Vanilloid Receptor-1 (VR1) is Widely Expressed on Various Epithelial and Mesenchymal Cell Types of Human Skin

To the Editor:

A subset of sensory neurons can be defined by their susceptibility to capsaicin and related vanilloids (Szallasi and Blumberg, 1999). The molecular target of these agents is the vanilloid receptor-1 (VR1), which functions as a calcium-permeable non-specific cation channel (Caterina *et al*, 1997). This receptor can also be activated by heat and acidosis, and by endogenous "endovanilloids" such as arachidonic acid derivatives and eicosanoids (Di Marzo *et al*, 2002). Therefore, VR1 was suggested as a key integrator molecule of various nociceptive stimuli.

In addition to its presence on sensory neurons, functional VR1s have also been identified on various non-neuronal cell types *in vitro*. We have previously shown that activation of VR1 in mast cells (Bíró *et al*, 1998b) and glial cells (Bíró *et al*, 1998a), similar to findings by others on bronchial (Veronesi *et al*, 1999) and uroepithelial cells (Birder *et al*, 2001), resulted in the onset of a variety of cellular processes such as changes in proliferation, apoptosis, differentiation, and cytokine release.

Very recently, a functional VR1 was also identified on human epidermal keratinocytes (NHEK, Denda *et al*, 2001; Inoue *et al*, 2002; Southall *et al*, 2003). It is not clear, however, whether VR1 is also expressed in normal human skin in addition to sensory neurons and keratinocytes. Therefore, in this study, our goal was to characterize VR1 immunoreactivity on epithelial and mesenchymal cells of normal human skin *in situ*.

Normal skin samples (n = 7; trunk, back), obtained during plastic surgery, were used as either frozen or formaldehyde-fixed sections embedded in paraffin (3–5 μm thickness in both cases). To detect VR1, a streptavidin–biotin-complex (SABC) three-step immunohistochemical technique (DAKO, Hamburg, Germany) was employed. Inhibition of endogenous peroxidase activity was performed using 0.5% H_2O_2 in 100% methanol. Non-specific binding of the antibodies was blocked by 2% bovine serum albumin (BSA, Sigma, St Louis, Missouri) in phosphate-buffered saline (pH 7.6). Sections were first incubated with an anti-VR1 goat primary antibody against the N-terminus of VR1 (1:20 dilution, Santa Cruz, Santa Cruz, California), then with a biotin-coupled anti-goat secondary antibody (1:500, DAKO), and, finally, with streptavidin conjugated with horseradish peroxidase (1:400, DAKO). To reveal the peroxidase activity, DAB (Vector, Burlingame, California) or VIP SK-4600 (Vector) was employed as chromogenes. Tissue samples were finally slightly counterstained with hematoxylin Gill I (Surgipath Europe, Peterborough, UK) and mounted with Aquatex (Merck, Vienna, Austria).

In control experiments, the specificity of VR1 staining was assessed by (1) omitting the primary antibody or by incubating the sections with the VR1 antibody pre-absorbed with a synthetic blocking peptide (Santa Cruz) (Fig 1*G*); (2) using another antibody against the C-terminus of VR1 (Santa Cruz), which resulted in an identical staining pattern (data not shown); and (3) performing VR1 immunostaining on frozen skin sections from wild-type C57BL/6J and VR1 knock-out (VR1/KO) B6.129S4-Trpv1 mice (The Jackson Laboratory, Bar Harbor, Maine) (Fig 1*J, K*). In this latter case, a fluorescein-isothiocyanate (FITC)-conjugated secondary antibody was used for visualization. Frozen sections of rat spinal cord were used as positive tissue controls (Fig 1*H, I*).

For double immunohistochemistry, frozen skin sections were first labeled to detect VR1 as described above and then were again blocked using 2% BSA. To detect mast cells or dendritic cells, sections were incubated with either a monoclonal mouse anti-human mast cell tryptase antibody (1:50, DAKO) or with a monoclonal mouse anti-CD1a (a dendritic cell-specific marker) antibody (1:20, Novocastra, Newcastle upon Tyne, UK), then with biotin-conjugated anti-mouse secondary antibody (1:500, DAKO), and, finally, with alkaline phosphatase-conjugated streptavidine (1:50 dilution, DAKO). Endogenous alkaline phosphatase activity was blocked using Levamisole (Sigma), and Fast blue BB (Sigma) was applied as a chromogene.

