Significance of *N*-Methyl-D-Aspartate (NMDA) Receptor-Mediated Signaling in Human Keratinocytes

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Increasing data suggest that glutamate might act as a cell-signaling molecule in nonneuronal tissues such as the skin. Here we demonstrate the presence of functional N-methyl-D-aspartate (NMDA)-type glutamate receptors in human keratinocytes. NMDA receptor expression strongly reflects the degree of cell-to-cell contact. Wounding polarizes the expression of NMDA receptors in keratinocytes involved in re-epithelialization, and the process of re-epithelialization is inhibited by NMDA receptor activation. We also demonstrate that squamous cell carcinomas lack NMDA receptors. Our data suggest that Ca^{2+} entry through NMDA receptors influences the cycle of keratinocyte proliferation, differentiation, and migration during epithelialization. Moreover, NMDA receptor activation might play a role in contact-mediated inhibition of growth, a process that is absent during neoplastic pathology. This receptor may serve as a pharmacological target for modulating keratinocyte behavior and treating cutaneous disorders. J. Cell. Physiol. 200: 309-317, 2004. © 2004 Wiley-Liss, Inc.

The N-methyl-p-aspartate (NMDA) receptor, a type of ionotropic glutamate receptor, plays a prominent role within the central nervous system (CNS). NMDA receptors are heteromeric complexes comprising some combination of NR1, the obligatory subunit for a functional receptor, and NR2 subunits (Monyer et al., 1992). This complex forms a channel that has high permeability to Ca²⁺ and displays voltage-dependent blockade by Mg²⁺ ions (Mayer and Westbrook, 1987). The influx of Ca²⁺ through the NMDA receptor can initiate a variety of intracellular second messenger cascades, and the precise nature of these second messenger cascades is in a large part determined by the amount and duration of NMDA receptor activation (Sattler and Tymianski, 2001). In the CNS, NMDA-mediated Ca²⁺ entry influences widespread events such as gene expression, circuit development, synaptic plasticity, and cell survival (Bliss and Collingridge, 1993; Choi, 1994; Scheetz and Constantine-Paton, 1994; Tsien et al., 1996; Dalva et al., 2000; Sattler and Tymianski, 2001; Takasu et al., 2002). Thus, the presence of NMDA receptors is important for regulating a myriad of Ca²⁺-mediated cellular events, and these events can be shaped by the number or location of NMDA receptors activated by glutamate (Sattler and Tymianski, 2001).

Recent data have raised the exciting possibility that glutamate receptor-mediated signaling can occur in non-neuronal tissues, including skin (Nordlind et al., 1993; Morhenn et al., 1994; Cooper et al., 1998; Genever et al., 1999; Skerry and Genever, 2001). Glutamate is concentrated at high levels in the skin with wounding or inflammation (Albina et al., 1993; Nordlind et al., 1993); an additional source of glutamate might be provided by glutamate spillover from peripheral innervation of the epidermis (Fagan and Cahusac, 2001). Previous findings demonstrate that intracellular changes in Ca²⁺

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concentration can help regulate the cycle of keratinocyte proliferation, differentiation, and migration that occur during epidermal renewal and wound re-epithelialization (Li et al., 1995; Martin, 1997). Currently, the mechanism of Ca²⁺ entry into keratinocytes is poorly understood, although there is evidence that Ca²⁺ entry may occur through non-specific cation channels (Bikle et al., 1996; Koegel and Alzheimer, 2001). We hypothesized that (1) human keratinocytes express functional NMDA receptors that serve as novel sites for Ca²⁺ entry, and (2) the activation and regulation of NMDA receptors might influence a number of Ca²⁺-dependent phenotypic keratinocyte alterations, such as those observed during wound repair and neoplastic events.

In the current study, we investigated the expression pattern of NMDA receptors on human keratinocytes and tested the possibility that these receptors could conduct Ca^{2+} -mediated currents. We demonstrate that human keratinocytes express functional NMDA receptors. Moreover, cell-to-cell contact initiated signaling appears to trigger the surface expression of NMDA receptors. We demonstrate that the expression of NMDA receptors in keratinocytes can be altered by injury in a human bioengineered skin construct (BSC), and the process of epithelial outgrowth can be inhibited by NMDA receptor activation. Finally, we demonstrate that cutaneous squamous cell carcinomas lack expression of the NMDA receptor. These data suggest that the NMDA receptor-mediated cell signaling pathway in keratinocytes may be involved in epithelialization and contact inhibition of growth. Thus, the NMDA receptor may serve as a pharmacological target for regulating keratinocyte biology and treating certain cutaneous disorders.

