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EFFECT OF N-METHYL-D-ASPARTATE RECEPTOR ANTAGONIST ON RADIATION-INDUCED GUT INJURIES IN MICE

Masatsugu Ohgami[1], Nobuhiko Takai[2*], Masahiko Watanabe[3], Koichi Ando[3], Akiko Uzawa[3], Ryoichi Hirayama[3]

[1]Department of Pharmaceutical Organic Chemistry, Faculty of Pharmaceutical Sciences, Nagasaki International University, 2825-7 Huis Ten Bosch, Sasebo, Nagasaki, 859-3298, Japan
[2]Department of Analytical Chemistry, Faculty of Pharmaceutical Sciences, Nagasaki International University, 2825-7 Huis Ten Bosch, Sasebo, Nagasaki, 859-3298, Japan
[3]Medical Physics Research Program, Research Center for Charged Particle Therapy, National Institute of Radiological Sciences (NIRS), 4-9-1 Anagawa, Inage-ku, Chiba-shi, Chiba, 263-8555, Japan

Abstract. *The intestinal crypt stem cells in gut have a high growth potential and radiosensitivity, it is dose-dependently reduced by heavy-ion irradiation and intestinal death occurs by arrest of epithelial cells supply in high dose area. The radiation to abdominal cancer, for example uterus and bladder, could give impairments not only on tumor, but also on gut nearby target. Therefore, the development of radioprotective agents for gut may contribute to more effective and less harmful heavy-ion therapy. N-methyl-D-aspartate receptor (NMDAR) is one of glutamate receptors and NMDAR antagonist has been reported to prevent the radiation-induced injuries in the central nervous system. Thus, we examined whether the peripheral NMDAR activation is a possible cause of gut injuries in mice irradiated with carbon-ion beam. We compared the dose-dependent change in the number of crypts after irradiation between treated MK-801 (0.1 mg/kg), a noncompetitive NMDAR antagonist, and untreated mice in order to confirm a MK-801 radioprotective effect on crypts. Compared with the sham group, the number of crypts in MK-801 group was significantly increased at 12.0 Gy or over. The radiolabeled [^{3}H]MK-801 was intravenously injected with C3H female mice received 9 Gy whole body irradiation (290 MeV/u, 20 keV/μm). The significant increase was observed in [^{3}H]MK-801 at 24 hr and 48 hr after irradiation, followed by decrease thereafter. These results suggest that intestinal NMDAR are most activated at 48 hr after carbon-ion irradiation. Thus, we suggested that radiation-induced gut injuries could be suppressed by NMDAR antagonists as radioprotective agents until 48 hr after carbon-ion exposure.*

Key words: *NMDA receptor, intestinal crypt stem cell, radiotherapy, MK-801*

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1. INTRODUCTION

Carbon-ion radiotherapy at the National Institute of Radiological Sciences (NIRS) using the Heavy Ion Medical Accelerator in Chiba (HIMAC) has been performed from 1994, and has treated more than 9,000 patients by March 2015 [1]. Charged particles such as carbon-ions are known to have a superior dose distribution associated with the sharp penumbra and Bragg peak, allowing for highly conformal irradiation of deep seated tumors, together with a higher biological effectiveness than X-rays or gamma-rays [2], [3]. On the other hand, radiation to abdominal cancer, for example the uterus and the bladder, would cause serious damages not only to the tumor, but also to the normal gut nearby target still. Radiation-induced gut injuries are most potent threat to the radiotherapy for abdominal cancer.

Intestinal crypt stem cells are highly sensitive to radiation due to their high proliferative potential. In the case of the exposure of the whole human body to more than 5 Gy radiation, the intestinal death may occur by the necrosis, due to the growth arrest of crypt stem cells and the following arrest of epithelial cells provision [4]. This radiosensitivity of the gut makes radiation therapy for the abdominal cancer difficult [5].