To detect VR1 mRNA expression, skin homogenates were pulverized in liquid N_2. Total RNA was then isolated using TRIzol (Invitrogen, Paisley, UK) and was reverse transcribed strictly following the procedure described before (Southall *et al*, 2003). PCR amplification was performed using human VR1-specific primers: sense, 5′-ctcctacaacagcctgtac-3′; antisense, 5′-aaggcccagtgttgacagtg-3′ (RT-PCR).

To detect VR1 protein expression, skin homogenates (60–80 μg protein) were subjected to SDS-PAGE as described before (Lázár *et al*, 2003). VR1 expression was determined by immunoblotting using the above goat anti-VR1 antibody, a horseradish peroxidase-conjugate rabbit anti-goat secondary antibody (BioRad, Wien, Austria), and enhanced chemiluminescence (Amersham, Little Chalfont, England). For the RT-PCR and western blot analyses, cultured NHEK and HaCaT keratinocytes were used as VR1-expressing positive controls (Denda *et al*, 2001; Southall *et al*, 2003). The study was approved by the

Abbreviations: VR1, vanilloid receptor-1; VR1-ir, VR1 immunoreactivity

Institutional Research Ethics Committee and adhered to Declaration of Helsinki guidelines.

Since we are currently investigating in detail VR1 expression and function in human hair biology in a separate study, hair follicle VR1 immunoreactivity patters were ignored in this manuscript (Bodó *et al*, manuscript in preparation).

Using immunolabeling on paraffin-embedded human skin samples, specific VR1 immunoreactivity (VR1-ir) was identified on several cell types of human skin (Table I). The specificity of VR1-ir was approved by using various positive and negative controls (Fig 1*G–I*), including skin sections of VR1/KO mice in which there was a complete lack of VR1-ir (Fig 1*J, K*). With respect to the epidermis, confirming previous data (Denda *et al*, 2001), VR1 was expressed in the epidermal keratinocytes. The VR1-ir pattern, however, was inhomogeneous; i.e., whereas a rather strong cytoplasmic and nuclear VR1-ir was detected in the basal and spinous layers, much weaker signals were found in the suprabasal layers (Fig 1*A*). Of great novelty, VR1 was also expressed in CD1a-positive epidermal Langerhans cells (Fig 1*B*). Epidermal melanocytes, instead, were negative for VR1 (data not shown).

In addition to sensory nerve fibers (which also served as positive controls, Fig 1*C*), we demonstrated VR1-ir, for the first time, on various cells populations of the dermis. A strong VR1-ir was observed on sebocytes (Fig 1*D*) and sweat gland epithelium (Fig 1*E*), on endothelial and smooth muscle cells of skin blood vessels (Fig 1*C–F*), on smooth muscles (Fig 1*D*), and on tryptase-positive dermal mast cells (Fig 1*F*). Connective tissue fibroblasts showed no VR1-ir (Fig 1*C*). There was no difference in the VR1 expression pattern of skin samples of different patients or of different body sites (data not shown). Finally, the presence of VR1 in human skin, both at the mRNA and protein levels, was also demonstrated using Western blotting (Fig 1*L*) and RT-PCR (Fig 1*M*).

The complex functional roles of VR1 signaling in human skin biology and pathology now await dissection and clarification. One straightforward possibility is that VR1, functioning as a calcium-permeable channel (Caterina *et al*, 1997; Szallasi and Blumberg, 1999), upon activation, leads to an increase in intracellular calcium concentration ($[Ca^{2+}]_i$) and hence may initiate calcium-mediated processes. Such calcium-coupled mechanisms were described for urinary epithelial cells (nitric oxide release) (Birder *et al*, 2001), glial cells (proliferation, differentiation, apoptosis) (Bíró *et al*, 1998a), and mast cells and epidermal keratinocytes (pro-inflammatory mediator release) (Bíró *et al*, 1998b; Southall *et al*, 2003). In addition, since most skin cell functions are strongly affected by $[Ca^{2+}]_i$ (Hennings *et al*, 1980; Bikle and Pillai, 1993; Vicanova *et al*, 1998), VR1 may possess a significant role, e.g., in the regulation of keratinocyte differentiation and proliferation. This is supported by our demonstration that the expression of VR1