MATERIALS AND METHODS Immunocytochemistry (ICC)

Cutaneous samples were harvested from excess tissue of inframammary reduction surgery and surgical cones of non-carcinomatous excisions. BSC was provided by Organogenesis (Canton, MA). The cutaneous squamous cell carcinoma sample was obtained from the pathology services of the Roger Williams Medical Center at Rhode Island. Normal human skin tissue, BSC, and squamous cell carcinoma specimens were allowed to fix in 10% buffered formalin for 24 h and were paraffin embedded. Paraffin-embedded blocks were cut on a microtome in 4 µm sections onto glass slides. Tissue specimens on slides were subsequently deparaffinized. Serial steps of methanol/ H₂O₂ block and antigen retrieval (0.01 M citric acid, pH 6.0 with steam for 20 min) were performed. For cultures, keratinocytes grown on glass slides were allowed to air dry for 24 h. Slides with keratinocytes were then placed in cold (4°C) acetone for 10 min, allowed to air dry, and rinsed with cold (4°C) PBS.

For both tissues section and cultured cells, a soak in one time PBS with blocking normal horse serum incubation was performed. Primary antibody (mouse monoclonal NR1, directed against amino acids 660–811, BD Pharmingen, San Diego, CA) incubation (1 h) and subsequent steps were performed at room temperature. Secondary antibody (biotinylated horse antimouse IgG; 1:200) incubation was carried out for 1 h.

Tertiary antibody with peroxidase conjugated streptavidin diluted 1:400 was incubated on the slides for 1 h. The peroxidase reaction was developed using a 3-amino-9-ethylcarbazole (AEC) substrate chromogen system, which produced a red color in the positively stained cells. A counter stain with hematoxylin for 10 sec was performed. Negative controls were produced by omission of the primary antibody.

Cultured keratinocytes

Cultured keratinocytes were harvested from neonatal foreskin explants, plated on glass slides in Defined Keratinocyte-SF media (Gibco, Grand Island, NY), and grown in either a confluent, semiconfluent, or nonconfluent manner as previously described (Hawley-Nelson et al., 1980; Morris, 2000). Confluent cells were defined as having their entire cell membrane surface in contact with other keratinocytes. Semiconfluent cells were defined as being in contact with at least one other keratinocyte but not in a confluent manner. Non-confluent keratinocytes were defined as having no cell-to-cell contacts.

Meshing

BSC was meshed with a standard mechanical mesher (1.5:1.0). Specimens were allowed to incubate for 24 h at 5% $\rm CO_2$ and 95% room air at 37°C. Specimens were harvested and fixed in 10% formalin for ICC.

Calcium imaging

Cultures were loaded with 4 µM fluo-4 AM (Molecular Probes, Eugene, OR) that was reconstituted in DMSO and Pluronic F-127 for final concentrations of 0.09% and 0.005%, respectively. After 45-60 min incubation in fluo-4, cells were placed in saline medium as above but with 2.8 mM CaCl2, no MgCl2, and 1 μM glycine. Frames were acquired every 250 msec for 30 sec on an Olympus Fluoview FV300 confocal laser scanning microscope equipped with a 40× objective (NA 0.6). The Ca²⁺ indicator was excited by an argon laser at 488 nm, reflected by an excitation dichroic mirror, and detected by a photomultiplier tuned to specimens with a wavelength shorter than 570 nm. Fluorescence, in arbitrary units, was measured and normalized to the average baseline fluorescence (F_0) collected over 4 sec. The postbaseline fluorescence (F₁) was measured before and after 4 sec of picospritzing saline media or 1 mM NMDA (made in saline media) through a borosilicate pipette positioned proximal to the analyzed keratinocytes. Some Ca²⁺ fluorescent measurements were carried out after NMDA application in the presence of bath applied (1 h) 100 µM APV (DL-2-amino-5-phosphono-valeric Acid; Sigma, St. Louis, Missouri) or 10 μM MK-801 (Sigma, St. Louis, MO). The change in fluorescence (F $_1\!/$ F_0) was used to assess Ca^{2+} responses. Phase contrast images were visualized using laser differential interference optics.

Statistical analyses were performed with StatView 5.0.1 (Abacus Concepts, Berkeley, CA). Potential group differences with respect to treatment group were tested by a one factorial ANOVA and follow-up, pair-wise contrasts were made using the Fisher's Protected Least Square Differences post-hoc comparison. Significance

was set at P < 0.05.