L-Glutamate acts as major excitatory neurotransmitters in the mammalian central nervous system (CNS) by stimulating or exciting postsynaptic neurons. Acting on glutamate receptors (GluRs), it plays a key role in nearly all aspects of normal brain function including learning and memory, movement, cognition, development, and synaptic plasticity [6], [7]. GluRs are classified into two groups, ionotropic receptors (iGluRs) and metabotropic receptors (mGluRs). Further, three forms of iGluRs have been identified: *N*-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-4-isoxazol-propionate (AMPA), and kainite. The NMDA receptor (NMDAR) has been the one most strongly implicated in excitotoxic, as well as neuroexcitatory events [8]. The

* n_takai@niu.ac.jp

overstimulation of NMDAR can be lethal to neurons, due to resulting in the excessive influx of intracellular Ca^{2+} and ultimately induces a series of toxic events leading to cell death in acute and chronic conditions, including epilepsy, ischemia, Huntington's chorea, Alzheimer's disease, and AIDS encephalopathy [9]-[13]. In order to detect these pathological conditions in the CNS, the development of numerous radioactive probes has been vigorously researched. Despite considerable efforts, no radioligands are currently available for human *in vivo* positron emission tomography (PET) or single photon emission tomography (SPECT) imaging of NMDAR [14]. It has been reported that NMDAR is expressed in various peripheral tissues such as small intestine, bronchus, and vascular endothelial cells [8], [15], [16]. Since peripheral NMDAR is closely related to pain, it has also been studied as an attractive therapeutic target for neuropathic pain [17]-[19]. More interestingly, based on the report that NMDA and AMPA antagonists enhanced tumoricidal effects of cytostatic drugs *in vitro* by inhibiting tumor cell proliferation and enhancing tumor cell death [20], peripheral NMDAR antagonists are expected as a potential anticancer drug. As described above, it plays diverse physiological and pathological roles, so we have assumed that the peripheral NMDAR activation is a possible cause of injuries to normal tissues after irradiation, because NMDAR antagonist has been reported to prevent radiation-induced brain injuries [21].

Our objectives are to demonstrate a role of NMDAR in radiation-induced gut injuries, and to investigate the possibility of NMDAR antagonist as a protective agent for the gastrointestinal system. Here we report about the radioprotective effect of MK-801, an open channel blocker of NMDAR [22], [23], and about time and dose at which Ca^{2+} aberrant influx that induces cell death occurs.

2. Materials and methods

2.1. Animals and irradiation procedure

C3H/HeMsNrsfICR female mice aged 14 weeks were used for the gut study. The mice were produced and maintained in the specific pathogen-free (SPF) facilities at the NIRS in Japan. They were housed in groups of 5 mice per cage under controlled conditions of 12 h dark/light cycles with free access to food and water. Mice were fixed on an acryl plate under the unanesthetized conditions and the whole body was irradiated with the carbon-ion beam. Carbon ions were accelerated by the HIMAC synchrotron up to 290 MeV/u. Lucite absorbers with 43 mm thickness were used to obtain the selected LET of 20 keV/μm. The exposure rate was approximately 1 Gy/min. Sham-irradiated mice were used as a control group. The animals involved in these studies were procured, maintained and used in accordance with the Recommendations for Handling of Laboratory Animals for Biomedical Research, compiled by the committee on the Safety and Handling Regulations for Laboratory Animal Experiments of NIRS.

2.2. Crypt survival assay

(+)-MK-801 hydrogen maleate (Sigma-Aldrich Japan K.K., Tokyo, Japan) was used as a radioprotective agent. (+)-MK-801 solution was intraperitoneally administered to mice 30 min before carbon ion irradiation (0.1mg/kg). Mice in the control group were injected with saline. The crypt survival assay was performed as described previously [24]. In brief, the jejunums of mice were removed and fixed in formalin 84 hr after irradiation. Historical preparations were treated, and then stained with hematoxylin and eosin (HE). The number of crypts per transverse circumference was counted microscopically for 10 through 12 histological sections of each mouse. The average number of crypts was plot on a semilogarithmic scale against the dose. The crypt survivals were fitted on an exponential function. D_0 and D_{10} values were calculated from the dose-survival curve. D_0 and D_{10} is a reciprocal slope of a survival curve and a dose that the curve meets 10 crypts, respectively. The dose reduction factor (DRF) was determined by the following formula:

DRF = [D_0 (or D_{10}) value for MK-801] / [D_0 (or D_{10}) value for saline]

2.3. Radiochemicals and tracer experiments

(+)-[3-3H]MK-801 (1.25 TBq/mmol, Biotrend, Cologne, Germany) was intravenously administered to mice 30 min before sacrifices to detect the NMDAR activation. Mice were sacrificed by decapitation followed by tracer injection. The plasma was obtained from centrifuged whole blood. To obtain gut samples, 3-cm segments of the proximal jejunum were removed at 5-6 cm from the pylorus of stomach. All samples were weighed and completely dissolved in 1 mL of tissue solubilizer (Soluene-350, PerkinElmer Japan, Yokohama, Japan), and 10 mL of scintillation cocktail (Hionic-Fluor, PerkinElmer Japan, Yokohama, Japan) was subsequently added in each vial. Radioactivity in the tissue sample was measured with a liquid scintillation counter (LS-6000TA, Beckman Coulter, California, USA). The results were expressed as % injected dose per gram of tissues.

2.4. Statistical analysis

The results were expressed as means ± standard deviation (SD). Statistical analyses were performed by unpaired t-test or by non-parametric multiple comparison (Dunnett's test). A p-value of less than 0.05 was considered to be statistically significant.

3. Results and discussion

3.1. Crypt survival assay

To determine whether MK-801 has radioprotective effect on the gut, the crypt survival was investigated at 84 hr after carbon-ion irradiation. Based on the report [21], we selected the content of MK-801 (0.1 mg/kg) that prevented radiation-induced brain injuries without adversely affecting the central nervous system. Compared with the control groups, the survived number of crypts in MK-801 groups was significantly increased at 12.0 Gy or over (Fig. 1). The D_0 value of MK-801 administered groups, 1.32 Gy was significantly

larger than that of saline administered groups, 1.09 Gy. The D_{10} value of MK-801 administered groups, 13.9 Gy was also significantly larger than that of saline administered groups, 12.3 Gy. The DRF of D_0 and D_{10} were 1.21 and 1.12, respectively. The results of Histological examination are shown in Fig. 2. The remarkable difference was observed between the control and MK-801 groups after 13.8 Gy irradiation. These results suggest that NMDAR antagonists such as MK-801 would be function as radioprotectants.

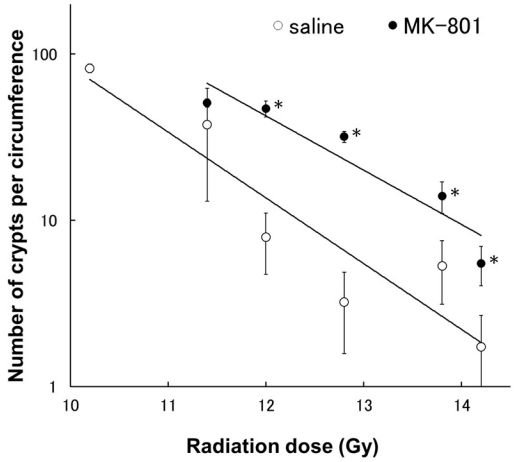

Figure 1. The number of survival crypt at 84 h after irradiation. Data are given as means ± SD (n = 4, * $p < 0.05$, Comparison of MK-801 vs saline analyzed by unpaired t-test)

3.2. In vivo [³H]MK-801 biodistribution

When administered intraperitoneally NMDAR antagonist, we found the effect on radiation-induced gut injuries in mice. In order to elucidate this mechanism of radiation protection, we used [³H]MK-801 as a radiotracer for experiments (Fig. 3 and 4). First of all, it was necessary to investigate the time to maximize accumulation of MK-801 in gut by intravenous administration (Fig. 3). After determining the administration time of MK-801, the time after carbon beam irradiation in which accumulation in the gut was examined (Fig. 4).

