Neuroprotective effects of an extract from the inflamed skin of rabbits inoculated with vaccinia virus on glutamate-induced neurotoxicity in cultured neuronal cell line

Tatsuki Itoh*, Takao Satou*, Shozo Nishida*, Masahiro Tsubaki*, Shigeo Hashimoto¥ and Hiroyuki Ito*

*Department of Pathology, Kinki University School of Medicine, Osaka, Japan
¥Division of Hospital Pathology, Hospital of Kinki University School of Medicine, Osaka, Japan
Division of Sports Medicine, Institute of Life Science, Kinki University, Osaka, Japan
Department of Pathology, PL Hospital, Osaka, Japan
Kinki University School of Pharmaceutical Sciences, Osaka, Japan

Objective: Protein-free extracts from the inflamed skin of rabbits inoculated with vaccinia virus (Rosemorgen® and Neurotropin®) are widely employed to combat chronic pain and treat allergic conditions in human subjects in Japan. However, the pharmacologic mechanisms of Rosemorgen® and Neurotropin® remain unclear.

Methods: In this study, we examined the effects of Rosemorgen® on L-glutamic acid (Glu)-induced cell death in N18-RE-105 neural cell line, which only possessed non-N-methyl-D-aspartate (NMDA)-type receptors.

Results: There were many large cytoplasmic cells and elongation of fivers in phosphate-buffered saline (PBS) additional group without Glu. In PBS and Glu simultaneous additional group, the survival ratio was decrease significantly compared with PBS alone group. Moreover, there were dead cells which did not have cytoplasm and aggregated nucleus. The Glu-induced cell death of N18-RE-105 cells was inhibited by both pre-treatment (24 hours before Glu treatment) and simultaneous treatment with Rosemorgen®. There were many large cytoplasmic cells and elongation of fivers in Rosemorgen® group.

Discussion: From this finding in N18-RE-105 cells, Rosemorgen® was concluded to inhibit Glu-induced cell death via non-NMDA type receptors. One of the pharmacologic mechanisms of Rosemorgen® has been clear. These results suggest that Rosemorgen® depresses allodynia and chronic pain through interaction with non-NMDA type receptors. [Neurol Res 2008; 30: 430–434]

Keywords: N18-RE-105; non-NMDA-type receptor; L-glutamic acid; vaccinia virus; chronic pain

INTRODUCTION

L-glutamic acid (Glu) acts as an excitatory neurotransmitter in the mammalian central nervous system. Its action on the long-term potentiation of synapses is mediated through glutamate receptors on the neurons. However, high concentrations of Glu cause neuronal cell death1-4.

In recent years, much attention has been focused on the glutamate receptor due to evidence that it may be related to the induction of chronic pain, such as causalgia5,6 and allodynia5-9. Therefore, the sensitization to chronic pain and hypersensitivity reactions of the sympathetic nervous system are associated with the N-methyl-D-aspartate (NMDA)-type and non-NMDA-type receptors of neurons. In addition, we presumed that prevention of neuronal cell damage due to various stimulations through glutamate receptors would be effective for relief from chronic pain such as causalgia and allodynia.

Rosemorgen® and Neurotropin®, protein-free extracts from the inflamed skin of rabbits inoculated with vaccinia virus, are widely employed to combat chronic pain, such as lumbago, cervicobrachial and syndrome symptomatic neuralgia, and treat allergic conditions, such as cutaneous pruritus and allergic rhinitis, in human subjects in Japan10-14. However, the pharmacologic mechanisms of Rosemorgen® and Neurotropin® remain unclear, although their effects on pain have been experimentally and clinically clarified10,13,14.

Murphy et al.15-17 established that the N18-RE-105 neural cell line was produced by hybridization of the mouse neuroblastoma clone N18TG-2 with Fisher rat (18 days old) embryonic retinal neurons. It has reported that N18-RE-105 hybrid cells, which only have non-NMDA-type receptors, were induced cell death by Glu15-17.

In this study, to investigate the action of Rosemorgen® to nervous system, we study effect of Rosemorgen® on Glu-induced cell death in N18-RE-105 hybrid cells.
MATERIALS AND METHODS

Materials
Rosemorgen® (Lot No. 13508B) was donated by Fujimoto Diagnostics Inc. (Osaka, Japan). Dulbecco’s modified Eagle’s medium (DMEM), fetal calf serum (FCS) and HAT medium were purchased from Gibco (St Louis, MO, USA). Glu was purchased from Wako (Osaka, Japan). All other chemicals were obtained from standard sources at the highest purity available.

