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CHANGES IN VARIOUS AMINO ACID CONCENTRATIONS IN THE SMALL INTESTINE AND PATHOGENESIS OF INTESTINAL INJURY CAUSED BY CARBON ION IRRADIATION

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Abstract. *The intestinal crypt stem cells in the gut have a high growth potential and radiosensitivity that is dose-dependently reduced by carbon-ion irradiation, and intestinal death occurs by the arrest of epithelial cells supply in high-dose areas. Therefore, the development of intestinal radioprotection methods may contribute to more effective and less harmful carbon-ion radiotherapy. We have demonstrated that N-methyl-D-aspartate (NMDA) receptor antagonists reduce radiation-induced intestinal injury and that the activation of NMDA receptors significantly increased 24 hours after irradiation. In this study, we investigated the association with amino acid concentration that activates NMDA receptors in intestinal injury in irradiated mice. To investigate changes in amino acid concentration in mouse small intestine by carbon ion irradiation, we developed the HPLC method for the determination of six amino acids and related compounds—glycine (Gly), serine (Ser), aspartic acid (Asp), glutamic acid (Glu), taurine (Tau), and γ-aminobutyric acid (GABA). C3H/He female mice were abdominally irradiated with carbon ion at doses of 9 Gy (20 keV/μm, 290 MeV/u, accelerated by Heavy-Ion Medical Accelerator in Chiba synchrotron at National Institute of Radiological Sciences, Japan). After carbon-ion irradiation, the concentration of Tau significantly decreased with time. Tau, a sulfur-containing amino acid-related compound, has been reported to have a radioprotective effect. Therefore, the decrease in Tau concentration was inferred to be a decrease in radioprotective ability in the mouse's intestine. On the contrary, the concentration of Glu significantly increased with time dependence by the irradiation. These results suggested that the increase in glutamate concentration after irradiation induces the activation of NMDA receptors; thus, radiation-induced intestinal injuries could be suppressed by NMDA receptor antagonists as radioprotective agents after carbon-ion irradiation.*

Keywords: *NMDA receptor, intestinal crypt stem cell, radiotherapy, MK-801, carbon-ion radiotherapy*

1. INTRODUCTION

Carbon-ion radiotherapy at the National Institute of Quantum Science and Technology (QST) using the Heavy-Ion Medical Accelerator in Chiba (HIMAC) has been performed since 1994 and has been used to treat more than 10,000 patients by March 2016 [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 the highly conformal irradiation of deep-seated tumors, together with higher biological effectiveness than X-rays or gamma-rays [2, 3]. However, the irradiation of intra-abdominal cancer (for example, cancer of the uterus and bladder) would cause serious damage not only to the tumor but also to the normal intestine near the target organ. Radiation-induced intestinal injuries are the most potent threat to 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 of radiation, intestinal death may occur via necrosis due to the growth arrest of crypt stem cells and the ensuing arrest of epithelial cell provision [4]. This radiosensitivity of the gut makes radiation therapy for intra-abdominal cancer difficult [5].

L-Glutamate acts as a major excitatory neurotransmitter 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

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implicated in excitotoxic and neuroexcitatory events [8]. The overstimulation of NMDAR can be lethal to neurons due to the resultant 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]. It has been reported that NMDAR is expressed in various peripheral tissues such as the small intestine, bronchus, and vascular endothelial cells [8, 14, 15]. Since peripheral NMDAR is closely related to pain, it has also been studied as an attractive therapeutic target for neuropathic pain [16, 17]. More interestingly, based on the report that NMDA and AMPA antagonists enhanced the tumoricidal effects of cytostatic drugs *in vitro* by inhibiting tumor cell proliferation and enhancing tumor cell death [18], peripheral NMDAR antagonists are expected to be potential anticancer drugs. As described above, it plays diverse physiological and pathological roles, so we have assumed that peripheral NMDAR activation is a possible cause of injuries to normal tissues after irradiation because NMDAR antagonists have been reported to prevent radiation-induced brain injuries [19]. We also reported that NMDAR inhibitors prevented small bowel injury induced by carbon-ion irradiation [20]. NMDARs have been reported to be regulated by endogenous neurotransmitters such as Glu, Gly, Ser, and GABA. In addition to glutamate, aspartate is another excitatory neurotransmitter, while GABA and Gly are the main inhibitory neurotransmitters in the brain, and GABA is an important neurotransmitter for the synaptic sites in the CNS [21]. Furthermore, Tau, a sulfur-containing amino acid-related compound, has been reported to have a radioprotective effect. Tau is a regulator of crypt stem cells and plays an important regulatory role in intestinal cell survival and proliferation [22]. In this study, to investigate the activation mechanism of NMDAR involved in radiation-induced small bowel injury, we investigated the time course of various amino acid concentrations in the small intestine after carbon-ion irradiation.

