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| Original Article |

The Effect of Differential Modulation of the N-methyl-D-aspartate Receptor on Growth and Intracellular Calcium Ion Concentration in Normal Human Oral Keratinocytes

Insoo Kim[1]*, Ki-Suk Paik[2], Mi-Sook Chang[2], and Seung-Pyo Lee[2]*

[1]*Div. of Oral and Maxillofacial Surgery, Dept. of Dentistry, Uijeongbu St. Mary's Hospital, The Catholic University of Korea, Gyeonggi-do, 480-717 Korea*

[2]*Department of Oral Anatomy, School of Dentistry, Seoul National University, Seoul, 110-749 Korea*

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Abstracts : The purpose of this study was to examine the interaction between the agents [N-methyl-D-aspartate (NMDA), arachidonic acid (AA), and Nitric Oxide Synthase Inhibitor (NOS-I)] and cultured normal human oral keratinocytes (NHOK) in order to elucidate the mechanisms by which epithelial growth and regeneration are regulated. We also examined whether AA and NOS-I could protect NHOK from glutamate cytotoxicity and the change of intracellular calcium ion concentration. NHOK were obtained from gingival tissue of 20 individuals aged 20 to 29, and third passage (P3) cells were used for this study. Cell viability was measured by the MTT assay and DNA synthesis by the BrdU assay. The microscopic features of NHOK were observed and changes in intracellular calcium ion concentration were measured. NMDA and NNA induced an initial increase in cell number. Low concentration of AA induced an increase in cell number while high concentrations of AA induced a decrease in cell number. The decrease in cell number induced by NMDA at the seventh day was abolished by the addition of low concentrations of AA or NOS inhibitors. Low concentrations of AA or NMDA with high concentrations of AA significantly increased the DNA synthesis rate at four hours. NMDA and AA both induced an increase in intracellular calcium ion concentration, and AA enhanced NMDA-induced intracellular calcium ion in a concentration-dependent manner. NMDA-induced NHOK death was associated with intracellular calcium ion change and the promotion of cell differentiation. Low concentrations of AA protected NHOK cells from NMDA-induced death.

Key words: *N-methyl-D-aspartate, intracellular calcium ion, normal human oral keratinocyte, arachidonic acid, nitric oxide synthase inhibitor*

1. Introduction

Normal human oral keratinocytes (NHOK) are derived from keratinized stratified squamous epithelium. Proliferation of both oral and skin epithelium is primarily accomplished by division of basal cells in the lowest epithelial layer. However, oral epithelium differs from general skin epithelium in several ways, including hairlessness, lack of sweat glands and always being wet, and has characteristics intermediate between general skin and intestinal mucosa. After the epithelium has been disrupted by tissue injury, re-epithelialization must occur as rapidly as possible in order to re-establish tissue integrity.[1] Healing of damaged oral epithelium is a complex process involving a series of controlled events including the formation of a provisional extracellular matrix composed mainly of fibrin, fibronectin and vitronectin and the migration of epithelial cells from the edges of the wound.[2] The proliferation and differentiation of epithelial cells play an important role in cell migration, and the ability to control these processes might be useful in the treatment or reconstruction of pathologic and traumatic wounds, in the control of neoplasm, in the reconstruction of pathologic or embryological defects, and in dental implant treatment. Most epithelial wounds require rapid repair to protect the tissue. However, in certain cases of bone defects or dental implants, rapid growth of epithelium could lead to an

*Tel: +82-31-820-3184; Fax: +82-31-847-2894
e-mail: omskim@catholic.ac.kr (Insoo Kim)
*Tel: +82-2-740-8671; Fax: +82-2-762-6671
e-mail: orana9@snu.ac.kr (Seung-Pyo Lee)

unfavorable outcome, such as bone insufficiency or the interposition of soft tissue between the implant and bone, as a result of the restriction of bone growth or disturbance of the osseointegration between implant and bone.

Glutamate is the major excitatory neurotransmitter at synaptic junctions within the central nervous system. Glutamate receptors are divided into two groups: G protein coupled metabotropic receptors and ionotropic glutamate gated ion channels [classified as N-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA), and kainate receptors according to their pharmacologic responsiveness to these synthetic agonists]. Glutamate has a potent neurotoxic effect and the loss of glutamate transporter function is implicated in the pathogenesis of neurodegenerative disease. Glutamate signaling is predominantly associated with excitatory neurotransmission in the CNS; however, there is increasing evidence that non-neuronal tissues also express glutamate receptors. Keratinocytes and brain are related embryonically, both being derived from the neural tube. The observation that skin denervation significantly inhibits keratinocyte proliferation supports a neuronal influence on keratinocyte function.[3-5] There are several opinions concerning the pathway of epithelial growth and the agents involved in that pathway, such as, stimulation of the sensory nerves in the epithelium by neurotransmitter[6] or regulation of the glutamate receptors participate in the proliferation and differentiation of the epithelium.[3, 7] This suggests potentially novel therapeutic targets for the treatment of skin disease and enhancement of wound healing. However, some investigators insist NMDA influence only on the differentiation of keratinocytes but not on the proliferation.[8] The NMDA receptors in keratinocytes may involve in not only re-epithelialization but also neoplastic pathology.[9, 10] All of these functions may derive from the regulation of the intracellular calcium ion influx by NMDA.[3, 7-10] The exhaustion of the calcium in the endoplasmic reticulum (ER), the reservoir of intracellular calcium, stimulates the proliferation of keratinocyte.[11] Intracellular free calcium concentration is a powerful signal for keratinocyte differentiation and cell cycle withdrawal.[11-14]