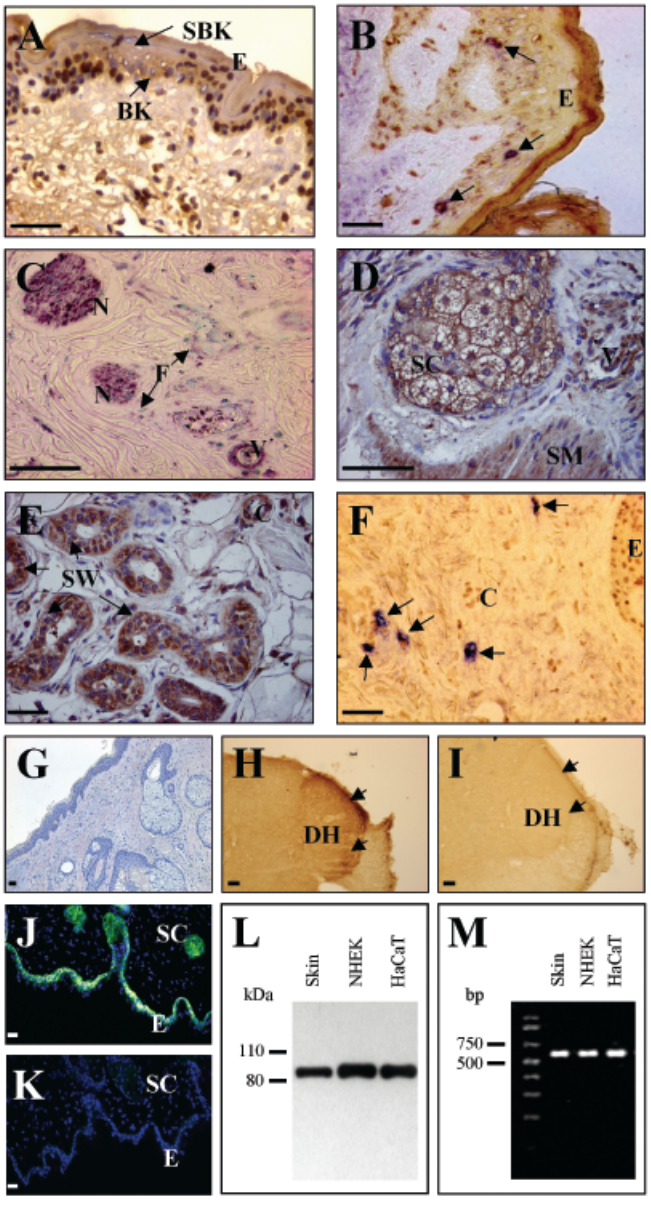

Figure 1
VR1 immunoreactivity and protein and mRNA expression on human skin. (*A*) Immunoreactivity for VR1 (VR1-ir) on epidermal keratinocytes. Note the stronger staining observed on basal (BK) than on suprabasal (SBK) keratinocytes (E, epidermis). (*B*) Co-localization of VR1 (brown) and CD1a (blue) on Langerhans cells (arrow) of the epidermis (E), as revealed by double immunolabeling. (*C*) VR1-ir on nerve fibers (N) and endothelium and smooth muscle cells of dermal blood vessels (V). Note the lack of VR-ir on dermal fibroblasts (F, *arrows*). (*D*) VR1-ir on sebocytes (SC), endothelial and smooth muscle cells of blood vessels (V), and on smooth muscle of dermis (SM). (*E*) VR1 expression on sweat gland epithelium (SW, *arrows*) (C, capillary). (*F*) Co-localization of VR1 (brown) and mast cell-specific tryptase (blue) on dermal mast cells (*arrows*) (E, epidermis; C, capillary). (*G*) Negative control. Specificity of staining was assessed by incubating skin sections with the VR1 antibody pre-absorbed with a synthetic blocking peptide. (*H*) Positive control. VR1-ir (*arrows*), as observed on the dorsal horn (DH) of rat spinal cord. (*I*) Negative control. Lack of VR1-ir (*arrows*) on dorsal horn (DH) of rat spinal cord when stained with the VR1 antibody pre-absorbed with a synthetic blocking peptide. (*J, K*) VR1-ir on skin of wild type C57BL/6J (*J*) and VR1/KO (*K*) mice (an FITC-conjugated secondary antibody was used for visualization). E, epidermis; SC, sebocytes. (*A, C, D, E, G*). Paraffin-embedded sections. (*B, F, H, I–K*) Frozen sections. In most cases, DAB was used to develop VR1-ir, except for *C*, where VIP SK-4600 was applied as a chromogene; and *C*, where VIP SK-4600 was applied as a chromogene. Original magnifications, *A–F*: × 400; *G*: × 40; × *H–K*: × 100. (*L*) Western blot analysis of VR1 protein expression (approximately 90 kDa) in human skin homogenates and in NHEK and HaCaT keratinocytes. (*M*) RT-PCR analysis of VR1 mRNA expression (predicted size of approximately 680 base pairs, bp) in human skin homogenates and in NHEK and HaCaT keratinocytes.

Table I. Vanilloid receptor-1 (VR1) immunoreactivity (VR1-ir) on various cell types of human skin

Cell	Type	VR1-ir
Epidermis		
Basal keratinocytes	E	+ + +
Suprabasal keratinocytes	M	+
Melanocytes	M	–
Langerhans cells	M	+ +
Dermis		
Mast cells	M	+ +
Sweat gland epithelium	E	+ + +
Sebocytes	E	+ + +