Epiboly assay

Six-millimeter punch biopsies of a BSC were placed into 6-well plates with 3 ml of AIM-V serum-free media (Invitrogen Corp., Grand Island, NY) and incubated at 37° C with 5% CO $_2$ for 72 h. The wafers of BSC were incubated in the AIM-V media with various concentrations of Ca $^{2+}$ (0.02, 0.15, and 1.8 mM) and with and without the presence of 100 μ M NMDA. Tissue specimens were formalin-fixed and paraffin-embedded. Specimens were subsequently sectioned at 4 μ m onto glass slides and stained with hematoxylin and eosin.

RESULTS NMDA receptor expression pattern in human cutaneous tissues

To characterize the expression pattern of NMDA receptors, we performed ICC using a monoclonal antibody to the NR1 subunit of the NMDA receptor in specimens of normal human skin, cultured foreskin keratinocytes, and BSC. Notably, Western blot analyses demonstrated that the NR1 antibody recognized the same molecular weight protein both in keratinocytes and CA1 pyramidal cells from the brain (data not shown). In normal human skin, we observed strong NR1 staining in the stratum malpighii of the epidermis, eccrine structures, sebaceous glands, and selected fibroblasts. NR1 staining appeared granular and concentrated along the cellular periphery, with the greatest staining intensity in the basal cell layer (Fig. 1A). Confluently grown cultured human keratinocytes also displayed pronounced cell membrane NR1 expression. In these cultured cells, there was increased focal NMDA receptor expression at sites of cell-to-cell contact (Fig. 1B). The epidermis of BSC exhibited a similar pattern of NR1 expression to that found in human epidermis. Strong granular NR1 staining of the viable epithelial keratinocytes was observed in the epidermis of BSC, while specific staining was absent in the non-viable cells of the stratum corneum (Fig. 1C). Negative controls (produced by omission of the NR1 antibody) for normal human skin, cultured keratinocytes, and BSC exhibited no staining (Fig. 1D–F). These data demonstrate that the expression of NR1, the obligatory NMDA receptor subunit, is similar in human keratinocytes both in vitro and in vivo. Moreover, the pattern of NR1 labeling in vivo is recapitulated in the BSC, which has been used as a model for studying keratinocyte outgrowth and wound healing (Falanga and Butmarc, 2002; Falanga et al., 2002).

Cell-to-cell contact increases cell surface NR1 expression in human keratinocytes

Previous studies in neuronal cultures demonstrated changes in the subcellular distribution of NMDA receptors with cell-to-cell contact and activity-dependent processes (Rao and Craig, 1997; Rao et al., 1998; Okabe et al., 2001). Therefore, we examined the pattern of expression of NR1 with ICC in cultured foreskin keratinocytes grown in a confluent, semiconfluent (at least two cells touching each other, but not entirely confluent), and non-confluent (isolated cells) manner. Confluently grown keratinocytes display very high levels of cell surface NR1 expression. Moreover, there is a clustering of cell surface NR1 expression at sites of cell-to-cell contact (Fig. 2A). Cultured keratinocytes grown in a semiconfluent fashion display less generalized cell surface NR1 expression as compared with the levels found in confluently grown keratinocytes. However, some of these cells express NR1, especially at areas

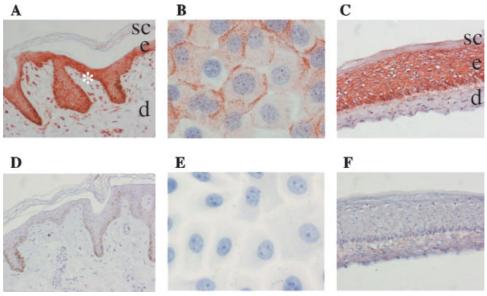
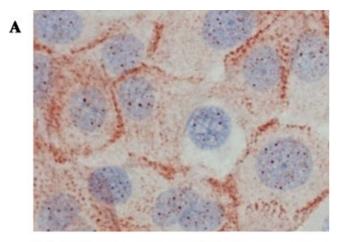
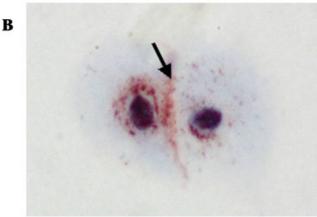
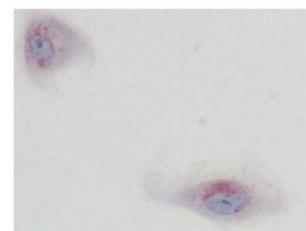


