N-methyl-D-aspartate 수용기의 다양한 조절이 일차 배양된 정상사람구강각화세포의 생존에 미치는 영향

김인수 · 이 원 · 김성훈* · 최 봄**
가톨릭대학교 의정부성모병원 치과 구강악안면외과, *교정과, **보철과

Abstract

THE EFFECT OF DIFFERENTIAL MODULATION OF N-METHYL-D-ASPART ATE RECEPTOR ON THE VIABILITY OF PRIMARY CULTURED NORMAL HUMAN ORAL KERATINOCYTES

Insoo Kim, Won Lee, Seong-Hun Kim*, Bohm Choi**
Division of Oral and Maxillofacial Surgery, Department of Dentistry,
**Division of Orthodontics, Department of Dentistry,*
***Division of Prosthodontics, Department of Dentistry,*
Uijeongbu St. Mary's Hospital, The Catholic University of Korea

In the present study, I 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 viability of cultured primary normal human oral keratinocytes (NHOK). Specifically, we examined whether AA and NOS-I could protect primary NHOK from glutamate cytotoxicity. The purpose of this study was therefore the preliminary study for the examination of the interaction between these agents and NHOK in order to elucidate the mechanisms by which epithelial growth and regeneration are regulated.

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.

NMDA and NNA, a calcium dependent NOS inhibitor, induced an initial increase in cell number, which subsequently decreased by the 7th day. Low concentration of AA (0.5 μM & 1 μM) induced an increase in cell number while high concentrations of AA (5 μM & 10 μM) induced a decrease in cell number. The decrease in cell number induced by NMDA at the 7th day was abolished by the addition of low concentrations of AA (0.5 μM & 1 μM) or NOS inhibitors. Low concentrations of AA (0.5 μM & 1 μM) or NOS inhibitors may protect the NHOK from NMDA induced cytotoxicity. These reactions might be related to the NMDA receptor in the cell and the change of the intracellular calcium ion concentration.

Key words : N-methyl-D-aspartate, Viability, Normal human oral keratinocyte, Arachidonic acid, Nitric oxide synthase inhibitor

Ⅰ. INTRODUCTION

Normal human oral keratinocyte (NHOK) is keratinized stratified squamous epithelium, and its proliferation is primarily accomplished by division of basal cell which exists in the lowest layer like the epithelium of the general skin. The oral epithelium has different characteristics from epithelium of general skin such as hairlessness, no sweat glands, and always being wet. It shows

intermediate figures of general skin and intestinal mucosa. After the epithelium has been disrupted by tissue injury, re-epithelialization must be occurred as rapidly as possible in order to re-establish tissue integrity[1]. In the case that oral epithelium was damaged, wound healing is a complex phenomenon that involves series of controlled events including the formation of a provisional extracellular matrix mainly composed of fibrin, fibronectin and vitronectin and the migration of epithelial cells from the edges of the wound[2]. The proliferation and differentiation of epithelium carry an important meaning on the migration and the control of these procedures might be useful in the treatment or reconstruction of traumatic wounds, in the reconstruction of pathologic or embryological defects, and in the dental implant treatment.

Most of the epithelial wounds need rapid repair due to protection of the tissue. However, in some defects of bone or dental implant case, rapid growth of epithelium could bring out the unfavorable outcome, such as, the insufficiency of bone or the interposition of the soft tissue between implant and bone, as a result of the restriction of the bone growth or the disturbance of the osseointegration between implant and bone. Ientile *et al.*[3] applied the NMDA to the nerve cell and they insisted it result in fetal damage to the cell by increasing of the tissue transglutaminase (tTG). Moreover, the application of the NMDA inhibitor to the epithelium suppressed the growth of the epithelial cell[4]. The regulation of the intracellular calcium ion influx by the NMDA was known to the cause of both experimental results. Recent experiment showed that the exhausting of calcium in ER, the reservoir of the intracellular calcium, caused stimulation of keratinocyte proliferation[5]. It was suggested that NMDA receptor activity in basal cell keratinocytes should play a role in determining their proliferative and differentiative fate, and that NMDA signaling may contribute to the maintenance of the undifferentiated basal cell phenotype. While it can trigger glutamate release and excitotoxic cell death[4].