Protection by Rosemorgen® against Glu-induced cell death in N18-RE-105 cells
N18-RE-105 cells were grown in 75 cm² flasks as previously described. Cytotoxicity works were carried out according to a previously described method. Briefly, the culture medium was removed and phosphate-buffered saline (PBS) was added to the flask. The flask was allowed to stand for 10 minutes before the cells were desquamated by trituration and collected in centrifuge tubes. The tubes were centrifuged for 5 minutes at 1500 rpm and the supernatant was removed. Culture medium was added to the tubes and the cells were dissociated by trituration. The single-cell suspension was plated on 35 mm culture dishes in DMEM supplemented with 10% FCS, 2 mM glutamine, 21 mM glucose, 38 mM bicarbonate and HAT at a density of 20,000 cells/dish, and maintained at 37°C in a humidified 5% CO₂ atmosphere for 24 hours. Next, the medium was removed and the cells were immediately washed with culture medium. The medium was replaced with medium containing 10 mM Glu and the N18-RE-105 cells were then cultured in a humidified 5% CO₂ atmosphere for 24 hours. For control cultures, 10 μl PBS was used instead of Glu. The effect of Rosemorgen® on Glu action was investigated by two types of treatment. In the first, Rosemorgen® was added to the culture dishes simultaneously with Glu. In the second, Rosemorgen® was added to the culture dishes 24 hours before the Glu treatment. After 24 hour exposure to Glu, the cytotoxicity was routinely monitored by phase-contrast microscopy. Because the N18-RE-105 cell line was a pure cell preparation, evaluation of the survival ratio of the cells was possible by assaying the cytosolic enzyme lactate dehydrogenase (LDH). The LDH level was measured according to a previously described method and the survival ratio was calculated. Briefly, the LDH activities in the culture medium and cell lysates were measured spectrophotometrically, and the percentage of surviving cells was calculated using the following formula

Surviving cells(%) = 100 -

\[
\frac{\text{LDH activity in the medium}}{\text{LDH activity in the medium} + \text{LDH activity in cell lysates}} \times 100
\]

Statistical analysis
All biochemical data were tested for significant differences by Dunnett’s test. \( p < 0.05 \) was considered to be significant.

RESULTS

Protection by Rosemorgen® against Glu-induced N18-RE-105 cell death
The protection by Rosemorgen® against Glu-induced N18-RE-105 cell death is shown in Figure 1A. There were many large cytoplasmic cells and elongation of fivers in PBS alone group (Figure 2A). We set survival ratio of this group as 100% for comparisons with test groups. The cell survival rate of N18-RE-105 cells was 45.7 ± 8.8% after Glu addition alone. The cell survival ratio was significantly decreased. There were many atrophic cells, globular changed cells and aggregated cells in Glu addition group (Figure 2B).

Whereas the survival rates after simultaneous treatment with Glu and Rosemorgen® (10 and 20 μl/ml) were 66.0 ± 9.5 and 57.5 ± 3.5%, respectively. These increases in the survival rate were both significant (\( p < 0.05 \)). There were many large cytoplasmic cells and elongation of fivers in Glu and Rosemorgen® simultaneous treatment group (Figure 2C).

The protection by Rosemorgen® pre-treatment (24 hours before Glu treatment) on Glu-induced N18-RE-105 cell death is shown in Figure 1B.
Figure 2: Rosemorgen® protects against L-glutamic acid-induced cell death in N18-RE-105 cells. Phase-contrast photomicrographs of cultures after 24 hours of L-glutamic acid toxic exposure are shown. Cells survive in the control culture (+PBS, -L-glutamic acid) (A), while L-glutamic acid (10 mM) induces cell death (B). Rosemorgen® (10 μl/ml) simultaneous treatment (C) and pre-treatment (D) protect against cell death and the cells survive similarly to the control culture. Scale bars=50 μm
survival rate of N18-RE-105 cells was 45.7 ± 8.8% after Glu addition alone. The cell survival after Rosemorgen® (10 µl/ml) pre-treatment was significantly increased to 76.2 ± 10.3% (p<0.01). In addition, there were many large cytoplasmic cells and elongation of fivers (Figure 2D). However, the survival rate after Rosemorgen® (20 µl/ml) pre-treatment, i.e. twice the concentration, was increased, but the effect was not significant.