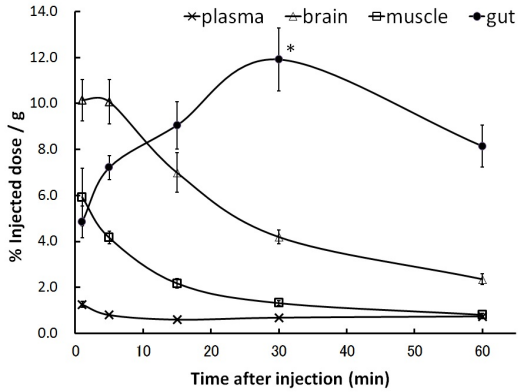

Figure 2. [³H]MK-801 accumulation in plasma, brain, muscle and gut. Data are given as means ± SD (n = 3, * $p < 0.05$, Comparison of 30 min to the other time points analyzed by Dunnett's test)

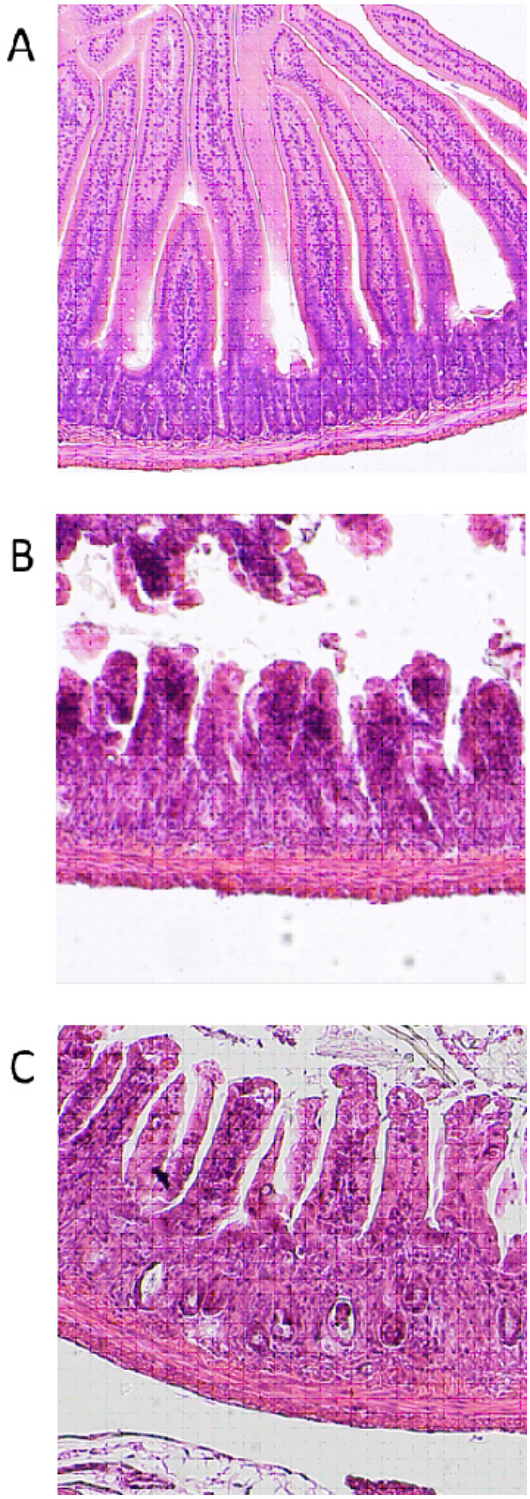

Figure 3. HE stained histology of jejunum transvers sections. (A) Unirradiated jejunum; jejunum irradiated with 13.8 Gy carbon ion after (B) saline and (C) MK-801 (0.1 mg/kg p.o.). (Original magnification: A × 100, B and C × 200)

The biodistribution of MK-801 in mice without irradiation are shown in Fig. 3. Immediately after administration, [³H]MK-801 accumulated in brain and muscle, then the activity decreased with time after

injection. On the other hand, the accumulation in gut gradually increased and reached a maximum at 30 min after tracer administration. These results indicate that the optimum time for gut sampling is 30 min after [3H]MK-801 injection.

