helsinki Principles. RNA was extracted and *CAMP* expression determined with Real-Time RT-PCR as described above. Although varying between probands, a clear tran-

Table I. Putative VDR binding sites within the hCAP18 promoter

Putative VDRE	Sequences	Coordinates of binding sites
VDRE 1 (+)	CTGCTTCCCG **GGTTCA** ATG **GGTTCA** AGTGATTCTC	−494/−480
	cca	
VDRE 2 (−)	TCTCAGGTGA **TCCGCC** CACCT **TGGCCT** CCCAAAGTGC	−344/−328
VDRE 3 (+)	ATCTCCGGTC **AGGTCT** GGG **AGGTTG** TCAGAGATGA	−104/−90

Displayed are the putative binding sites including 10 bp of the flanking sequences, +/− indicating the sequence orientation. The core binding motifs are indicated in bold. Underlined sequences were deleted in the *in vitro* mutagenesis studies, and the small letters show the sequences introduced as point mutations.
VDRE, vitamin D responsive elements.

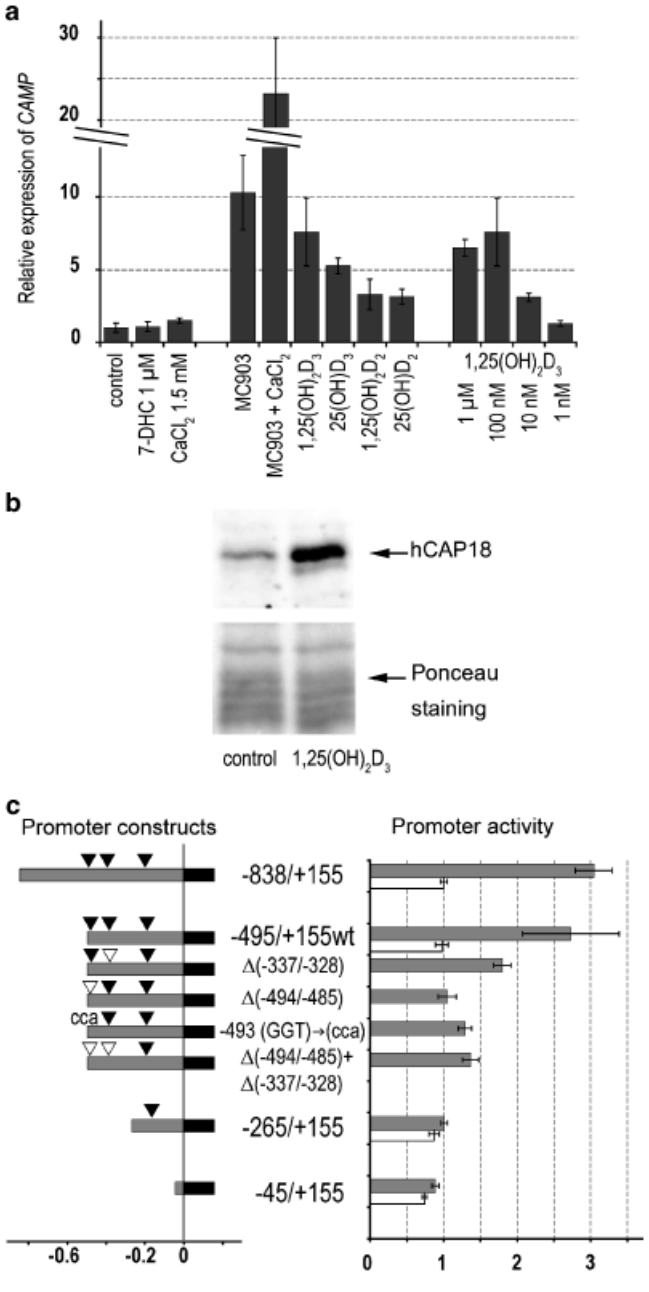

Figure 1
Stimulation of *CAMP* expression by vitamin D in human keratinocytes. (*a*) Expression of *CAMP* RNA in primary keratinocytes as quantified by Real-Time RT-PCR is shown relative to the untreated control. Keratinocytes were grown and treated as described above, for vitamin D analogs at 100 nM if not stated otherwise. The samples were evaluated in triplicates, the SD indicated with bars, and normalized by quantification of 18S-RNA (Assay on Demand, Applied Biosystems). (*b*) Western blot analysis of hCAP18 expression after 24 h treatment with 100 nM $1,25(OH)_2D_3$. Protein was extracted in SDS-containing sample buffer, separated on a 15% Tris-Glycine gel and electroblotted onto nitrocellulose according to standard protocols (Ausubel *et al*, 2003). Filters were reversibly stained with a 3% Ponceau S (Sigma, Sigma-Aldrich, St. Louis, Missouri) solution in 3% TCA before incubating with affinity-purified anti-LL-37 antiserum (Heilborn *et al*, 2003) at a 1:1000 dilution. Signals from horseradish peroxidase-conjugated secondary IgG (Santa Cruz Biotechnology, Santa Cruz, California) using enhanced chemiluminiscence (Amersham Biosciences, Piscataway, New Jersey) were captured with a CCD camera (LAS 1000, Fujifilm, Tokyo, Japan). (*c*) Determination of the Vitamin D responsible regions in the *CAMP* promoter. The fragments assayed are shown with their position relative to transcription start site +1, referring to Ensembl Gene ID ENS G00000164047. The position of putative vitamin D responsive elements (VDRE) is indicated as black triangles. White triangles indicate deleted VDRE and mutations are indicated as text. Freshly trypsinized and suspended HaCaT cells were mixed with 2 μg of plasmid DNA precomplexed with Fugene 6 (Roche, Indianapolis, Indiana) according to the manufacturer's description, and seeded in a six-well plate at 1/5 confluence. After 24 h, the cells were treated with $1,25(OH)_2D_3$ dissolved in DMSO, or DMSO in control assays. After a further 24 h, the cells were lysed and luciferase measured in assay systems according to the manufacturer's instructions (Promega). Luciferase activity for each construct in HaCaT cells, unstimulated (white bars) or stimulated with 1 μM $1,25(OH)_2D_3$ (gray bars), is shown relative to the basic activity of the full-length fragment. The basal activity of wild-type and mutagenized fragments −495/+155 is identical and thus shown for wild-type only. The activities are normalized against β-galactosidase, expressed from 100 ng of cotransfected plasmid (pEF1/LacZ, Invitrogen, Paisley, UK). The results represent the mean of three experiments.