**DISCUSSION**

Recently, it has been suggested that the degeneration of neurons mediated by Glu may have some relationship with allodynia2–9, while other reports have indicated that increased depolarization of glutamic acid receptors in the posterior horn of the spinal cord is involved in the mechanism of persistent pain after nerve injury, such as causalgia4–5.

It has been reported that Glu binds to non-NMDA-type receptors, followed by increased Ca\(^{2+}\) influx in N18-RE-105 cells through the non-NMDA-type receptors. Subsequently, cell degeneration and cell death are induced by the oxidative stress due to the abundant Ca\(^{2+}\) in neural cell line N18-RE-105 cells, which possess only non-NMDA-type receptors.15–17. In the present study, we examined the suppressive effect of Rosemorgen® on Glu-induced cell death in cultured N18-RE-105 cells. The results revealed that both simultaneous treatment and pre-treatment with Rosemorgen® significantly suppressed Glu-induced cell death in N18-RE-105 cells (p<0.05). This finding indicates that Rosemorgen® affects non-NMDA-type receptors. A recent study reported that the AMPA-type receptor, which is one of the non-NMDA-type receptors, is an ion channel-type receptor involving the passage of Na\(^{+}\) and Ca\(^{2+}\) ions. Ca\(^{2+}\) ions inflow is progressed into cells through voltage dependent Ca\(^{2+}\) ions channel by binding to non-NMDA-type receptors of Glu. Subsequently, cell degeneration and cell death are enhanced14–19. And then it was thought that Ca\(^{2+}\) ions inflow into cell was inhibited by action of Rosemorgen® to non-NMDA-type receptors and Rosemorgen® prevented cell death.

Src-family kinases, G-proteins and mitogen-activated protein kinase (MAPK) are involved downstream of this non-NMDA-type receptor. These signaling cascades are activated by normal volume glutamic acid and biologically active substances acting on AMPA-type receptors, resulting in neuroprotective effects. As a consequence, these reactions inhibit the neuronal toxicity induced by high concentrations of Glu and have important roles in recovery from cell damage.1,20–23. In addition, a study using cultured rat hippocampal slices reported that the expression of brain-derived neurotrophic factor (BDNF), a nerve growth factor, is promoted by AMPA-type receptor-mediated activation of the MAPK signaling pathway and correlated with neuronal survival.21,24,25. Therefore, we cannot deny that Rosemorgen® affects the non-NMDA-type receptors, and may activate signal transduction resulting in BDNF expression, which in turn inhibits Glu-induced neurotoxicity.

The survival ratio was not increased significantly in Rosemorgen® pre-treatment (20 µl/ml) group. It was thought that binding to non-NMDA-type receptors of Rosemorgen®, pre-treatment (10 µl/ml) was saturated and more protective effect of Rosemorgen® pre-treatment (20 µl/ml) could not be shown. In addition, although action of Rosemorgen® on non-NMDA-type receptors is clear, action of Rosemorgen® on NMDA-type receptors is not clear in this study. Thus, to investigate action on NMDA-type receptors of Rosemorgen®, we should demonstrate the possible mechanism that action of Rosemorgen® directly inhibits NMDA-type receptors using the cultured cortical neurons which have NMDA-type receptor and non-NMDA-type receptor in the future.

Although Rosemorgen®, which is protein-free extracts from the inflamed skin of rabbits inoculated with vaccinia virus, is employed to combat chronic pain, one of the pharmacologic mechanisms of Rosemorgen® has been clear. It was whether Rosemorgen® bound to non-NMDA-type receptor or competed with Glu in this study. Rosemorgen® was effective for inhibiting Glu-induced cell death via non-NMDA-type receptor. In view of this finding, it is likely that Rosemorgen® may inhibit allodynia and chronic pain induced by nerve degeneration via non-NMDA-type receptors. Furthermore, when we consider the analgesic effect on allodynia and chronic pain, the action via non-NMDA-type receptors is more important.

**ACKNOWLEDGEMENT**

The authors thank Mari Machino and Emi Watanabe for technical assistance.

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