Fig. 1. Human cutaneous tissues express NR1 NMDA receptor subunits. A: Normal human skin demonstrates strong NR1-immuno-reactivity in keratinocytes of the epidermis (e) and selected fibroblasts of the dermis (d). The expression is granular and is most intense in the epidermal basal cell layer (marked by asterisk). Note that stratum corneum (sc) lacks NR1 staining. B: Confluently grown cultured

keratinocytes express strong cell membrane NR1 receptor subunit expression. Note the enhanced NR1 expression at focal areas of cell-to-cell contact. C: BSCs show strong epidermal NR1 subunit expression, while the stratum corneum lacks staining. **D**: Negative control for normal human skin, (**E**) cultured keratinocytes, and (**F**) the BSC. (Copyrighted, V. Falanga, 2003.)







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Fig. 2. The level of NR1 NMDA receptor subunit expression reflects the extent of confluency in cultured human keratinocytes. A: Confluent keratinocytes display strong cell surface NR1 expression. Focal increases in expression are seen at sites of cell-to-cell contact. B: Semiconfluent keratinocytes express NR1 immunoreactivity, especially at sites of cell-to-cell contact (denoted by arrow). C: NR1 immunoreactivity is almost entirely limited to perinuclear regions in non-confluent keratinocytes. (Copyrighted, V. Falanga, 2003.)

of cell-to-cell contact (Fig. 2B). Although, these isolated (non-confluent) keratinocytes do show a perinuclear staining pattern with the NR1 antibody, every isolated cell failed to exhibit cell surface NR1 (Fig. 2C).

NMDA-evoked Ca²⁺ responses in human keratinocytes

We next turned to Ca²⁺-imaging techniques to more thoroughly characterize NMDA receptor functionality and to investigate Ca²⁺ entry through NMDA channels. We used the fluorescent Ca²⁺ indicator fluo-4 to detect Ca²⁺ responses in cultured human keratinocytes before and after a picospritzed (pressure) application of 1 mM NMDA (Fig. 3). Every confluent keratinocyte that we imaged demonstrated a robust Ca²⁺ response after a 4-sec application of 1 mM NMDA (averaged peak F₁/ $F_0 = 4.62 \pm 0.14$, n = 11). To verify that the observed Ca^{2+} responses were specific to NMDA application, we analyzed Ca^{2+} responses before and after application of the saline vehicle alone. We failed to observe a change in fluorescence when the same cells that exhibited NMDA-evoked Ca^{2+} responses were treated with the saline vehicle alone (averaged peak $\mathrm{F}_1/$ $F_0 = 0.96 \pm 0.02$, n = 11; inset Fig. 3E), indicating that the observed Ca^{2+} responses were specific to NMDA (e.g., the responses were not caused by the saline vehicle or a result of mechanical influences from the picospritzing technique). To ensure that these Ca²⁺ responses were specific to the NMDA receptor and not a consequence of non-specific pH-sensitive Ca²⁺ channels, we examined the fluorescent responses in confluently grown keratinocytes after application of 1 mM NMDA (4-sec application) in the presence of bath applied MK-801 (non-competitive antagonist) or APV (competitive antagonist). We failed to detect a change in the fluorescence with NMDA when the same keratinocytes that exhibited NMDA-evoked Ca²⁺ responses were bath treated (for 1 h) with either MK-801 or APV (Fig. 3F).

Because we failed to observe significant surface expression of NR1 in non-confluent keratinocytes, we tested whether NMDA-evoked Ca^{2+} responses could be measured in non-confluent cells. Consistent with our anatomical data, we failed to observe NMDA-evoked Ca^{2+} responses in non-confluent keratinocytes (averaged peak $F_1/F_0=1.03\pm0.02,\,n=12;\,Fig.\,3F).$ To assess the requirements for expression of functional NMDA receptors, we also analyzed Ca^{2+} responses in semiconfluent keratinocytes. NMDA-evoked Ca^{2+} responses were seen in some semiconfluent keratinocytes, but others exhibited no functional NMDA receptors (averaged peak $F_1/F_0=1.66\pm0.28,\,n=11;\,Fig.\,3F).$

ANOVA demonstrated a significant effect of treatment across the four groups: confluent cells after saline application, confluent cells after NMDA application, semiconfluent cells after NMDA application, and nonconfluent cells after NMDA application (F(3,47) = 66.6, P < 0.0001). Post-hoc analyses suggested that the Ca²+ responses were greatest after NMDA application to confluent keratinocytes as compared to the other groups (all P-values < 0.0001). Moreover, Ca²+ responses after NMDA application in semiconfluent cells were significantly greater than Ca²+ responses after NMDA application in non-confluent cells or after saline application in confluent cells (both P-values < 0.03). These data suggest that functional NMDA receptors begin to be expressed on cultured keratinocytes as they become more confluent.