In addition to triggering glutamate release and excitotoxic cell death, nitric oxide (NO) can also prevent caspase activation and switch cell death from apoptosis into necrosis.[3] The elevated intracellular Ca^{2+} activates nitric oxide synthesis and that the resulting synthesis of NO reduces the Ca^{2+} response to NMDA, while arachidonic acid (AA) augments these responses. Therefore, arachidonic acid and nitric oxide may play a role in synaptic plasticity, and may differentially modulate MNDA-mediated Ca^{2+} entry.[15] NO is thought to be involved in the fine regulation of NMDA receptors[16-17] and the apoptosis-necrosis switch.[18] NO is an extremely versatile messenger in biological systems, and has been implicated in a number of different physiopathological roles, such as smooth muscle relaxation, platelet inhibition, neurotransmission, immune regulation, cell differentiation, tissue morphogenesis and cytotoxicity. Through S-nitrosylation or nitration of proteins, NO can be a bifunctional modulator of cell death, capable of either triggering or inhibiting cell death. In the excitotoxic death of cultured neurons, NO-triggered apoptosis requires a Ca^{2+} signal triggered by the activation of the NMDA receptor channels.[18, 19] It is well known that the neuronal glutamate-NO pathway modulates several important physiological processes and that NO is an intermediary in the action of glutamate. Activation of NMDA receptors increases intracellular Ca^{2+} in the postsynaptic neuron; this calcium binds to calmodulin and activates NOS, stimulating the formation of NO.[20]

Many researchers have studied the relationships between the change in intracellular calcium ion concentration, glutamate receptors and epithelial growth; however, there are some controversies and no one clarified the relationships between intracellular calcium ion concentration, glutamate receptors, and the growth of oral keratinocytes.

In the present study, we investigated the effects of N-methyl-D-aspartate (NMDA), arachidonic acid (AA) and nitric oxide synthase inhibitor (NOS-I), alone or in combination, on the survival rate, proliferation rate, morphology, and intracellular calcium ion concentration of cultured primary NHOK. We further examined whether AA and NOS-I could protect cultured primary NHOK from glutamate cytotoxicity. An understanding of the relationship between these factors may help elucidate the mechanism of regulation of epithelial growth and regeneration.

2. Materials and Methods

2.1 Cell Culture of Primary NHOK

Primary NHOK (P0) were cultured as described previously.[21] Briefly, NHOK were prepared from keratinizing oral epithelial tissue from 20 healthy volunteers, who gave informed consent, aged 20-29 years undergoing oral surgery. The Institutional Review Board of Uijeongbu St. Mary's Hospital, the Catholic University of Korea approved these experiments and all patients signed an informed consent. The tissue samples were thoroughly washed three times with calcium- and magnesium-free Hank's balanced salt solution (CMF-HBSS). To separate the epithelium from the underlying submucosa, the tissues were incubated in CFM-HBSS containing collagenase (Type II,

1.0 mg/ml; Sigma Chemical Co., St. Louis, MO) and dispase (grade II, 2.4 mg/ml; Boehringer-Mannheim, Indianapolis, IN) for 90 min at 37°C in 95% air and 5% CO_2. The cells were cultured in keratinocyte growth medium (KGM; Clonetics, Cambrex, Walkersville, MD) containing a low level (0.15 mM) of Ca^{2+} and supplementary growth factor bullet kit. Cells were seeded onto culture dishes, allowed to proliferate until approximately 60-70% confluence and then subcultured. The third passage (P3) cells were used for the study. Trypsinized second passage (P2) keratinocytes were seeded on 8-well chamber slides for morphoologic evaluation with H-E satin, on 96-well plates for MTT and BrdU assays, and on sterile coverslips for intracellular calcium measurement.[19]

2.2 Drug Administration

Cells were divided into test groups and control groups. The control groups were cultured in pure KGM and the test groups in KGM with drugs. Reagents were added after the cells had attached (about two days after seeding), and maintained at that concentration through subsequent media changes.

2.2.1 Reagents

Drug concentrations used were: 100 μM NMDA; 0.5, 1, 5 and 10 μM AA; 10 and 100 μM NNA (neuronal and endothelial constitutive NOS inhibitor); 10 and 100 μM NAME (methyl ester form of NNA; the nonselective NOS inhibitor).

2.3 MTT Assay

Cell viability was assessed by the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide) assay. P2 NHOK were seeded at about 1×10^4 cells/well (5×10^4 cells/ml X 200 μl) in 96-well plates and 10 wells were used for each test and control group. The assays were performed on the first, second, third, fourth, and seventh days after drug administration. MTT was dissolved in phosphate buffered saline (PBS) at a concentration of 2 mg/ml. 50 μl of MTT solution was added to each well and the cells were incubated for four hours at 37°C in 95% air and 5% CO_2. The medium in the wells was carefully removed leaving about 30 μl / well. To each well was added 150 μl dimethyl sulfoxide (DMSO), and the plates were shaken gently for about 10 minutes until the formazan crystals were dissolved. Optical density was measured using a microplate reader at a wavelength of 540 nm.

2.4 BrdU Assay

For the assessment of DNA synthesis, cells were immunostained with anti-BrdU antibodies using the BrdU cell proliferation assay kit (Oncogenre Reaearch Products, Cambridge, MA). P2 NHOK were seeded at about 1×10^5 cells/well (5×10^5 cells/ml X 200 μl) in 96-well plates and 10 wells were used for each test and control group. The assays were performed four and twenty-four hours after BrdU label administration. Briefly, 20 μl/well BrdU label was added immediately after replacing the medium with 100 μl medium containing the test drug or pure medium, and the plates were incubated for four or twenty-four hours in the tissue culture incubator. After removing the medium, 200 μl fixative/ denaturing solution was added to each well and the cells were incubated for minutes at room temperature. After removal of the fixative/denaturing solution, 100 μl anti-BrdU antibody was added to each well and the cells were incubated for one hour at room temperature. The wells were washed with wash buffer and 100 μl peroxidase Goat Anti-Mouse IgG HRP conjugate was added to each well, and the cells were incubated for 30 minutes at room temperature. The wells were washed with wash buffer and total of 100 μl substrate solution was added to each well, and the cells were incubated in the dark at room temperature for 15 minutes. Stop solution (100 μl) was added to each well and optical density was measured within 30 minutes using a microplate reader at dual wavelength of 450-540 nm.

2.5 Morphological Changes of Keratinocytes

P2 NHOK were seeded at about 1×10^5 cells/chamber (5×10^5 cells/ml X 200 μl) on 8-well chamber slides. The cells were fixed with 4% paraformaldehyde solution at the first, second, fourth, and seventh days after drug administration and stained with hematoxylin-eosin (H-E). The microscopic features were observed with an Olympus BX50 (Olympus Optical Co., Ltd. Japan) optical microscope and ProgRes C12 (JENOPTIK Laser, Optick, systeme GmbH Business Unit Sensor Systems, Germany) digital camera.