Endothelial cells	M	+ +
Smooth muscle cells	M	+ + +
Connective tissue fibroblasts	M	–

Intensity of VR1-ir: –, no; +, weak; + +, medium; + + +, strong. E, neuroectodermal; M, mesenchymal.

among epidermal keratinocytes appeared to be linked to their distinct differentiation status (Fig 1*A*). In fact, activation of VR1 by capsaicin in cultured HaCaT keratinocytes results in a concentration-dependent inhibition of proliferation (Bíró *et al*, unpublished observations).

Our findings may possess even therapeutic significance. The VR1 agonist capsaicin was previously described to act exclusively indirectly on non-neuronal cells of skin via the release of various neuropeptides from sensory neurons (Bíró *et al*, 1998b; Szallasi and Blumberg, 1999; Townley *et al*, 2002). This study, however, clearly argues for that activators of VR1 signaling can directly target cutaneous structures other than sensory neurons. Therefore, the "dual" activation of VR1 by exogenous capsaicin or "endovanilloids" on neuronal and non-neuronal cell types of the skin likely results in the simultaneous release of neuropeptides from sensory axons and of other mediators (e.g., histamine, pro-inflammatory cytokines) from keratinocytes, mast cells, or endothelial cells. This could activate a complex, multi-directional signaling cascade augmenting the action of the VR1 agonist. No wonder, therefore, that capsaicin application was found to be most effective in the treatment of chiefly histamine-dependent and/or neurogenic pruritic skin disorders (Greaves and Wall, 1996; Bíró *et al*, 1997; Ständer *et al*, 2001, 2003).

In conclusion, in this study, we presented evidence that VR1-ir is expressed not only on epidermal keratinocytes of normal human skin (Denda *et al*, 2001) but also by neuroectodermal and mesenchymal cell types such as Langerhans cells, sebocytes, sweat gland epithelium, endothelial and smooth muscle cells of skin blood vessels, and mast cells. This widespread, but certainly not ubiquitous, VR1 protein expression pattern suggests multiple, previously unappreciated additional functions for VR1-mediated signaling, well beyond nociception.

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Note added in proof: During the revision process of this manuscript, we were intrigued to learn that a similar study was performed by Ständer *et al* (2004), confirming our findings presented above.

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