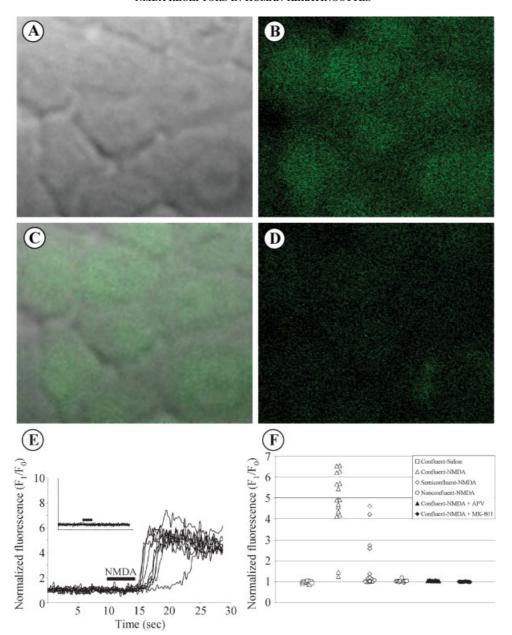


Fig. 3. Fluorescent Ca^{2+} imaging demonstrates that NMDA receptor-mediated entry of Ca^{2+} occurs in confluent but not isolated keratinocytes. A: Low-resolution phase contrast picture of confluent human keratinocytes imaged with fluorescence in $(\mathbf{B}-\mathbf{D})$. B: The summed fluo-4 fluorescent image taken after a 4-sec NMDA application. C: Overlay image of (A) and (B). D: The summed fluo-4 fluorescent image taken after a 4-sec application of saline vehicle. E: Normalized fluorescence intensity for individual confluent keratinocytes in response to NMDA application. Inset depicts fluorescence

intensity of individual keratinocytes before and after application of saline vehicle (indicated by black bar). **F**: Scatter plot of the 5-sec averaged peak fluorescent response of individual cells in six experimental conditions: confluent cells after saline application, confluent cells after NMDA application, semiconfluent cells after NMDA application, non-confluent cells after NMDA application, confluent cells after both NMDA and APV application, and confluent cells after both NMDA and MK-801 application. (Copyrighted, V. Falanga, 2003.)

Wounding in BSCs polarizes NMDA receptor expression in migrating keratinocytes

Recent studies have demonstrated that BSC can serve as a wounding model since it can re-epithelialize once it has been injured (Falanga et al., 2002). Moreover, phenotypic alterations of keratinocytes occur in stratified epithelium when they are pushed into a migrating

or re-epithelialization pattern. Using ICC for NR1 and the BSC wound model, we examined whether NMDA receptor expression pattern is altered in the migrating keratinocytes involved in re-epithelialization. Twenty-four hours after injury by meshing in BSCs, suprabasal keratinocytes at the migrating epidermal lip expressed NR1 in a polarized pattern (Fig. 4A); this is in obvious contrast to the normal uniform distribution of NR1 as

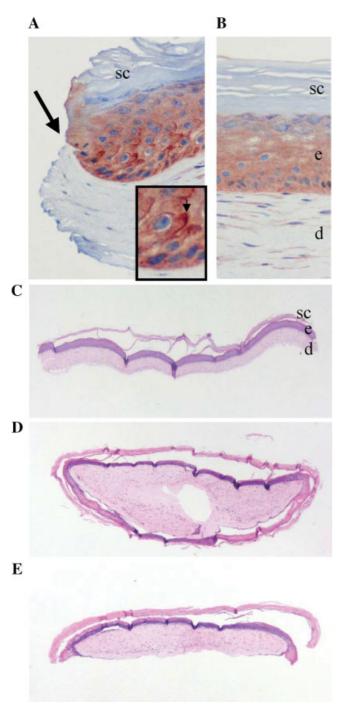


Fig. 4. Wounding polarizes NMDA receptor expression and NMDA receptor activation inhibits keratinocyte outgrowth. A: NR1-immunoreactivity at the wounded or cut surface (denoted by large arrow) of BSC (sc, stratum corneum). Inset is a high power magnification of suprabasal keratinocytes demonstrating polarized NR1 expression (denoted by small arrow). B: Representative portion of the BSC distal to the wound site. No evidence of polarization of NR1 is seen. (e, epidermis; d, dermis) C: A 6-mm circular wafer from a BSC. D: A BSC specimen after being incubated in serum-free media with low calcium concentrations for 72 h. Note keratinocyte outgrowth extends over the entire dermal surface. E: Chronic NMDA application blocks the keratinocyte outgrowth that normally occurs in the serum-free media. (Copyrighted, V. Falanga, 2003.)