2.6 Intracellular Calcium Ion Measurement

Intracellular calcium concentration in cultured normal human oral keratinocytes was measured with the membrane-permeant fluorescent calcium indicator dye fura-2/AM (Molecular Probes, Eugene, OR, USA), as described by Grynkiewicz et al.,[21] with minor modifications,[15, 19, 22, 23] Briefly, NHOK were incubated with 5 μM fura-2/AM for 1 h at 37°C. Before each measurement, cells were maintained at room temperature in normal Tyrode's solution (137 mM Nacl, 5.4 mM KCl, 0.5 mM $MgCl_2$, 1.8 mM $CaCl_2$, 11.8 mM HEPES - NaOH, 1g per liter glucose, pH 7.4) for 30 min to allow homogeneous distribution of the dye. All Ca^{2+} imaging

Table 1. MTT absorbance at 540 nm in control, NMDA, AA, and NMDA+AA groups

day	1	2	3	4	7
	M±SD	M±SD	M±SD	M±SD	M±SD
Control	0.668±0.066	0.801±0.057	1.015±0.077	1.128±0.061	1.235±0.059
NMDA(100 μM)	0.728±0.092	0.869±0.093	1.174±0.092	1.240±0.070	1.021±0.069
AA (0.5 μM)	0.649±0.075	0.933±0.078	1.136±0.074	1.144±0.075	1.333±0.108
AA (1 μM)	0.652±0.077	0.897±0.073	1.074±0.089	1.177±0.063	1.440±0.128
AA (5 μM)	0.588±0.052	0.654±0.088	0.829±0.065	0.928±0.076	1.108±0.090
AA (10 μM)	0.523±0.073	0.489±0.101	0.383±0.076	0.345±0.051	0.199±0.054
N+AA (0.5 μM)	0.654±0.075	0.918±0.088	1.115±0.057	1.177±0.083	1.339±0.075
N+AA (1 μM)	0.629±0.069	0.862±0.093	1.029±0.080	1.189±0.060	1.392±0.120
N+AA (5 μM)	0.568±0.055	0.644±0.092	0.750±0.100	1.136±0.054	0.969±0.077
N+AA (10 μM)	0.543±0.070	0.508±0.068	0.410±0.087	0.605±0.088	0.325±0.095

M: Mean, SD: Standard Deviation

was performed at room temperature and within about one hour of loading. The coverslip was mounted on a fluorescence microscope (IX70, TS Olympus, Tokyo, Japan). Intracellular calcium ion concentration ($[Ca^{2+}]_i$) was measured using Photo Multiplier Tube (PMT) detectors coupled to a microscope and software (Felix, PTI, Lawrenceville, NJ, USA) on a Pentium computer. The cells were illuminated with a 75 W xenon arc lamp and excitation wavelengths (340/380) were selected by a filter changer. The drugs were applied by gravity through a bath perfusion system.

Intracellular calcium ion levels can be calculated from the ratio ($R = F_{340}/F_{380}$) of the fluorescence intensities measured with excitation wavelengths of 340 nm (F_{340}) and 380 nm (F_{380}).[19] The fluorescence intensity ratio R is used in this study as an indicator of intracellular calcium ion level.

2.7 Statistical Analyses

All values are expressed as mean±standard deviation and experiments were repeated at least five times. Data sets were analyzed by Mann-Whitney U-test to determine differences between the two groups (test groups and control group) using the SPSS 10.0 program. A significance level of $p < 0.05$ was considered significant.

3. Results

3.1 MTT Assay for Cell Viability

The value of MTT absorbance at 540 nm in control (C), NMDA (N), AA (AA) and NMDA + AA (N+AA) groups are indicated in Table 1.

3.1.1 Comparison of Test Groups Grown in the Presence of NMDA only (N) and AA only (AA) with the Control Group Grown in Medium Alone (C) (Fig 1).

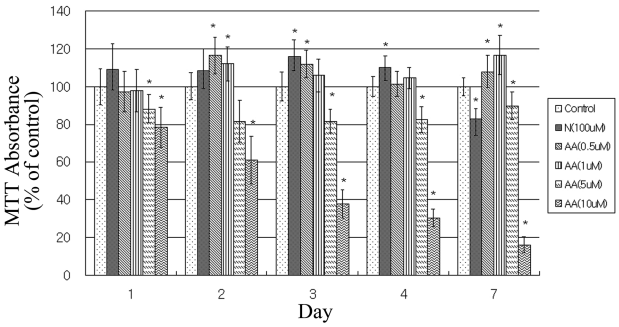

Figure 1. The effect of NMDA & AA on the viability of NHOK. MTT absorbance values were converted to % of control at each time point. * P<0.05: Significantly different from the control group of the same day.

3.1.1.1 Comparison of Control Group (C) with the NMDA Group (N): Effect of NMDA on NHOK Viability

In the presence of NMDA, the number of viable cells increased relative to the control group until the fourth day. However, at the seventh day the number of viable cells in the NMDA group was significantly decreased compared with the control group.

3.1.1.2 Comparison of Control Group (C) with the AA Groups (AA): Effect of AA on NHOK Viability

At low concentrations of AA (0.5 μM & 1 μM) the number of viable cells was increased compared with the control group, while at high concentration of AA (5 μM & 10 μM) the number of viable cells was decreased relative to the control group

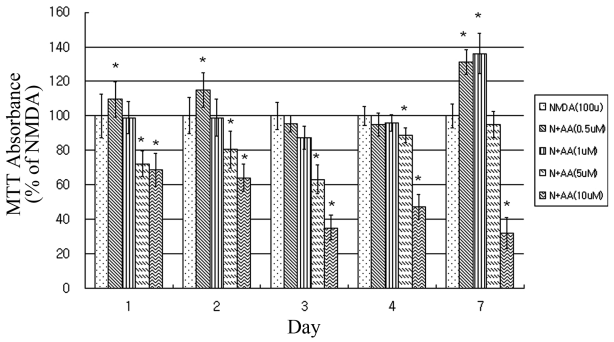

Figure 2. The effect of AA on NMDA cytotoxicity. MTT absorbance values were converted to % of NMDA only at each time point. * P<0.05: Significantly different from the NMDA group of the same day.

throughout the whole experimental period.

