Effects of High Linear Energy Transfer Radiation on the Cochlea of C3H/He Mouse During Postnatal Developmental Course

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Cochlear degeneration/SEM/High linear energy transfer radiation/Mouse/Neutron.

To investigate the biological effectiveness of neutrons at energies below 1MeV with regard to ear toxicity, we exposed mice to 1.0 Gy of monoenergetic neutrons (1.026 MeV) or $^{137}$Cs gamma rays at 7 days of age, and observed subsequent morphological changes in the inner ear with light and scanning electron microscopes. Monoenergetic neutrons, but not gamma rays, caused acute changes in the ear. The epithelium of the greater epithelial ridge in the organ of Corti showed degeneration around 6 hours and disappeared by 72 hours post-irradiation. The apoptotic cell death of the epithelium of the greater epithelial ridge was inducible by the radiation at 3 or 4 days of age. The hair cells formed the protrusion structures by 72 hours post-irradiation. Neutron-irradiation also caused acute otitis media until 10 weeks of age.

INTRODUCTION

Radiotherapy administered to manage head and neck tumors and brain malignancies causes hearing impairment. This is because of the inevitable exposures with high doses when the organ of cochlea is involved in the radiation field. Epidemiological studies indicate that post-irradiation hearing loss occurs in about one-third of patients.1,2) The relative risk of irreversible hearing loss increased by 3.7-fold in the group exposed to 50 Gy compared with the group exposed to <30 Gy.3) High-resolution computed tomography scanning, surgery and postmortem examination indicated that the vascular insult to the inner ear structures caused progressive degeneration and atrophy of the sensory structure and fibrosis of the inner ear fluid spaces by 3 to 4 months after irradiation. The rate of patients carrying permanent hearing loss with cochlear fibrosis increased to 36% for the dose of 60 Gy.4,5)

This study aimed to investigate the biological effectiveness of radiations of high linear energy transfer (LET), especially neutrons of below 1.0 MeV using the mouse model.6,7) As the energies of neutrons emitted from the Hiroshima and Nagasaki bombs were different: 10-2~1.0 and >1.0 MeV, respectively,8) the causal relationship of the neutron energy to the relative biological effectiveness has been re-investigated experimentally. The higher susceptibility of oocytes to neutrons was demonstrated by the parameter of apoptotic index.7) Effects of neutrons to shorten the lifespan and the tumorigenicity of neutrons were reported by the comparison with those of the reference radiations, the low LET radia-
tions.6,7) Here we showed effects of neutrons on the cochlea during its postnatal developmental course, as cochlear potentials of mice appear with narrow range at 7 days of age and increase until 14 days of age when the hearing responses attain to adult values.9)

MATERIALS AND METHODS

Radiation
Monoenergetic neutrons were generated by a $^7$Li(p, n)$^7$Be reaction at the Hiroshima University Radiobiological Reactor (HIRRAC).10) Gamma rays were generated by the $^{137}$Cs source (Shimadzu biotech, Japan). The average energy of neutrons (Table 1), and the irradiated dose and dose rate were referred to our previous reports.6,7)
Table 1. Experimental groups, radiation quality, dose and dose rate used for the experiment.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Radiation</th>
<th>Radiation component (cGy)</th>
<th>Dose rate</th>
<th>Age at exposure</th>
<th>Number of mice examined</th>
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<td>Source</td>
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<td>γ-ray</td>
<td>(cGy/min)</td>
<td>(days old)</td>
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</table>

*SEM: scanning electron microscopy

Fig. 1. Cross-section of the organ of Corti at 6, 24 or 72 hours after the neutron-irradiation at 7 days of age (B, D, F) and the age-adjusted control (A, C, E). The nuclear pyknosis at the great epithelial ridge (GER) (bracket) at 6 hours after neutron-irradiation (B). Disappearance of the epithelial cells at the GER (bracket) and atrophy of the tectorial membrane (TM) were prominent at 24 and 72 hours post neutron-irradiation (D, E). Cells facing the internal surface of the scala tympani (ST) became atrophic and decreased in number at 24 and 72 hours post neutron-irradiation (arrow in F) when compared to the non-irradiated control (arrow in E). H&E staining. The pyknotic cells at the GER and of the ST are TUNEL positive in the serial section counterstained with methyl green (arrow in the insert in B). OHC: outer hair cells; IHC: inner hair cells; Bar: 50 µm.
Fig. 2. The mid-modiolar cross-sections through cochleas of mice at 10 weeks of age (77 days old) post neutron-irradiation at 7 days of age and the age-adjusted control (B and A, respectively). Inflammatory cells infiltrated the tympanic cavity (\* in B). Pictures of the high magnification of the stria vascularis (SV) are in A’ and B’. The structure of neutron-irradiated SV is in normal appearance. Cross-sections of the organ of Corti at 12 months post neutron-irradiation at 7 days of age and the age-adjusted control (D and C, respectively). Pictures of the high magnification of the SV are in D’ and C’. Deposition of lipofuscin (arrow) is prominent in the neutron-irradiated SV. H&E staining. Bar: 100 µm.