NO can also prevent caspases activation and switch cell death from apoptosis into necrosis[4]. Richalrds *et al.*[6] suggested that elevated intracellular Ca^{2+} activate nitric oxide synthesis and the resulting synthesis of nitric oxide (NO) depress the Ca^{2+} response to NMDA, while arachidonic acid (AA) augments these responses. Therefore, arachidonic acid and nitric oxide implicated in synaptic plasticity differentially modulate MNDA-mediated Ca^{2+} entry into hippocampal neurons.

There are several opinions concerning the pathway of epithelial growth and the agents involved in that pathway. Hsieh *et al.*[7] proposed that growth of keratinocytes involves stimulation of the sensory nerves in the epithelium by neurotransmitter, while Genever *et al.*[4] reported that glutamate receptors in the basal cell participate in the proliferation and differentiation of the epithelium.

Geneve *et al.*[4] have identified *in vivo* expression of several regulatory molecules associated with glutamate signaling in keratinocytes, and their data provide strong evidence to support a role for glutamate in the control of epidermal renewal. This suggests potentially novel therapeutic targets for the treatment of skin disease and enhancement of wound healing. Keratinocytes express several classes of glutamate receptors, clustering proteins, and glutamate transporters *in vivo*. Expression of the glutamate receptor is dependent on the differentiation state, and changes during epidermal wound healing and embryonic development. These findings provide evidence that glutamate-mediated signaling may be intimately involved in major aspects of keratinocyte biology.

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[4,8,9].

Topical application of L-glutamic acid, L-aspartic acid (non-specific glutamate receptor agonists) and NMDA (NMDA type receptor agonist) delayed the barrier recovery rate after barrier disruption with tape stripping. Conversely, topical application of (+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine hydrogen maleate (MK-801) and D(-)-2-amino-5-phosphonopentanoic acid (D-AP5, NMDA-type receptor antagonists) accelerated the barrier repair. The non-NMDA type receptor agonist D,L-α-amino-3-hydroxy-5-methylisoxalone-4-propionic acid (AMPA) did not affect the barrier recovery. Topical application of MK-801 also promoted the healing of epidermal hyperplasia induced by acetone treatment under low environmental humidity. Secretion of glutamic acid from skin was significantly increased immediately after barrier disruption in skin organ culture. In untreated normal skin, glutamate was localized in the upper layer of the epidermis; this localization disappeared following barrier disruption and some of the glutamate was relocalized or regenerated in the basal layer of the epidermis. These results suggest that glutamate in the epidermis might play an important role as a signal of cutaneous barrier homeostasis and epidermal hyperplasia induced by barrier disruption. Different types of glutamate receptors might differentially regulate skin barrier homeostasis and other metabolic systems in the epidermis[10].

NO is thought to be involved in the fine regulation of NMDA receptors[11,12] and the apoptosis-necrosis switch[13]. 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. NO is a hydrophobic molecule and a highly diffusible free radical that is generated from the oxidation of L-arginine to L-citrulline by a family of constitutive or cytokine-inducible nictotinamide adenine dinucleotide phosphate-dependent isoenzymes, the NOS. Through S-nitrosylation or nitration of proteins, NO can be a bifunctional modulator of cell death, capable of either triggering or inhibiting cell death. S-nitrosylation of the NMDA receptor reduces neuronal death due to excitotoxicity. In the excitotoxic death of cultured neurons, NO-triggered apoptosis requires a Ca^{2+} signal triggered by the activation of the NMDA receptor channels[13,14]. Zhu *et al.*[15] investigated the ionic dependence of burst firing induced by NMDA in neurons of the subthalamic nucleus (STN) in slices of rat brain, and demonstrated that the NMDA-induced burst firing in STN neurons requires activation of either a Ca^{2+}-activated non-selective cation cannel (ICAN) or a Na^{+}-Ca^{2+} exchanger. Among the many types of glutamate receptors, activation of the NMDA-receptor has been most frequently linked to induction of burst firing in central neurons. 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[16].

Kawasaki *et al.*[17] investigated whether AA could protect retinal ganglial cells (RGCs) from glutamate neurotoxicity and showed that low concentrations of AA could protect RGCs from glutamate-induced RGC death by decreasing the calcium influx through non-NMDA ionotropic receptors. Nontoxic concentrations of peroxynitrite promote mitochondrial permeability transition (MPT)-dependent toxicity in various cell types, and this process is inhibited by exogenous AA.