3.1.2 Comparison of Test Groups grown in the Presence of NMDA and AA (N+AA) with the NMDA only Group (N) (Fig 2).

In the presence of NMDA and low concentration of AA (0.5 μM & 1 μM), the number of viable cells increased more than in the NMDA-only group, except at the third and fourth days when the number of viable cells in the NMDA-only group increased significantly more than that in the control group. In particular, at the seventh day, when the number of viable cells in the NMDA-only group was significantly reduced compared with the control group, significantly more cells survived in the group treated with NMDA and a low concentration of AA (0.5 μM & 1 μM) than in the NMDA-only group and the control group (P<0.05).

However, the number of viable cells was significantly decreased in the group treated with NMDA with a high concentration of AA (5 μM & 10 μM) compared with the NMDA-only group throughout the whole experimental period. The decrease in surviving cell numbers in the group with NMDA and high-concentration AA was lower than in the group with a high concentration of AA alone; however, this difference was not significant.

The result of the comparison of the AA with NMDA groups with the control group was similar to that of the comparison of the AA-only groups with the control group for each AA concentration.

The value of MTT absorbance at 540 nm in control (C), NMDA (N), NOS-I (NNA, NAME) and NMDA + NOS-I (N+NNA, N+NAME) groups are indicated in Table 2.

3.1.3 Comparison of Test Groups Grown in the Presence of NMDA only (N) and in the Presence of Nitric Oxide Synthase Inhibitors (NOS-I) (NNA and NAME) with the Control Group

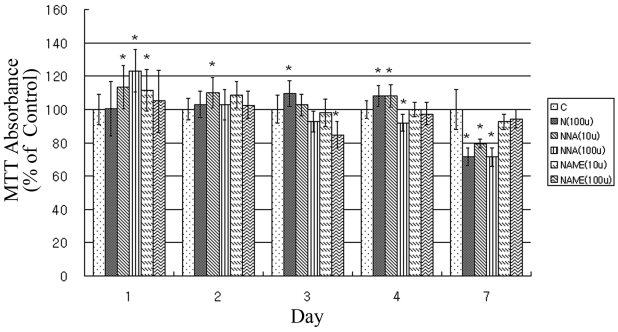

Figure 3. The effect of NMDA & NOS-I on the viability of NHOK. MTT absorbance values were converted to % of control at each time point. * P<0.05: Significantly different from the control group of the same day.

Table 2. MTT absorbance at 540 nm in control, NMDA, NOS-I and NMDA+NOS-I groups

day	1	2	3	4	7
	M±SD	M±SD	M±SD	M±SD	M±SD
Control	0.496±0.045	1.069±0.070	1.116±0.094	1.106±0.058	1.431±0.173
NMDA (100 μM)	0.498±0.080	1.101±0.085	1.222±0.085	1.197±0.069	1.027±0.074
NNA (10 μM)	0.563±0.065	1.177±0.095	1.148±0.072	1.196±0.072	1.135±0.038
NNA (100 μM)	0.619±0.073	1.221±0.083	1.184±0.075	1.132±0.065	1.020±0.076
NAME (10 μM)	0.612±0.064	1.101±0.096	1.035±0.069	1.019±0.055	1.326±0.067
NAME (100 μM)	0.543±0.072	1.063±0.085	1.001±0.072	0.998±0.047	1.353±0.080
N+NNA (10 μM)	0.619±0.141	1.221±0.117	1.184±0.075	1.132±0.059	1.330±0.118
N+NNA (100 μM)	0.543±0.094	1.063±0.079	1.001±0.043	0.998±0.029	1.278±0.079
N+NAME (10 μM)	0.540±0.049	1.082±0.076	1.096±0.064	1.067±0.040	1.382±0.163
N+NAME (100 μM)	0.509±0.078	1.072±0.094	0.880±0.041	1.125±0.082	1.368±0.195

M: Mean, SD: Standard Deviation

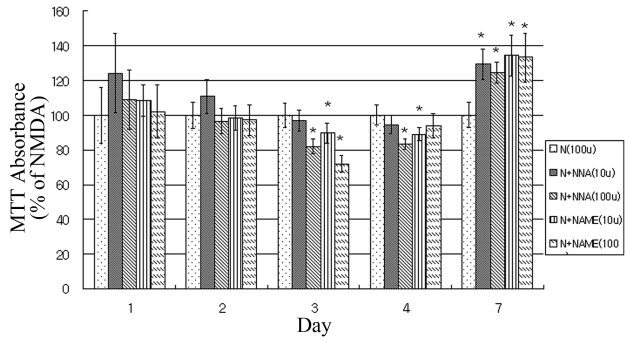

Figure 4. The effect of NOS-I on NMDA cytotoxicity. MTT absorbance values were converted to % of NMDA at each time point. * P<0.05: Significantly different from the NMDA group of the same day.

Grown in Medium Alone (C) (Fig 3).

When grown in the presence of the neuronal and endothelial constitutive NOS inhibitor NNA at a concentration of 10 μM, the number of viable cells increased significantly throughout most of the experimental period until the seventh day. In the presence of 100 μM of NNA, the numbers of viable cells increased at the first and the second days; however, they were decreased at the third day. At the seventh day, the number of viable cells decreased significantly for both concentrations of NNA.

The results for cells treated with the nonselective NOS inhibitor NAME showed a similar trend to those of the NNA groups; however, most were not significantly different from the control group.

3.1.4 Comparison of Test Groups Grown in the Presence of NMDA and NOS-I (N+NNA, N+NAME) with the NMDA only Group (N) (Fig 4).

In the presence of NMDA and NOS-I (N+NNA, N+NAME), the number of viable cells decreased compared with the NMDA only group (N) at the third and fourth days, when the number of viable cells increased in the NMDA group (N) relative to the control group (C).

At the seventh day, when the number of viable cells in the NMDA group (N) decreased significantly compared with the control group (C), there was a significant increase in the number of cells in all NMDA with NOS-I groups (N+NNA, N+NMDA) compared with the NMDA only group (N).

The number of viable cells in all NMDA with NOS-I groups (N+NNA, N+NAME) at the seventh day was not significantly different from that of the control group (C).