Fig. 3. Cross-section of the organ of Corti at 24 hours after the neutron-irradiation at 3 (B) or 4 (D) days of age, and the age-adjusted control (4 or 5 days old for A or C, respectively). The nuclear pyknosis at the great epithelial ridge (bracket) in B and D. H&E staining. Bar: 50 µm.
Mice

C3H/HeN mice were purchased from Charles River Inc., Japan to generate the offspring. Mice were kept in an animal facility at 22 ± 2°C under a 12-h light/dark cycle. Food and tap water were provided ad libitum. All procedures for treating mice were followed in accordance with the instructional guidelines of the Institute of Laboratory Animal Science, Hiroshima University.

For the light and scanning electron microscopic examinations, male mice were exposed to neutrons or gamma-rays at the age of 7 days and sacrificed at 6, 12, 24, 48, 72, 144 or 336 hours, 10 weeks or 12 months post-irradiation (Table 1). For the detection of apoptosis in situ, male mice exposed to neutrons or gamma rays at the age of three and 4 days were sacrificed at 24 hours post-irradiation. Age-matched non-irradiated mice were used for the control group for both experiments.

Histology

Animals were perfused with phosphate buffered saline containing 4% paraformaldehyde under the anesthesia with ethyl ether. The whole hemisphere of the head and the whole inner ear removed from another hemisphere of the head were fixed in 10% phosphate-buffered formalin, decalcified with 5% formic acid (Wako Pure Chemical Industries Ltd., Japan) for 24 hrs, dehydrated through a graded-alcohol series and embedded in paraffin. The tissues were sliced into 4 µm-thick serial sections. Four serial sections were mounted per glass slide. The even-numbered slides were stained with hematoxylin and eosin (H&E) to enumerate the pyknotic nuclei of cells at the greater epithelial ridge (GER) in the cochlea.

in situ apoptosis

The odd-numbered slides were used for the application of the TdT-mediated dUTP-biotin nick-end labeling (TUNEL) method (Wako Pharmacological Ltd, Osaka, Japan) to identify apoptosis.31

Scanning electron microscopy (SEM)

Inner ears were dissected under 2.5% glutaraldehyde prepared in 0.05M sodium cacodylate buffer at pH 7.2 and fixed for 2 hours at 4°C. The tissue was post-fixed with 1% OsO₄ prepared in 0.05M sodium cacodylate bu↵er for 1 hr at room temperature,11 then dehydrated, critical point dried, sputtered with platinum and examined with a JEOL6400 Winsem at 6kV.

RESULTS

Histopathological changes of the inner ear and nasal cavity

The inner ears and the whole mounts of nasal cavity were compared between irradiated with neutrons or gamma rays and non-irradiated controls (Table 1). Gross morphological abnormalities were not observed.

The cross-sections of neutron-irradiated cochlea revealed the degeneration of epithelia at the GER in the organ of Corti. Pyknotic cells detected at the GER at 6 hours post irradiation were TUNEL positive (Fig. 1-B, insert). The cells disappeared at 72 hours post neutron-irradiation (Fig. 1-D, F). The cells facing internal surface of the scala tympani...
decreased in number (Fig. 1-F). The tectorial membrane appeared progressively atrophic (Fig. 1-D, F). These changes were observed neither in the gamma-irradiated group nor in the control group.

Acute otitis media occurred in the tympanic cavity at 144 hours post irradiation. It remained until 10 weeks post irradiation (77 days old), however, the structure of capillaries in the stria vascularis was normal in appearance (Fig. 2-A, B). The structure of the organ of Corti was normal at 12 months post irradiation (Fig. 2-C, D). Lipofuscin deposited most of epithelia in the stria vascularis was more prominent when compared with the control (Fig. 1-A’, B’ C’, D’). These changes were neither in the gamma-irradiated group nor in the control group.

To examine the radiation susceptibility of epithelial cells at the GER at younger ages than 7 days old, mice were exposed to neutrons at 3 or 4 days of age. The cross-sections of neutron-irradiated cochlea at 24 hours later showed the nuclear piknosis and the condensation of epithelium at the GER in the organ of Corti (Fig. 3).