Sub-toxic concentrations of peroxynitrite inflict a potentially lethal damage that would result in MPT-dependent necrosis in the absence of the AA-dependent protective signaling. Thus, the protective signaling triggered by AA plays a pivotal role in promoting the survival of peroxynitrite-producing cells[18].

In the present study, we investigated the effects of N-methyl-D-aspartate, arachidonic acid and nitric oxide synthase inhibitor (NOS-I) itself or its combination on the survival rate of cultured primary NHOK in different concentrations. Moreover, we studied whether AA and NOS-I could protect NHOK from glutamate cytotoxicity using cultured primary NHOK. Therefore, the purpose of this study is the preliminary study for the examination of the interaction between these agents and NHOK in order to elucidate the mechanisms by which epithelial growth and regeneration are regulated.

Ⅱ. MATERIALS AND METHODS

1. Cell culture of primary NHOK

Primary NHOK (P0) were cultured as described previously[29]. Briefly, NHOK were prepared from keratinizing oral epithelial tissue from 20 healthy volunteers aged 20-29 years undergoing oral surgery. 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℃ 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 96-well plates[14].

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. The test groups were consisted of NMDA (100 ㎛), AA (0.5 ㎛), AA (1 ㎛), AA (5 ㎛), AA (10 ㎛), NMDA (100 ㎛) + AA (0.5 ㎛), NMDA (100 ㎛) + AA (1 ㎛), NMDA (100 ㎛) + AA (5 ㎛), NMDA (100 ㎛) + AA (10 ㎛), NNA (10 ㎛), NNA (100 ㎛), NAME (10 ㎛), NAME (100 ㎛), NMDA (100 ㎛) + NNA (10 ㎛), NMDA (100 ㎛) + NNA (100 ㎛), NMDA (100 ㎛) + NAME (10 ㎛), and NMDA (100 ㎛) + NAME (100 ㎛).

Reagents were added after the cells had attached (about 2 days after seeding), and maintained at that concentration through subsequent media changes.

Reagents

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

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 × 200 ㎕) in 96-well plates and 10 wells were used for each test and control group. The assays were performed on the 1st, 2nd, 3rd, 4th, and 7th days after drug administration. MTT was dissolved in phosphate buffered saline (PBS) at a concentration of 2 mg/ml. 50 ㎕ of MTT solution was added to each well and the cells were incubated for 4 hours at 37℃ in 95% air and 5% CO_2. The medium in the wells was carefully removed leaving about 30 ㎕ / well. To each well was added 150 ㎕ dimethyl sul-

foxide (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.

4. 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.

Ⅲ. RESULTS

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 1, Fig. 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 4th day. However, at the 7th day the number of viable cells in the NMDA group was significantly decreased compared with the control group.

(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 throughout the whole experimental period.

2. Comparison of test groups grown in the presence of NMDA and AA (N+AA) with the NMDA only group (N) (Table 2, 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 3rd and 4th days when the number of viable cells in the NMDA-only group increased significantly more than that in the con-

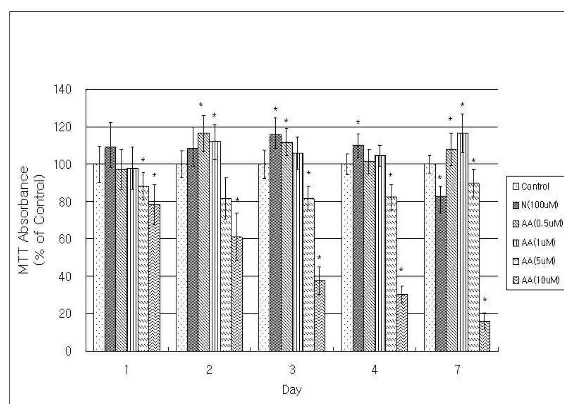

Fig. 1. The effect of NMDA & AA on the viability of NHOK.
MTT absorbance values were converted to % of control at each time point. * P<0.05: Significantly different from the control group of the same day.

Table 1. MTT Absorbance at 540 nm in Control, NMDA and 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*

M: Mean, SD: Standard Deviation
* P<0.05, Significant difference from the control group.

trol group. In particular, at the 7th 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.

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 grown in medium alone (C) (Table 3, Fig. 3).