Table 3. BrdU absorbance at 450 and 540 nm in control, NMDA, AA & NMDA + AA groups

hours	4	24
	M±SD	M±SD
Control	0.948±0.108	0.652±0.138
NMDA (100 μM)	1.011±0.118	0.611±0.066
AA (1 μM)	1.055±0.023*	0.597±0.055
N + AA (1 μM)	0.958±0.027	0.668±0.099
AA (10 μM)	1.032±0.091	0.467±0.098
N + AA (10 μM)	1.304±0.065*	0.405±0.052

M: Mean, SD: Standard Deviation

* P<0.05, Significant difference from the control group of the same hour.

3.2 BrdU Assay

In all experimental groups, the rate of DNA synthesis was reduced more significantly at twenty-four hours than at four hours. Therefore, it was known that the DNA synthesis increased immediately after exchanging medium and decreased before twenty-four hours.

3.2.1 Comparison of Test Groups Grown in the Presence of NMDA only (N) and AA only (AA) with the Control Group Grown in Medium Alone (C) (Table 3).

Four hours after exchanging the medium, DNA synthesis activity was greater in all test groups (NMDA only, AA only, and AA with NMDA) than in the control group. In particular, a statistically significant increase in DNA synthesis was observed in the groups treated with a low concentration of AA (AA 1 μM) and those treated with NMDA and a high concentration of AA (N+AA 10 μM). However, at twenty-four hours DNA synthesis rates were generally lower than in the control group.

3.2.1.1 Comparison of Control Group (C) with the NMDA only Group (N): Effect of NMDA on DNA Synthesis in NHOK

Although it was not statistically significant, the addition of NMDA caused the DNA synthesis rate of NHOK to increase at four hours and to decrease at twenty-four hours.

3.2.1.2 Comparison of Control Group (C) with AA only Groups (AA): Effect of AA on DNA Synthesis in NHOK

Addition of low concentrations of AA (1 μM) caused a significant increase in the DNA synthesis rate at four hours; however, there was no effect at twenty-four hours. Addition of high concentrations of AA (10 μM) caused an increase in the

Table 4. BrdU absorbance at 450 and 540 nm in control, NMDA, NOS-I and NMDA+NOS-I groups

hours	4	24
	M±SD	M±SD
Control	0.613±0.106	0.338±0.129
NMDA (100 μM)	0.660±0.087	0.267±0.049
NNA (10 μM)	0.933±0.101*	0.227±0.047
N + NNA (10 μM)	0.960±0.157* +	0.253±0.069
NAME (10 μM)	0.744±0.354	0.306±0.110
N + NAME (10 μM)	0.826±0.024* +	0.303±0.119

M: Mean, SD: Standard Deviation
* P<0.05, Significant difference from the control group of the same hour.
+ P<0.05, Significant difference from the NMDA group of same hour.

DNA synthesis rate at four hours, although this was not significant, but suppressed DNA synthesis within twenty-four hours.

3.2.1.3 Comparison of AA only Groups (AA) with N+AA Groups (N+AA): Effect of NMDA on the Activity of AA

The stimulation of DNA synthesis by low concentration of AA was suppressed by NMDA and the inhibition of DNA synthesis by the high concentration of AA was enhanced by NMDA; however, these results were not statistically significant.

3.2.2 Comparison of Test Groups Grown in the Presence of NMDA and AA (N+AA) with the NMDA only Group (N): Effect of AA on NMDA.

The addition of AA with NMDA suppressed the effect of NMDA at low concentration and significantly enhanced the effect of NMDA at high concentration.

3.2.3 Comparison of Test Groups Grown in the Presence of NMDA only (N) and Nitric Oxide Synthase Inhibitor (NOS–I) only (NNA, NAME) with the Control Group Grown in Medium Alone (C) (Table 4).

In NKOK cells treated with NMDA only (N), the rate of DNA synthesis was higher than in the control group (C) at four hours and lower than in the control group at twenty-four hours. However, these differences were not statistically significant.

When cells were grown in the presence of NOS inhibitor, DNA synthesis was significantly increased at four hours and decreased at twenty-four hours, although this was not statistically significant either. In particular, the change in DNA synthesis over time was larger in the NNA 10 μM group than in the NAME 10 μM group. The rate of DNA synthesis was greater in the NOS-I with NMDA groups (N+NNA, N+NAME) than in the NOS-I only groups (NNA, NAME), although the differences were not statistically significant.

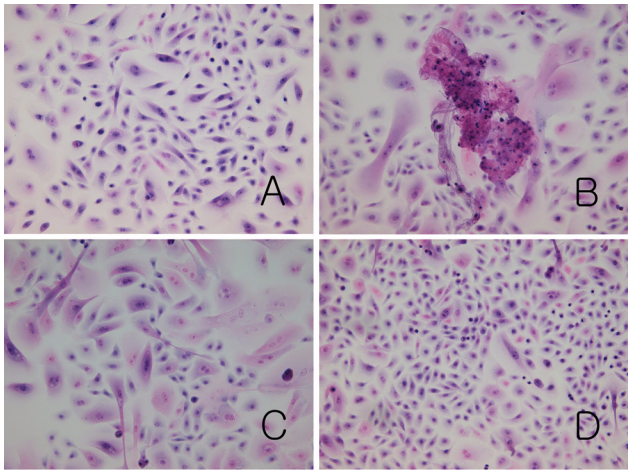

Figure 5. Micrographs of NHOK stained with Hematoxylin-Eosin 4 days after drug administration (× 20).
A. Control group, B. NMDA (100 μM) group, C. AA (1 μM) group, D. AA (1 μM) + NMDA (100 μM) group.

3.2.4 Comparison of Groups Grown in the Presence of NMDA and NOS-I (N+NNA, N+NMDA) with the NMDA only Group (N) (Table 4).

At four hours, the DNA synthesis rate was significantly greater in the NOS-I with NMDA groups than in the NMDA only group, while there was no significant difference at twenty-four hours.

3.3 Morphology of Keratinocytes

Squamous cell morphology was maintained in most cells at the first or second day after drug administration, but was lost by the seventh day. Therefore, morphological features were compared on the fourth day after drug administration (Fig 5).