**SEM observation of hair cells**

The protrusions appeared on the apical surface of hair cells in every coil at 6 hours and remained until 72 hours after the neutron-irradiation at 7 days of age (Fig. 4). The diameter of spherical protrusions was less than 5 μm. Fig 4-B showed the fourth row of the outer hair cells in mice. The sensory cilia of the outer hair cells showed the normal appearance at 144 hours after the neutron exposure (Fig. 4-D).

**DISCUSSION**

The early change in the post neutron-irradiated inner ear was the protrusions on the apical surface of hair cells by SEM. The formation of protrusion was reported in the endotelial cells of vessels or lymphocytes after the exposure to ionizing radiations.\(^{12,13}\) Polymerization of filamentous actin accompanied with the radiation-induced structural damage in cells.\(^{14}\) A high LET radiation was found toxic to the ear when irradiated during the postnatal developmental course. The protrusion-like structures were observed in guinea pigs after treatment with aminoglycoside antibiotics.\(^{15}\) The free radicals produced in hair cells by the aminoglycoside caused the degeneration of sensory cilia to form the protrusion-like structure. The antioxidants inhibited the ototoxicity of aminoglycosides.\(^{16}\) The local application of aminoglycosides into the semicircular canal induced apoptosis of hair cells in the organ of Corti.\(^{17}\) Both reversible and irreversible hearing loss associated with acute intoxication or long term-administration of a large range of drugs involve biochemical changes in the inner ear and eighth cranial nerve impulse transmission.\(^{18}\) The toxicity of each drug was found in the individual target; aminoglycosides, anti-neoplastic agents and loop diuretics for the inner and outer hair cells, inner hair cells, and stria vascularis, respectively.\(^{19}\)

Another change observed in the inner ear following irradiation was the disappearance of the cells at the GER, which was the remnant of the primordial organ of Corti. During normal maturation of the inner ear, the GER disappearance starts soon after birth and complete at 2 weeks of age.\(^{20}\) The mechanism of disappearance of the cells at GER was the caspase-dependent apoptosis.\(^{17,21}\) The neutron-irradiation at 7 days of age caused the epithelial disappearance at GER by 72 hours. This apoptotic cell death occurred by the neutron-irradiation at 3 or 4 days of age as well. It should be noted that a high LET radiation quickened the programmed cell death during the process of organ development. As the target cells were the remnant of the primordial organ of Corti, the radiations did not influence on the morphology of the organ of Corti. However, the accelerated disappearance of the GER cells might affect on its dysfunction. One possibility is the loss of the GER-cell ability to replace the hair cells damaged by radiation or other agents at the stages of newborn. Some lower vertebrates are capable of regenerating sensory hair cells from the GER cells even at adult ages.\(^{22,23,24}\) Another possibility is the termination of nutrients and tropic factors from the GER to hair cells, by which the hair cells damaged. A prenatal gamma-irradiation induced extent expression of intermediate filaments in cochlear hair cells\(^{25}\) and the missing of both inner and outer hair cells.\(^{20}\) However, there has been no description about epithelia at the GER.

It is interesting to assess the hearing ability of mice during the period of showing the radiation-induced protrusions. Ten days of age is too early to perform hearing tests; the external auditory meatus does not open until 12 days of age in C3H/He mice.\(^{20}\) The youngest age at which auditory brainstem response had been tested in mice was 1-month-old.\(^{26}\) The standard age for the behavioral test including the test for hearing ability was 6 weeks of age in the N-ethyl-N-nitrosourea mutagenesis program,\(^{27}\) or not until the adult stage in a genetically modified mice showing loss of hair cells\(^{28}\) and in the senescence-accelerated mice showing apoptosis of the spinal ganglion cells.\(^{29}\) Even if a hearing test is available, the neutron exposure causes otitis media subsequently, and the inflammation continues for at least 10 weeks after the exposure. Therefore, it is not clear whether the result of the hearing test reflects an effect from the primary damage of the hair cells or from the secondary inflammation. A prenatal exposure to ~2 Gy of gamma rays induced hearing inability at adult ages.\(^{30}\)

Degeneration of the stria vascularis, epithelia of which actively were involved in the inner ear fluid homeostasis, was not obvious, when 1.0Gy of neutron was irradiated at the age of 7 days. The deposition of lipofuscin was more obvious in the neuron-irradiated group than in the control group.\(^{31}\) It is likely that the exposure to neutrons quickens the physiological hearing impairment in mice at later ages.
The proportion of patients carrying permanent hearing loss with fibrosis at the stria vascularis was over one-third among the patients exposed to 60 Gy of gamma rays for tumor therapy.15

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REFERENCES