seen in the middle of the construct (Fig. 4B). After meshing, NR1 expression clustered on the membrane surface opposite to the wounded site, such that NR1 was localized opposite to the direction in which the keratinocytes were migrating.

NMDA application inhibits epiboly

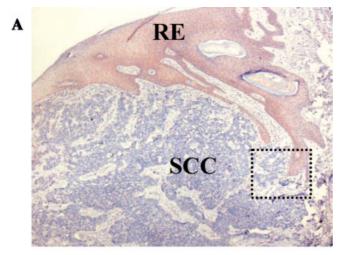
The process of keratinocyte outgrowth, known as epiboly, has been recently studied using a BSC epithelial migration assay. We used this BSC model to examine the role of NMDA receptors in keratinocyte outgrowth. Six-millimeter circular wafers of BSC (Fig. 4C) exhibit robust epidermal outgrowth after 72 h in serum-free media containing various concentrations of Ca²⁺ (0.02, 0.15, and 1.8 mM; n = 4 per group; Fig. 4D). Three days of NMDA treatment (100 µM) in serum-free media dramatically inhibited epiboly occurring in 0.02-1.8 mM Ca^{2+} as compared to controls (Fig. 4D,E; n=4 per group). Although, there was some minimal basal migration after NMDA application, there was no evidence of strong epidermal outgrowth (Fig. 4E demonstrates lack of epiboly with NMDA treatment in 0.15 mM Ca²⁺). These data suggest that NMDA receptor activation prevents epidermal outgrowth and migration.

Cutaneous squamous cell carcinomas lack NMDA receptor expression

In order to determine whether the NMDA receptor pathway is dysfunctional in a malignant tumor of epithelial keratinocytes, we examined the expression pattern of NR1 in a primary cutaneous squamous cell carcinoma. We found that the body of this squamous cell carcinoma lacked NR1-IR (Fig. 5). However, the reactive epithelium (non-neoplastic) surrounding the squamous cell carcinoma showed strong NR1 expression. Thus, NR1 staining clearly delineated the boundaries between the reactive and malignant epithelium. These data suggest that NMDA receptor signaling may be important for keratinocyte differentiation and also play a role in contact-mediated inhibition of growth that is lacking in most cutaneous neoplastic processes. However, these data cannot distinguish between the possibilities that a loss of NMDA receptor expression is a cause or a consequence of the malignant state.

DISCUSSION

The results shown in this report demonstrate the presence and describe the expression pattern of NMDA receptors in keratinocytes of normal human skin, foreskin cultures, and human-derived BSCs. We provide evidence that these NMDA receptors can conduct currents and serve as a novel site of Ca²⁺ entry into keratinocytes. The expression of functional NMDA receptors on keratinocytes is influenced by cell-tocell interactions; increasing cell-cell contact promotes NMDA receptor expression. Wounding alters the expression of NMDA receptors in suprabasal keratinocytes that are involved in re-epithelialization, and NMDA receptor activation inhibits keratinocyte outgrowth necessary for such epithelialization processes. Finally, we demonstrate that primary squamous cell carcinomas lack NMDA receptor expression. We suggest that Ca² entry through NMDA receptors might influence the cycle of keratinocyte proliferation, differentiation, and



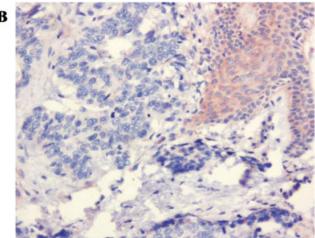


Fig. 5. Squamous cell carcinomas lack NR1-immunoreactivity. A: Pattern of NR-immunoreactivity in a primary squamous cell carcinoma. Note that the invasive aspect of the carcinoma, which lacks NR1 staining, can be clearly differentiated from the reactive surrounding epithelium (SCC, squamous cell carcinoma; RE, reactive epithelium). B: High magnification from the border of the carcinoma (boxed area shown in A). (Copyrighted, V. Falanga, 2003.)

migration during epithelialization and may play a role in contact inhibition of growth.