2. Materials and Methods

2.1. Animals and irradiation procedure

C3H/HeMsNrsfICR female mice aged 12–14 weeks were used for the intestine study. The mice were produced and maintained in specific pathogen-free facilities at the NIRS in Japan. They were housed in groups of five 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 anesthetized conditions (Secobarbital sodium 50 mg/kg i.p.), and their abdomen were irradiated with the carbon-ion beam. Carbon ions were accelerated by the HIMAC synchrotron up to 290 MeV/u. Lucite absorbers with a thickness of 43 mm 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 the control group. Four animals were used in each group for the experiment on the concentration of various amino acids in the small intestine after irradiation. The animals involved in these studies were procured, maintained, and used per 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 the QST.

2.2. HPLC procedure

Sample preparation and HPLC conditions were performed from Refs 25-26 with some modifications.: Mouse intestine was weighed and homogenized in 20 volumes of methanol. The homogenate was centrifuged at 1,200x g for 10 min, after which the resultant supernatant was filtered through a 0.2-μm membrane filter and diluted 5-fold with methanol. Derivatization conditions: To 20 μL of the diluted supernatant, 20 μL each of borate buffer (pH 8.0), 10 mM NBD-F in acetonitrile, and water were added. The mixed solution was heated at 60 ºC for 3 min, and 140 μL of 0.1% (v/v) TFA in water was added. The obtained reaction mixture was further diluted with 0.2% (v/v) TFA in water, then 10 μL of the solution was injected into the HPLC system.

Instruments and conditions: The HPLC system consisted of a degasser (DG-2080-53, JASCO Corporation, Tokyo, Japan), two pumps (PU-2085 plus, JASCO), a column oven (CO-2060 plus, JASCO), an FP-2025 fluorescence detector (JASCO), and an LC-NetII/ADC interface box (JASCO). Chromatographic separation was performed on a COSMOSIL 5C18-MS-II (150 mm × 4.6 mm, 5 μm) column (Nacalai tesque, Kyoto, Japan). The column temperature was 25 °C. A mixture of 0.05% TFA aqueous solution (solvent A) and acetonitrile containing 0.05% TFA (solvent B) was used in the mobile phase, and the total flow rate was set at 0.5 mL/min. The gradient elution was programmed as follows: 0–16 min (15% B), 16–20 min (18% B), 20–25 min (40% B), and 25–30 min (15% B). The column temperature was set at 25 °C, and eluates were monitored at 470 nm (λex) and 530 nm (λem).

3. Results and Discussion

3.1. Measurement of amino acid concentrations in the small intestine

Amino acids in the mouse intestine were analyzed by HPLC-fluorescence analysis using NBD-F. Six amino acids (Tau, Ser, Asp, Gly, Glu and GABA) were well separated and detected (Figure 1). The absolute calibration methods of those amino acids showed good linearity ($r \geqq 0.997$). Therefore, we developed the HPLC method for the determination of 6 amino acids and related compounds in mouse intestine with pre-column derivatization with fluorogenic reagent, NBD-F [23, 24].

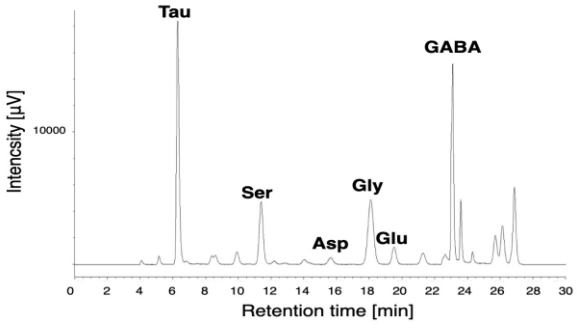


Figure 1. Chromatogram of non-irradiated mouse small intestine

Table 1. Calibration curves and detection limits of amino acids in the small intestine.