At the fourth day, the NMDA (100 μM) group showed aggregation of dead cells detached from the culture slide. The AA (1 μM) group showed more enlarged and elongated cytoplasm than the control group. The AA (1 μM) + NMDA (100 μM) group displayed the most typical keratinocyte morphology and retained an undifferentiated phenotype.

3.4 Measurement of the Intracellular Calcium Ion Change

In control cells grown in KGM only, the calcium ion ratio increased by only 0.05, but increased about 0.6 when 100 μM

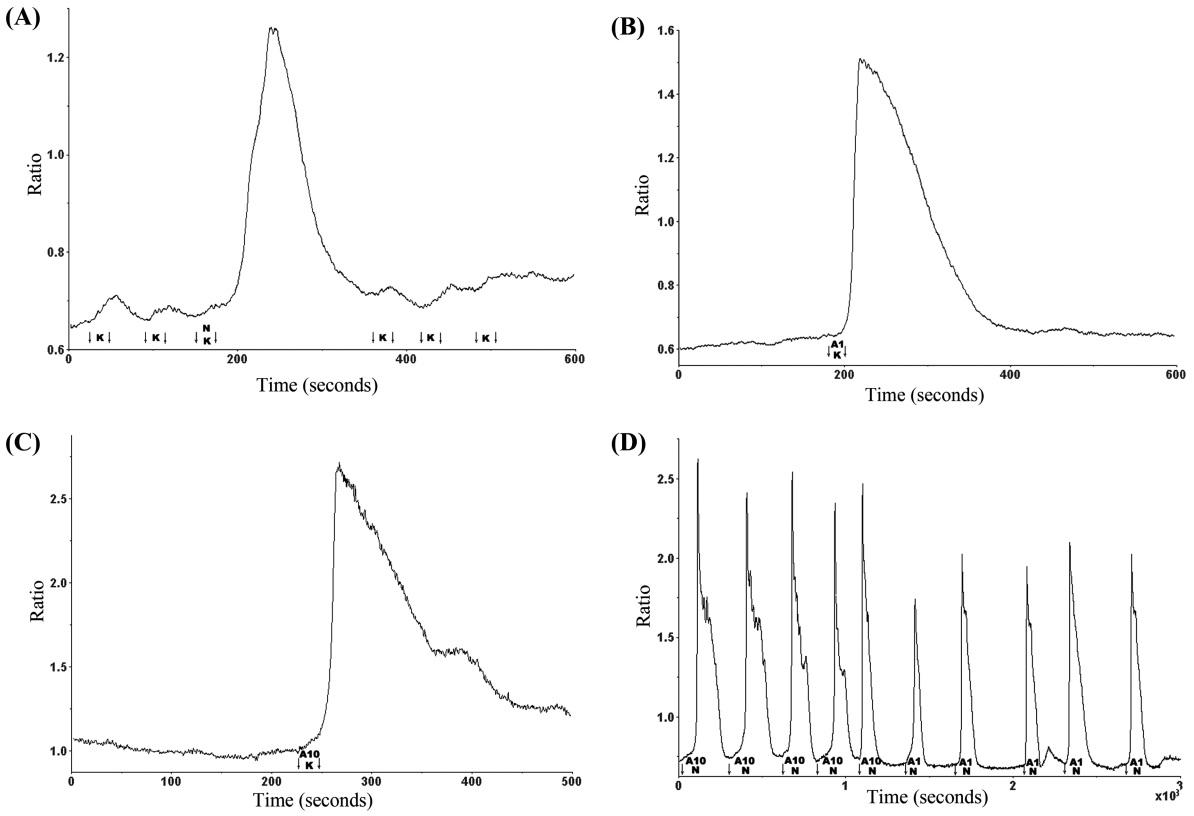

Figure 6. The intracellular calcium ion change ratio (F340/380) in NHOK. **(A)** Comparison of the effects of KGM medium alone (K) and KGM medium with NMDA (N). **(B)** The effect of 1 μM of AA. **(C)** The effect of 10 μM of AA. **(D)** Comparison of the effects of different concentrations of AA on the ion influx induced by NMDA.

of NMDA was added to the KGM medium (Fig 6A). Addition of 1 μM of AA increased the ratio by about 0.85 (Fig 6B), and 10 μM of AA increased the ratio by about 1.75 (Fig 6C). When 1 μM of AA and 100 μM of NMDA were added together, the ratio increased by about 1.21 (Fig 6D), which was 0.36 more than 1 μM of AA only (Fig 6B) and 0.61 more than 100 μM of NMDA only (Fig 6A). When 10 μM of AA and 100 μM of NMDA were added together, the ratio increased by about 1.75 (Fig 6D), which was not different from the addition of 10 μM of AA only (Fig 6C), but 1.15 more than the addition of 100 μM of NMDA only (Fig 6A).

AA can enhance NMDA-evoked intracellular calcium ion and which was affected by the concentration of the AA.

The addition of 1 μM AA to NMDA increased the calcium ion influx to twice that induced by NMDA alone, and adding 10 μM of AA to NMDA caused a three-fold increase. Therefore, the increase in calcium ion influx induced by NMDA was enhanced by AA in a concentration-dependent manner. However, the addition of NMDA to a high concentration of AA did not have any effect, although adding NMDA to a low concentration of AA increased the calcium ion influx slightly (by 0.36). The effect of adding NMDA was therefore weaker than the effect of adding AA.