When grown in the presence of the neuronal and endothelial constitutive NOS inhibitor NNA at a concentration of 10μM , the number of viable cells increased significantly throughout most of the experimental period until the 7th day. In the presence of 100 μM of NNA, the numbers of viable cells increased at the 1st and the 2nd days; however, they were decreased at the 3rd day. At the 7th 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.

4. Comparison of test groups grown in the presence of NMDA and NOS-I (N+NNA, N+NAME) with the NMDA only group (N) (Table 4, 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 3rd and 4th days, when the number of viable cells increased in the NMDA group (N) relative to the control group (C).

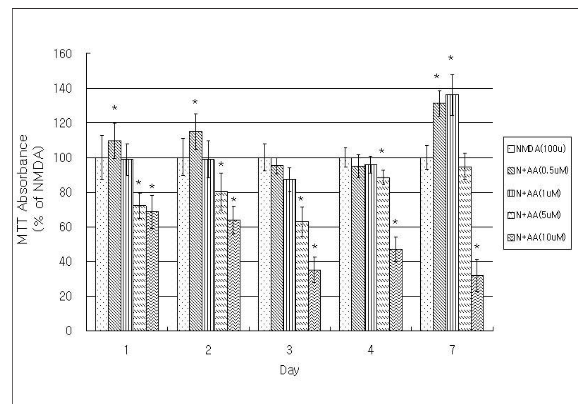

Fig. 2. The effect of AA on NMDA cytotoxicity. MTT absorbance values were converted to % of NMDA only at each time point. * $P<0.05$: Significantly different from the NMDA group of the same day.

Table 2. MTT Absorbance at 540 nm in NMDA and NMDA+AA groups

day	1	2	3	4	7
	M±SD	M±SD	M±SD	M±SD	M±SD
NMDA (100 μM)	0.728±0.092	0.869±0.093	1.174±0.092	1.240±0.070	1.021±0.069
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
* $P<0.05$, Significant difference from the NMDA group.

At the 7th 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 7th day was not significantly different from that of the control group (C).

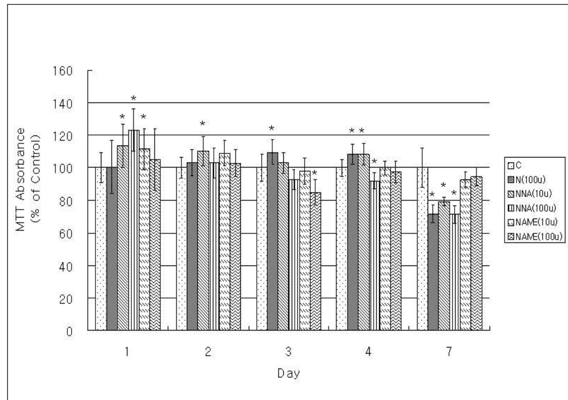

Fig. 3. The effect of NMDA & NOS-I on the viability of NHOK.
MTT absorbance values were converted to % of control at each time point. * P<0.05: Significantly different from the control group of the same day.

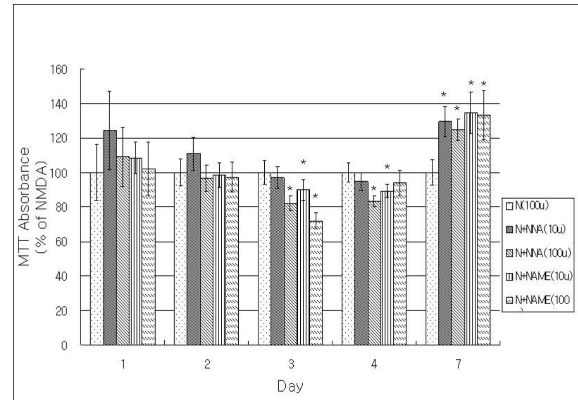

Fig. 4. The effect of NOS-I on NMDA cytotoxicity.
MTT absorbance values were converted to % of NMDA at each time point. * P<0.05: Significantly different from the NMDA group of the same day.

Table 3. MTT Absorbance at 540 nm in Control, NMDA and 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

M: Mean, SD: Standard Deviation
* P<0.05, Significant difference from the control group.

Table 4. MTT Absorbance in NMDA and NMDA+NOS-I groups

day	1	2	3	4	7
	M±SD	M±SD	M±SD	M±SD	M±SD
NMDA (100 μM)	0.498±0.081	1.101±0.085	1.222±0.085	1.197±0.069	1.027±0.074
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
* P<0.05, Significant difference from the NMDA group.