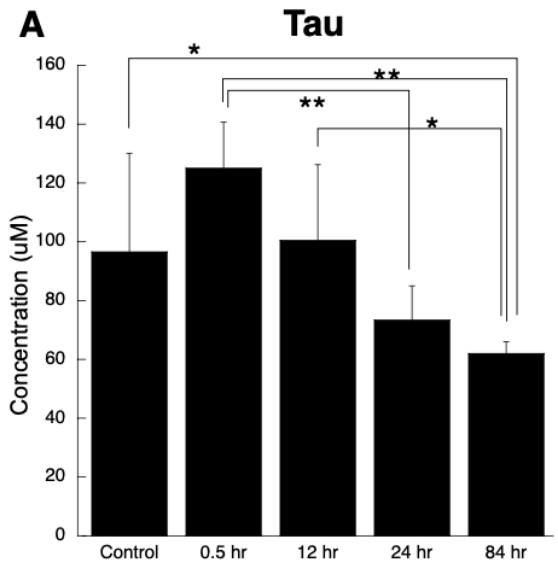
Amino acids	Calibration equation[1)]	Linear range (μM)	Correlation coefficient (r)	Limit of detection S/N =3
Gly	Y = 416.66X + 6316.4	5 – 200	0.997	1.898
Ser	Y = 323.05X + 3111.4	5 – 200	0.999	1.648
Asp	Y = 46.67X + 200.5	50 – 200	0.999	15.06
Glu	Y = 76.77X + 721.33	10 – 200	0.998	8.147
Tau	Y = 512.84X + 14639	5 – 200	0.991	0.686
GABA	Y = 464.67X + 10809	5 – 200	0.999	0.492

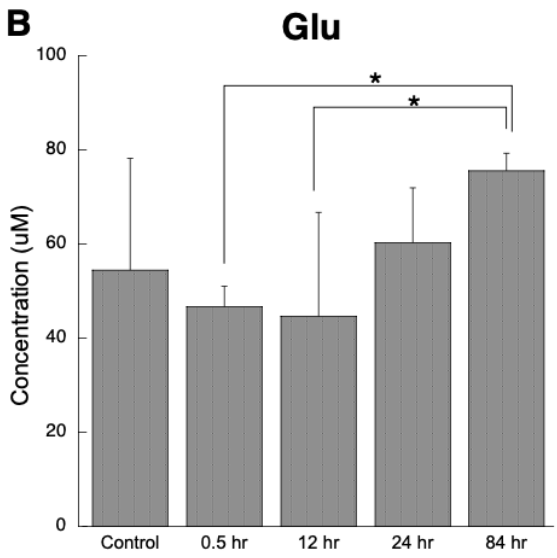
[1)] Y = Peak area, X = Concentration of amino acid (μM)

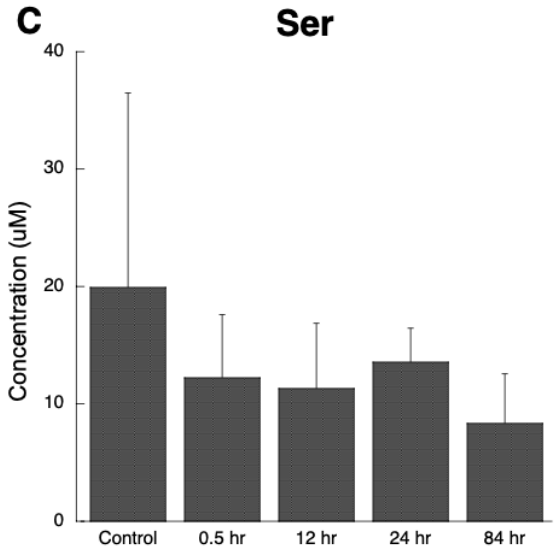
3.2. Various amino acid concentrations in the small intestine after irradiation

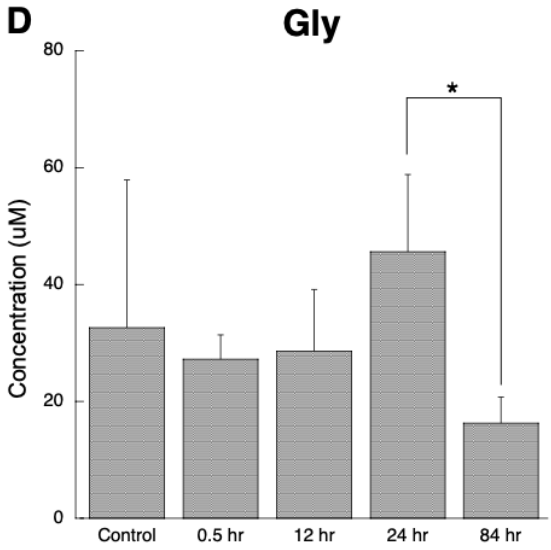
Tau showed a slight increase after 0.5 hours; however, the Tau concentrations decreased significantly in a time-dependent manner (Figure 2A). It was found that Tau decreased with time and radiation protection effects decreased. On the contrary, Glu increased significantly from 0.5 hours to 84 hours after irradiation (Figure 2B). It was suggested that Glu may increase with time and activate NMDAR. Gly increased only at 24 hours after irradiation (Figure 2D), while Ser and Asp were not affected by irradiation (Figures 2C and 2E). GABA decreased significantly only 0.5 hours after irradiation (Figure 2F).