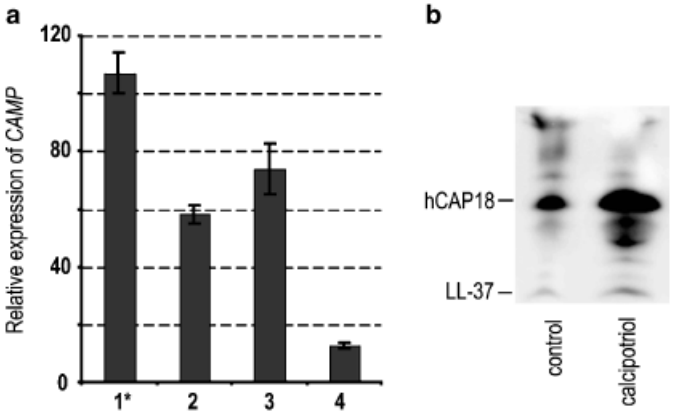

Figure 2
The expression of *CAMP* is upregulated by calcipotriol in human skin *in vivo*. (*a*) Real-Time RT-PCR expression analysis was performed on cDNA from skin biopsies of four probands locally treated with calcipotriol as described above. The stimulation of *CAMP* expression after treatment is shown relative to the respective untreated sample, which is set as 1 (not shown). The biopsy also analyzed by Western blot analysis is marked with an asterisk. (*b*) Western blot analysis on protein extracts of skin biopsies obtained as described above.

scriptional upregulation in all cases up to two magnitudes was observed (Fig 2*a*). For one of the probands, the expression was also assayed by Western blot analysis, which confirmed the upregulation of hCAP18 on the protein level with evidence of the cleaved peptide LL-37 (Fig 2*b*). Topical treatment with $1,25(OH)_2D_3$ also produced upregulation of hCAP1*8* in skin *in vivo* (not shown).

Our results confirm the findings of Wang *et al* (2004) and in addition delineate the effects exerted by different vitamin D compounds. Most importantly, we here demonstrate the upregulation of hCAP18 in human skin *in vivo* following a single application of vitamin D compounds topically. These findings reveal an intriguing mechanism to enhance skin protection, linking the UVB-dependent photochemical vitamin D with hCAP18, involved in wound repair and antimicrobial defense. Under normal conditions, skin is the major source of vitamin D in humans and its production is dependent on ultraviolet radiation. UVB acts on the precursor 7-DHC and forms provitamin D_3, which is thermally transformed to vitamin D_3. Further hydroxylation steps to produce $1,25(OH)_2D_3$ occur in the liver and kidney, but also skin keratinocytes contain the enzymatic machinery to fully hydroxylate vitamin D_3 and produce their own $1,25(OH)_2D_3$ (Bikle *et al*, 1986). If exposure to sunlight is limited, the body depends upon dietary intake of vitamin D_3 from animal food and vitamin D_2 from plants. Since all vitamin D metabolites including vitamin D_2 displayed significant activity, this suggests that vitamin D regulation of hCAP18 may be a conserved mechanism and not restricted to the skin.

Günther Weber,*† Johan D. Heilborn,* Clara I. Chamorro Jimenez,* Anna Hammarsjö,*† Hans Törmä,‡ and Mona Ståhle*
*Unit of Dermatology, Department of Medicine and †Department of Molecular Medicine, Karolinska Institutet, Stockholm, Sweden; ‡Department of Medical Science/Dermatology, University of Uppsala, Uppsala, Sweden

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Address correspondence to: Mona Ståhle, MD, PhD, Unit of Dermatology, Department of Medicine, Karolinska Institutet, S-17176 Stockholm, Sweden. Email: mona.stahle@medks.ki.se

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Reporting Hardy–Weinberg Tests in Case–Control Studies: Reasons for Caution but not for Panic Reactions

To the Editor:

In this journal, Györffy *et al* (2004) recently criticized the failure to report results from statistical tests of Hardy–Weinberg equilibrium (HWE) in some case–control studies, also citing our paper (Richter-Hintz *et al*, 2003). The authors claimed that information about results from testing HWE should be obligatory because deviations of the genotype