Ⅳ. Discussion

Glutamate receptors play an important role in the excitatory synaptic action of the central nervous system (CNS). 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. Morhenn *et al.*[9] suggested evidence for an NMDA receptor subunit in human keratinocytes and proposed that agonists and antagonists of NMDA do not act in the same way as in the brain because these proteins have the same morphological effect on keratinocytes.

For the comparison of cells grown in the presence of NMD 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 1st or 2nd day). However, long-term exposure caused a decrease in the number of viable cells by the 7th 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.

These results are consistent with those of Kawasaki *et al.*[17] who investigated the effects of AA on purified RGCs (retinal ganglion cells) of rats by incubating cultured cells with 1 to 50 μM AA. Increasing concentrations of AA caused a dose-dependent increase in cell death after 3 days of culture with an ED50 of 22.0 μM. The survival of RGCs was significantly reduced by AA at concentrations of 20 μM and 50 μM. They also showed that the neurotoxic effect of 25 μM glutamate was significantly ameliorated by 3 μM, but not 1 μM, AA.

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 3rd and 4th 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 μM 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 μM 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.

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[28]. NNA is a neuronal and endothelial constitutive inhibitor and NAME is a nonselective NOS inhibitor. Shim *et al.*[16] compared a variety of NOS inhibitors and NMDA receptor antagonists in nicotine-induced behavioral sensitization and suggested that constitutively produced NO may play an important role in the development of nicotine-induced behavioral effects and that the expression of behavioral sensitization of nicotine may be mediated by the activation of NMDA receptors. 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[29]. It has been suggested that the constitutive and inducible forms of NOS have distinct functions in mediating the physiological processes of drug dependency[30]. NO has been proposed to be a retrograde neurotransmitter and may diffuse from the postsynaptic membrane to the presynaptic membrane[31].

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.

In this study, we investigated the effects of NMDA, AA, and NOS-I, alone or in combination, on the viability of cultured primary NHOK; however, further study is needed to study the DNA synthesis for the proliferation and the intracellular calcium ion change.

Ⅴ. CONCLUSIONS

This study examined the effects of NMDA, AA and NOS-I on the viability of cultured primary NHOK.

The summary of this study are as follows:

1. Low concentrations of AA (0.5 μM & 1 μM) induced an increase in cell number and high concentrations of AA (5 μM & 10 μM) induced a decrease in cell number.
2. The decrease in cell number caused by NMDA at 7th day was reduced by low concentrations of AA (0.5 μM & 1 μM) or NOS inhibitors.
3. NMDA induced the death of NHOK and this appeared to be inhibited by low concentrations of AA (1 μM). Low concentrations of AA (1 μM) alone appeared to induce differentiation of NHOK, although the effect is smaller than that observed for NMDA.
4. NMDA and NNA (the calcium-dependent NOS inhibitor) induced an initial increase in viable cell number followed by a decrease in viable cell number at the 7th day.
5. NAME, the methyl ester form of NNA and a calcium-independent, nonselective NOS inhibitor, did not decrease the viable cell number at the 7th day. Therefore, the decrease of cell number induced by NMDA and NNA might be due to a change in intracellular calcium ion concentration.

Low concentrations of AA (0.5 μM & 1 μM) or NOS inhibitors may protect the NHOK from NMDA induced cytotoxicity. These reactions might be related to the NMDA receptor in the cell and the change of the intracellular calcium ion concentration.

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저자 연락처
우편번호 480-717
경기도 의정부시 금오동 65-1
가톨릭대학교 의정부성모병원 치과 구강악안면외과
김 인 수

Reprint Requests
Insoo Kim
Division of Oral and Maxillofacial Surgery, Department of Dentistry, Uijeongbu St. Mary's Hospital, The Catholic University of Korea
#65-1 Geumo-dong, Uijeongbu-si, Gyeonggi-do, 480-717, Republic of Korea
Tel: +82-31-820-3184 Fax: +82-31-847-2894
E-mail: omskim@catholic.ac.kr; omskim@yahoo.co.kr

원고 접수일 2006년 5월 9일
게재 확정일 2006년 7월 24일

Paper received 9 May 2006
Paper accepted 24 July 2006