It has been reported that NMDARs are regulated not only by Glu but also by Gly, D-Ser, and GABA. The changes in various amino acid concentrations after carbon irradiation may be associated with the activation of NMDAR and damage to the small intestine. The exposure of neuronal cell to relatively short durations or low concentrations of amino acid that activate NMDA receptors, induces a delayed form of neurotoxicity predominated by apoptotic features. In contrast, long-term exposure to high concentrations of amino acid that activate NMDA receptor induces necrotic cell damage characterized by acute swelling and lysis. [27] We found that Tau concentration decreased in a time-dependent manner, the concentration of Glu significantly increased with time dependence by the irradiation. It was assumed that these are involved in intestinal necrosis.









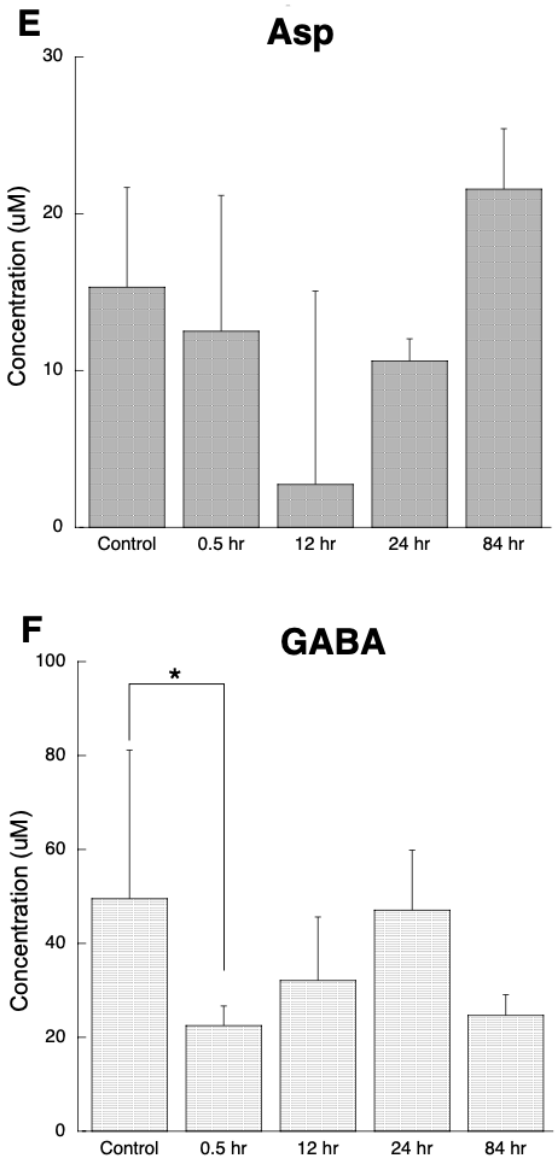


Figure 2. Changes in various amino acid concentrations in the small intestine after irradiation. Data are presented as mean values ± SD (n = 4, * $p < 0.05$, * * $p < 0.01$). Statistical analyses were performed via non-parametric multiple comparisons (Scheffe's test)

4. Conclusion

To probe into the mechanism of damage to the small intestine after carbon-ion irradiation, we developed a technique to measure the concentration of various amino acids after carbon-ion irradiation. Our results indicate that intestinal damage involves not only a time-dependent increase in Glu levels that activate NMDA receptors but also a significant decrease in Tau levels and transient changes in Gly and GABA levels. The measurement of amino acid concentrations in the small intestine by carbon-ion irradiation is important for the development of radioprotectants and optimal administration methods to protect the small intestine. We considered that this study provided important clues for the development of more effective and less harmful heavy-ion therapies.

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