Our demonstration of NR1-staining in human skin is consistent with the observation that NR1-labeling has been previously observed in rat skin (Genever et al., 1999). Notably, embryonic (E14-E16) rat skin displayed NR1-IR throughout all the keratinocyte layers in a manner similar to normal human skin, while neonatal and adult rat skin displayed NR1 labeling selectively in basal layers (Genever et al., 1999). Although, there have been many observations in murine epidermis that have proven to be valid in human epidermis (Byrne et al., 1994), the marked difference between NR1 staining patterns in adult rat and human skin compelled us to use only human cutaneous tissues for our characterizations of NMDA receptors.

Our findings support the evidence that cultured human keratinocytes have binding sites to MK-801 (NMDA receptor antagonist) and express NR1 mRNA (Morhenn et al., 1994). The BSCs also display strong levels of NR1 expression, and the expression pattern of NR1 is strikingly similar to that found in normal human skin. It was important to establish that keratinocytes in cultured human foreskin and BSCs have similar NR1 expression to that of human skin, because these preparations lend themselves to in vitro manipulation and allowed us to further examine NMDA receptor function in human keratinocytes.

Although NR2(A-D) subunits are innately involved in forming the heteromeric complexes of the NMDA receptor, we chose to examine the expression pattern of the NR1 subunit in human skin, because it is the fundamental subunit of NMDA receptors. It has been shown that NR1 subunits can form homomeric receptors that can flux current, while NR2 subunits cannot form functional receptors by themselves (Moriyoshi et al., 1991; Monyer et al., 1992). However, when NR2 are expressed with NR1 subunits they increase the current response by several orders of magnitude (Ozawa et al., 1998). Although we do not directly demonstrate the presence of NR2 expression in human keratinocytes, we speculate that human keratinocytes express heteromeric NMDA receptors, because we found strong NMDA-evoked responses with our cultured keratinocytes. In addition, the expression of colocalized expression of NR2 subunit with NR1 staining has been established in rat skin (Genever et al., 1999) also suggesting the presence of a heteromeric NMDA receptor

Interestingly, cell-to-cell contact promotes NMDA receptor expression; increasing cell-to-cell contact (confluency) in cultured keratinocytes was associated with greater levels of generalized and focal NMDA receptor expression. There is precedent that cell-to-cell contact can mediate the surface expression of receptors (Gebbink et al., 1995). This can be due to the fact that cell-to-cell contact promotes expression of new receptors, or that cell-to-cell contact can block the constitutive removal of surface expressed receptors. In neurons, there is evidence for both the regulated insertion and removal of cell surface NMDA receptors (Quinlan et al., 1999; Snyder et al., 2001). However, before being transferred to the cell membrane surface, recently made NMDA receptors must assemble and be delivered through the endoplasmic reticulum-Golgi secretory pathway. Although, we have no direct evidence of the subcellular localization of NMDA receptors, the perinuclear staining in isolated keratinocytes suggests that NMDA receptors may reside in a prepackaged form in the endoplasmic reticulum-Golgi apparatus. Perhaps, these receptors contain an endoplasmic reticulum retention signal, as has been observed with some neuronal NMDA receptors (Scott et al., 2001). We demonstrate that cell-to-cell contact can cause delivery and/or stabilization of NMDA receptors on the keratinocyte surface, not unlike that observed with neuronal synaptic maturation. The mechanisms underlying NMDA receptor surface expression in keratinocytes remain unclear and needs to be elucidated.

We felt that it was important to perform a direct test of the functionality of NMDA receptors. Therefore, we measured NMDA-evoked Ca²⁺ response. There have

been very few studies examining non-cancerous keratinocytes using whole-cell patch-clamping (Galietta et al., 1991; Mauro et al., 1997; Brouard et al., 1999). Calcium imaging techniques proved to be more reliable for assessing NMDA receptor function. Using this method, we demonstrated that human keratinocytes are functional, as NMDA application evokes Ca²⁺ responses and antagonists to the NMDA receptor block them. Moreover, NMDA-evoked Ca²⁺ responses correlate to the degree of cell-to-cell contact; increasing cell contact promotes greater NMDA-mediated Ca²⁺ responses. These data support our anatomical evidence that cell-to-cell contact increases surface expression of NR1. To our knowledge, the data shown here are quite novel in showing that Ca²⁺ entry can occur through NMDA receptors on keratinocytes. The finding that NMDA receptors can serve as a novel site of Ca²⁺ entry into keratinocytes is exciting; as currently, mechanisms of Ca²⁺ entry into keratinocytes are poorly understood (Bikle et al., 1996).