4. Discussion

Glutamate, one of the excitatory amino acids, is a typical neurotransmitter in the CNS that works through various receptors; the NMDA receptor is one of the ionotropic glutamate receptors. Many studies have been performed on the peripheral functions of glutamate and Fuziwara et al. demonstrated the specific localization and dynamics of glutamate in the epidermis. In normal skin, glutamate is localized in the uppermost epidermis; this localization disappears following barrier disruption.[24] Barrier disruption induces epidermal DNA synthesis in the basal layer of the epidermis.[25, 26] Glutamate might play a signaling role in the epidermal proliferative response induced by barrier disruption; however, the mechanism underlying these phenomena has not been clarified. As in the synaptic system, a transporter of

glutamate might play an important role in the localization of glutamate and its release from the epidermis following barrier disruption.[24] Several receptors originally found in nerve cells have been reported on keratinocytes.[27-31] Ectoderm derived keratinocytes and neurons showed similar expression of those receptors. Thus, different types of glutamate receptors might differentially regulate skin barrier homeostasis and other metabolic systems in the epidermis.[24]

In the evaluation of proliferation, viability and cytotoxicity, a high value in the MTT assay means that many cells were alive at that time, and could be interpreted as one, or a combination, of the following: First, increased proliferation rate leading to increased cell number. Second, suppression of programmed cell death, such as by reducing the cell differentiation rate, leading to increased viability. Third, the cytotoxicity of the experimental reagent was lower than other drugs tested or the medium itself. The first explanation can be tested by a BrdU assay to measure the DNA synthesis rate. The second, increased cell viability might be slowly shown over a long term while drug cytotoxicity might be shown continuously from an early stage.

For the comparison of cells grown in the presence of NMDA or AA with the control group grown in medium alone, our results were as follows: Adding 100 μM NMDA to the medium increased the number of viable cells initially (at the first or second day). However, long-term exposure caused a decrease in the number of viable cells by the seventh day. At low concentrations of AA (0.5 μM & 1 μM) the number of viable cells was increased compared with the control group, while at high concentration of AA (5 μM & 10 μM) the number of viable cells was decreased relative to the control group. The low concentration of AA might (1) increase the proliferation rate by stimulating cell division, (2) prolong the survival period (increasing viability) by suppressing differentiation, or (3) suppress the cytotoxicity of some environmental factor. The high concentration of AA might (1) decrease the proliferation rate by suppressing cell division, (2) decrease cell viability by stimulating the differentiation of NHOK and programmed cell death, or (3) have a cytotoxic effect on NHOK.

Low concentrations of AA were shown to protect NHOK cells from the cytotoxic effects of NMDA. It may be presumed that the low concentration of AA added to the NMDA increases cell viability and suppresses the cytotoxicity of long-term exposure to NMDA. If it had increased the proliferation rate, an increase in the number of viable cells would have been expected at the third and fourth days. High concentrations of AA (5 μM & 10 μM) reduced the number of viable cells, irrespective of exposure time and the presence of NMDA. Therefore, it is assumed that at high concentrations, AA exerts a cytotoxic effect on the primary cultured NHOK. The result of this experiment showed that the cytotoxic effects of long-term exposure to NMDA could be inhibited by adding low concentrations of AA (0.5 μM & 1 μM).

In the comparison of cells grown in the presence of NOS-I (NNA or NAME) with the control group grown in medium alone, the addition of 10 M NNA initially caused a significant increase in the number of viable cells; however, long-term exposure caused a decrease in the number of viable cells similar to that for 100 M NMDA. The methyl ester isoform of NNA, NAME, had a less pronounced effect on primary cultured NHOK.

In the comparison of test groups grown in the presence of NOS-I and NMDA (NMDA+NNA, NMDA+NAME) with groups grown in NMDA only, long-term exposure to NOS-1 significantly reduced the cytotoxicity of NMDA. Therefore, the cytotoxic effect of long-term exposure to NMDA could be inhibited by adding NOS-I or low concentrations of AA. Interestingly, NAME showed a significant protective effect on primary cultured NHOK when it was added with NMDA, even though it had little effect by itself.

In this study, the DNA synthesis rate generally increased 4 hours after changing media, but decreased within 24 hours. The test groups with a large initial increase in DNA synthesis rate also showed a large decrease in DNA synthesis rate at 24 hours. The change in the DNA synthesis over time was particularly large in cells treated with NMDA and 10 μM AA.

The action of NOS-I on DNA synthesis appeared to be activated by a mechanism other than the glutamate receptor. If NOS-I worked independently at the NMDA receptor, the difference between the control group and the NOS-I only group should be larger than the difference between the NMDA only group and the NOS-I with NMDA group. However, the opposite would be expected if NOS-I affected the NMDA receptor together with NMDA. In fact, the difference between the NMDA only group and the NOS-I with NMDA group and the difference between the control group and NOS-I-only-adding group were very similar. Moreover, the differences between the results of the BrdU assay in each group without NMDA and each group with NMDA were almost the same. Thus, it appears that the temporary increase in DNA synthesis following the addition of NOS-I is not related to the NMDA receptor.

The results of the BrdU assay suggest that low concentrations of AA initially increase DNA synthesis in NHOK and protect cells from the inhibition of DNA synthesis by long-term exposure to NMDA, while high concentrations of AA with NMDA initially increase the DNA synthesis rate and subsequently inhibit DNA synthesis. The effect of NOS

inhibitors in the initial increase of DNA synthesis was constantly shown with no respect to the NMDA addition. Therefore, it could be thought that NOS inhibitors do not show the competitive or synergic effect on the action of NMDA but show the additive effect on it: If NOS inhibitors make the synthesis of DNA increase by competing with NMDA at the NMDA receptor, the difference between control group and NOS inhibitors only group should be larger, being compared with the difference between NMDA only group and NMDA with NOS inhibitors group. Besides, if NOS inhibitors show the synergic action with NMDA to NMDA receptor, the opposite result should be expected.

In the study of NHOK morphology, at passage number 3 (P3) most cells maintained their squamous cell morphology. The NMDA only group showed the most differentiated features and the low concentration AA group was more differentiated than the control group. However, cells treated with NMDA and a low concentration of AA showed the least differentiated features and the largest number of cells. These observations are consistent with the results of the MTT assay. The previous study of the cell morphology of NHOK showed that primary NHOK (P0) and low passages of the cells (P1 and P2) displayed typical keratinocyte morphology and retained an undifferentiated phenotype. Most cells maintained their squamous cell morphology at passage number 3 (P3), although some cells were enlarged and flattened. The fourth (P4) and fifth (P5) passage cells displayed characteristics of terminal differentiation, with highly enlarged and elongated cytoplasm and cells rounding up and detaching from the culture dish.[32] Our observations are very consistent with these results. Exponentially replicating NHOK cells were small and polygonal in shape with large nuclei containing prominent nucleoli. Cells in the senescing phase were larger with a proportionately larger cytoplasmic compartment. The most notable change in cells entering senescence was a dramatic increase in the number and size of clear vacuoles that occupied most of the perinuclear cytoplasm.[33]