3.3. [3H]MK-801 accumulation in gut after irradiation

In order to investigate the time-course activation of NMDAR, we investigated the gut accumulation of [3H]MK-801 in irradiated mice (Fig. 4). The significant increase was observed in [3H]MK-801 accumulation at 24 hr and 48 hr after irradiation with 9 Gy carbon-ion, followed by decrease thereafter. These results suggest that NMDAR activation was caused by carbon-ion exposure until 48 hours after irradiation.

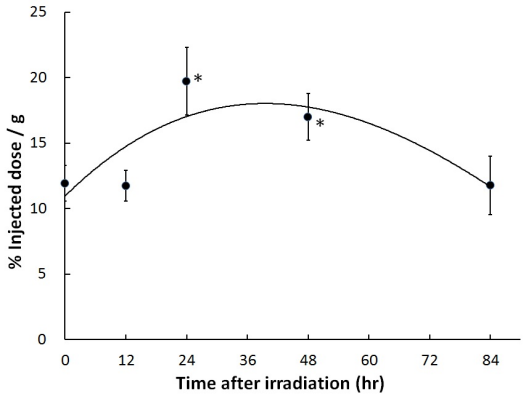

Figure 4. [3H]MK-801 accumulation in gut after 9 Gy irradiation. Data are given as means ± SD (n = 4, * $p < 0.05$, Comparison of 24 or 48 hr to the other time points analyzed by Dunnett's test)

In this study, we showed for the first time by *in vivo* experiments that it is possible to detect to radiation-induced gut injuries by investigating the activation of NMDAR. NMDAR includes several binding site for ligands, such as the glutamate, glycine, polyamine and phencyclidine (PCP). The PCP site is located on the inside of calcium channel pore, while the other sites are out of the channel. MK-801 can be bound to the PCP site when NMDAR is activated, because the calcium channel opens followed by the activation. Therefore, we can regard the [3H]MK-801 binding as an indicator for the NMDAR activation that undergoes the excessive Ca^{2+} influx and elicits the cell death such as apoptosis. The significant increase of [3H]MK-801 accumulation in gut show that the NMDAR activation is caused at 24 and 48 hr after irradiation (Fig. 4). It revealed that the activation of NMDAR is an early reaction of gut after carbon-ion irradiation. These results suggest that it would be possible to inhibit radiation-induced gut injuries by NMDAR antagonists until 48 hr after carbon-ion exposure.

As shown in Fig. 1, the radioprotective effects of MK-801 on gut were confirmed by the crypt survival assay. MK-801 binds to the PCP site and prevents Ca^{2+} flows through the pore. Since MK-801 has very high affinity for the PCP site of NMDAR, we consider that the crypt protection is induced by the antagonistic action of MK-801.

Though we demonstrated here the possibility of NMDAR antagonist as a radioprotective agent, it remains unclear whether MK-801 can be used in clinical application. PCP is known to cause the mental aberration like schizophrenia because of its high transition to human brain [25].Similarly, it is reported that MK-801 binds to the PCP site, causing learning impairments [26]. As shown in Fig. 3, MK-801 accumulates highly in the brain just after the injection. It is, therefore, essential to change the structure of MK-801 more hydrophilic by which its permeability to the BBB, and brain toxicity would be reduced.

4. Conclusions

We investigated the influence of NMDAR antagonist on radiation induced gut injuries in mice. NMDAR antagonists have been shown to be able to reduce intestinal radiation damage associated with radiotherapy and radiation disasters. Administration of NMDAR antagonist within 48 hr of radiation exposure may have protective effect of the small intestine.

Although it is still necessary to search for more optimal structure, NMDAR antagonist such as MK-801 would be useful and promising radioprotective agent for the small intestine. We considered that this study provide important clues to develop more effective and less harmful heavy ion therapy treatments.

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