Glutamate is present in the keratinocytes of normal skin and may be particularly expressed at high levels during skin wounding and inflammation (Albina et al., 1993; Nordlind et al., 1993), making it highly probable that functional NMDA receptors in keratinocytes are activated endogenously. The number and localization of NMDA receptors on the cell surface can be a mechanism for finely regulating Ca²⁺ entry into the cell and may, therefore, control keratinocyte biology. It was, therefore, of interest that the wounding of BSC polarizes expression of NR1 in migrating suprabasal keratinocytes at the advancing epidermal lip. This polarized expression pattern occurs 24 h after meshing the BSC. These suprabasal keratinocytes with polarized NMDA receptor expression are akin to those found in human wounds undergoing re-epithelialization. Notably, the process of re-epithelialization in human wounds involves a complex dissolution of hemidesmosomes and the expression of specific integrins on moving basal cells (Martin, 1997). There is a forward progression of basal cells over a wound, and there is evidence that suprabasal keratinocytes leapfrog over these basal cells contributing to wound re-epithelialization (Garlick and Taichman, 1994). These suprabasal cells are also found to express atypical integrins ($\alpha 2$, $\alpha 3$, $\alpha 6$, and $\beta 1$) that are only present during re-epithelialization (Hertle et al., 1992). Interestingly, the expression of certain integrins in neurons has been shown to modulate NMDA receptor expression (Chavis and Westbrook, 2001). One possibility is that the expression of particular cell surface adhesion molecules, such as a unique pattern of integrins, may promote the polarized expression of NMDA receptors that occurs during re-epithelialization.

A functional role for NMDA receptors in regulating keratinocyte migration is suggested by our finding that NMDA inhibits keratinocyte outgrowth. In this context, it is intriguing that cancerous skin completely lacks NMDA receptors. Perhaps the absence of NMDA receptor-mediated inhibition is partially responsible for the unregulated growth of the carcinoma. Future experiments will need to distinguish between the possibilities that a lack of NMDA receptors in the squamous cell carcinoma is the cause of the carcinoma's unregulated growth or is a phenotypic expression of the

unregulated growth. The highly invasive nature and lack of differentiation of this squamous cell carcinoma point to an absence of regulated signaling for such a neoplastic process. The fact that NMDA receptors are lacking in such a primary neoplasm suggests that NMDA receptor-mediated signaling between keratinocytes may play a role in contact inhibition of growth (which is deficient in malignancies) and may participate in keratinocyte differentiation. At the very least, we suggest that NR1-IR can serve as a valuable histological marker for Mohs micrographic surgery as a means to verify the complete surgical excision of primary squamous cell carcinomas.

We have established that NMDA receptor signaling in keratinocytes is important in epidermal migration, outgrowth, and possibly differentiation and contact inhibition. In addition to our direct demonstration of the importance of NMDA receptor activation in keratinocyte behavior, several indirect studies suggest that changes in keratinocyte behavior might be explained due to modulation of the NMDA receptor-mediated pathway. For example, topical Mg²⁺ application, which can antagonize NMDA receptor currents, greatly enhances cutaneous barrier recovery in a mouse model (Denda et al., 1999). In addition to Mg²⁺, several other modulators of NMDA receptor activity are also known to regulate keratinocyte phenotypic patterns, including polyamine synthesis, ultraviolet-B light, and EphrinB expression (Gilmour et al., 1999; Leszkiewicz et al., 2000; Takasu et al., 2002). Collectively, these data have led us to propose that the fine regulation of the NMDA receptor-mediated pathway can serve as pharmacologic targets for modulating keratinocyte behavior and altering cutaneous disorders. In the CNS, dysregulation of NMDA receptor-mediated transmission has been implicated in a wide variety of severe neurological diseases, and these pathological states can be caused to be either too much or too little NMDA receptor activation (Chojnacka-Wojcik et al., 2001; Wang et al., 2001; Vajda, 2002). The goal of cutaneous pharmacologic targets would be similar to those sought after in the CNS; that is, to prevent pathological activation of NMDA receptors, but allow their physiological response. Pharmacological targets include NMDA receptor antagonists, glycine site antagonists, NMDA subunit-selective antagonists, polyamine pathways, and changes in the concentration of divalent cations such as Mg²⁺ and Zn²⁺ ions (Parsons, 2001).

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