With respect to calcium ion influx, adding NMDA to a low concentration of AA caused the calcium ion influx to increase slightly, while adding NMDA to a high concentration of AA did not have any effect. Adding 1 μM of AA to NMDA increased the calcium ion influx two-fold compared with adding NMDA only, and adding 10 μM of AA to NMDA caused a three-fold increase. Thus, the effect of adding NMDA on the change in the calcium ion influx induced by increasing AA concentration was rather weak. However, the increase in calcium ion influx by NMDA was affected by the addition of AA in a concentration-dependent manner. These results suggest that the protective effect of low concentrations of AA on the cytotoxicity of long-term exposure to NMDA is not due to suppression of the calcium ion influx. It could be analogized that the activation of the NMDA receptor occurred through another mechanism, excluding or including a calcium channel. Zhe et al. showed that NMDA converts single-spike firing to burst firing, and this NMDA-induced bursting is dependent upon Na^+ influx, and upon the presence of raised levels of intracellular Ca^{2+}. Therefore, they suggested that the Na^+-Ca^{2+} exchanger may play a role in NMDA-induced burst firing.[34] The Na^+-Ca^{2+} exchanger creates a depolarizing current because it extrudes one divalent Ca^+ ion from the cell in exchange for moving three Na^+ ions into the cell,[35] and can be activated by group I metabotropic glutamic receptor stimulation.[36] NMDA has been shown to either activate the Na^+-Ca^{2+} exchanger as a consequence of Ca^{2+} influx through NMDA-gated channels,[37] or to inhibit or even reverse the exchanger by causing build-up of intracellular Na^+ and reducing the transmembrane Na^+ gradient.[38, 39]

Calcium is one of the most versatile second messengers involved in cell growth, differentiation, and programmed cell death,[40] and the presence of a physiological level of calcium in culture medium leads to apoptosis of primary human keratinocytes.[41] Activation of phosphatidylinositol-specific phospholipase C (PLC) increases the cellular calcium level and may participate in apoptosis.[32] The activation of phosphatidylinositol-specific PLC is linked to an increase in the free intracellular calcium level, resulting in epidermal maturation and differentiation.[42, 43] Inositol 1,4,5-trisphosphate induces release of calcium from the endoplasmic reticulum and diacylglycerol is a physiologic activator of protein kinase C. Protein kinase C activation, as well as calcium mobilization, is essential for the terminal phase of keratinocyte maturation.[32] An ionic Ca^{2+} concentration above 0.3 mM was identified as a potent differentiating agent for normal keratinocytes.[44, 45] Therefore, culture medium containing a low Ca^{2+} level was developed to permit long-term proliferation of cells.[45] Fuziwara et al. suggested that calcium ion influx into keratinocytes delays barrier repair and, conversely, that chloride ion influx accelerates barrier recovery. They also suggested that an abnormality in the calcium dynamics in epidermal keratinocytes may be an important cause of cutaneous diseases.[24] Although the relationship between the expression of the NMDA receptors and abnormal cellular metabolism has not been clarified, the NMDA receptor might also be associated with skin diseases. Thus, a new strategy for improving barrier repair may be available for human skin. This strategy could result in novel therapeutic approaches to treat cutaneous disorders caused by barrier damage or abnormal ion dynamics in keratinocytes. The psychological stress delayed skin barrier recovery and it seems to be mediated by glucocorticoid.[26]

There are three types of NOS: endothelial, neuronal and inducible. Endothelial cells and neuronal tissues contain constitutively expressed forms of NOS, which are Ca^{2+}/calmodulin-dependent, whereas inducible NOS is produced in macrophages and other cell types and is Ca^{2+}-independent.[46] NNA is a neuronal and endothelial constitutive inhibitor and NAME is a nonselective NOS inhibitor. It is well established that noncompetitive and competitive NMDA receptor antagonists can block psychostimulant-induced sensitization. Since NO is known to be formed as a result of the activation of NMDA receptors, followed by Ca^{2+} influx and stimulation of Ca^{2+}/calmoduline-dependent NOS, long-term behavioral changes produced by nicotine may be mediated by activation of NMDA receptors followed by the production of NO.[47] It has been suggested that the constitutive and inducible forms of NOS have distinct functions in mediating the physiological processes of drug dependency.[48] NO has been proposed to be a retrograde neurotransmitter and may diffuse from the postsynaptic membrane to the presynaptic membrane.[49]

All mature bone cells (osteoclasts and osteoblasts) express glutamate receptors, and the NMDA receptor appears to be the most highly expressed. MK-801, a blocker of the NMDA associated Ca^{2+} channel, inhibits the basal level of bone resorption.[50] NMDA receptors appear to be involved in the process of osteoblast maturation and osteoclastogenesis.[51] Regulation of intracellular calcium ion and glutamate receptor function could be involved in the control of bone resorption and regeneration, pain, psychological stress, drug sensitivity and skin disease as well as proliferation, differentiation and migration of keratinocytes. Furthermore, it may be important in the development of new medicines, the development of artificial skin, and reconstruction of tissue. Many pathologic or traumatic defects of the body require epithelial coverage after reconstruction; otherwise, there might be severe contraction or infection. However, in certain cases such as dental implant surgery, the rapid growth of gingival epithelium is an unfavorable condition. The ability to control the proliferation of the epithelium and bone may help overcome such problems. This study could be help to control the regeneration of the epithelium.

In this study, the relationships between respective test groups and a control group were compared; however, further study is needed to observe the comparisons within the test groups.

In conclusion, the NMDA-induced NHOK death was related to intracellular calcium ion change and induction of cellular differentiation. Low concentrations of AA induced an increase in cell number and high concentrations of AA induced a decrease in cell number. The decrease in cell number caused by NMDA was inhibited by low concentrations of AA or NOS inhibitors. Therefore, these agents may protect NHOK from NMDA-induced cytotoxicity. However, the protection from NMDA-induced NHOK death by low concentrations of AA might not be due to decreased calcium influx. NMDA appeared to induce differentiation of the NHOK and this induction seemed to be inhibited by low concentrations of AA. However, low concentrations of AA itself appeared to induce the differentiation of NHOK.

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