Effect of Monoenergetic Neutron Irradiation on the Postnatal Development of the Cochlea in C3H/HeN Mice

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(Received 26 October 2004/Accepted 28 February 2005)

ABSTRACT. To investigate the toxic effect of neutrons at energies of approximately 1 MeV on the ear, we exposed 7-day-old mice to 1.0 Gy of monoenergetic neutrons (1.026 MeV) or 137Cs gamma rays, and assessed subsequent morphological changes in the inner ear by light and scanning electron microscopy. Monoenergetic neutrons, but not gamma rays, caused acute changes in the ear. The epithelium of the greater epithelial ridge in the organ of Corti had disappeared by 72 hr post-irradiation, as a result of epithelial apoptosis observed 6 hr post-irradiation. Radiation could induce apoptotic cell death of the epithelium of the greater epithelial ridge at 3 or 4 days of age. Prominent structures were detected on the surface of the hair cells by 72 hr post-irradiation. The neutron-irradiation also caused the apoptotic cell death of epithelial cells at the nasal conchae, and subsequent acute otitis media continued until 10 weeks of age.

KEY WORDS: cochlear degeneration, high linear energy transfer radiation, mouse, neutron, scanning electron microscopy.

To date, there have been no epidemiological studies on hearing disability in A-bomb survivors. Epidemiological studies were deemed relevant because the absorbed doses were less than 1 Sv for survivors, and because the main component of the A-bomb radiation was gamma rays, which are low linear energy transfer radiation. Large doses of gamma rays, however, induce hearing impairment. Transient hearing loss occurs in one-third of patients treated with radiotherapy for head or neck tumors [10, 14]. The rate of patients suffering from permanent hearing loss was 36% upon treatment with a 60 Gy dose of gamma rays [2, 3]. However, the pathogenesis of radiation-induced transient or permanent hearing loss has not been well studied due to a lack of a good experimental model, and it is not fully understood.

The A-bomb radiation output included high linear energy transfer radiation, especially neutrons. Survivors from the Hiroshima bomb were exposed to heavier neutron irradiation, with energies between 10−2 and 1.0 MeV, than survivors from the Nagasaki bomb [28]. This difference in energy spectra of neutrons has been quoted to explain differences in data obtained in epidemiological studies of microcephaly, early menopause, chromosomal aberrations or leukemia for the two cities [12]. Investigating mechanisms of damage to the mammalian genome by neutrons and how target cells respond to the damage, will give us a better understanding of the A-bomb disaster.

The relative biological effectiveness (RBE) of neutrons with the energy between 10−2 and 1.0 MeV to gamma rays was 20 according to the radiation weighting factor [4]. The relationship of radiation energy to RBE is linear for gamma rays, but not neutrons, for which it is highest at 0.5–1.0 MeV with regard to cell survival and chromosomal damage [25]. We have used monoenergetic neutrons to establish a good experimental model to evaluate the RBE of neutrons to gamma rays [18]. In a previous report, we showed that oocytes are more susceptible to neutron than to gamma ray irradiation, and we demonstrated the life-shortening effect and tumorigenicity of neutrons in mice of different ages [18, 19].

Radiation is teratogenic in animals exposed to it during organogenesis [27]. Here we report that the inner ear is highly susceptible to damage by neutron irradiation in mice exposed at 7 days of age. The time chosen for irradiation coincides with the developmental process of the greater epithelial ridge (GER), a remnant of the primordial organ of Corti [13]. A higher susceptibility of cells to radiation at the time of developmental processes has been reported for the predigital regions, at which the inter-digital tissue degenerates in a way of programmed cell death [16].

MATERIALS AND METHODS

Radiation: Monoenergetic neutrons were generated by a 7Li(p,n)7Be reaction at the Hiroshima University Radiobiological Reactor (HIRRAC) [8]. The average energy of neutrons, components of radiation, irradiation dose and dose rate (Table 1) have been described previously [18, 19].
Gamma rays were generated from a $^{137}$Cs source (Shimadzu Biotech, Japan).

Mice: C3H/HeN mice were bred in our Animal Facility from breeding pairs purchased from Charles River Inc., Japan. Mice were kept at 22 ± 2°C under a 12 hr-light/dark cycle. Food and tap water were provided ad libitum. All animal procedures were in accordance with the instructional guidelines of the Institute of Laboratory Animal Science, Hiroshima University.

Irradiation protocol: For the light and scanning electron microscopic studies, 7-day-old male mice were exposed to neutrons or gamma rays and sacrificed at various times post-irradiation, as indicated in Table 1. To further assess the susceptibility of the GER epithelium to radiations, male mice were exposed to neutrons or gamma rays at the age of 3 and 4 days and sacrificed 24 hr later. Age-matched non-irradiated mice were used as controls for both experiments.

Histology: Animals were perfused with phosphate buffered saline containing 4% paraformaldehyde under with ethyl ether anesthesia. One whole hemisphere of the head and the whole inner ear removed from the other hemisphere were fixed in 10% phosphate-buffered formalin, decalcified with 5% formic acid (Wako Pure Chemical Industries Ltd., Japan) for 24 hr [5], 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 to enumerate the pyknotic nuclei of cells at the nasal epithelium and the GER in the cochlea. The odd-numbered slides were used to demonstrate in situ apoptosis by the TdT-mediated dUTP-biotin end-labeling (TUNEL) method (Wako Pharmacological Ltd, Osaka, Japan) [19].

Scanning electron microscopy: Inner ears were dissected under 2.5% glutaraldehyde prepared in 0.05 M sodium cacodylate buffer at pH 7.2, and fixed for 2 hr at 4°C. The tissue was post-fixed for 1 hr at room temperature with 1% OsO$_4$ prepared in 0.05 M sodium cacodylate buffer [9], dehydrated, air-dried, sputtered with platinum, and examined with a JEOL6400 Winsem at 6 kV (JEOL Ltd., Tokyo).

RESULTS

Histopathological changes of the inner ear and nasal cavity: The inner ears and whole mounts of the nasal cavity of mice irradiated with neutrons or gamma rays were compared with non-irradiated controls (Table 1). No gross morphological abnormalities were observed.

The cross-sections of neutron-irradiated cochlea revealed degeneration of the epithelium at the GER in the organ of Corti. TUNEL-positive pyknotic cells were detected at the GER 6 hr post-irradiation (Fig. 1B and insert). These cells were no longer detected 72 hr after neutron-irradiation (Fig. 1D, F). The number of endothelial cells of the scala tympani decreased (Fig. 1F), and the tectorial membrane showed progressive atrophy (Fig. 1D, F). These changes were not observed in the gamma-irradiated or control groups.

The longitudinal section of nasal conchae revealed epithelial pyknosis 6 hr after neutron irradiation (Fig 2A, D). Pyknotic cells were TUNEL-positive (Fig. 2D insert). Acute otitis media was seen in the tympanic cavity from 6 days post-irradiation until 10 weeks post-irradiation; capillaries in the stria vascularis appeared structurally normal (Fig. 2B, E). The structure of the organ of Corti was normal at 12 months post-irradiation (Fig. 2C, F). Increased lipofuscin deposition was observed throughout most of the epithelium of the stria vascularis in irradiated mice (Fig. 2C', F'). These changes were not observed in the gamma-irradiated or control groups.

To examine the susceptibility of GER epithelial cells to neutron radiation in mice younger than 7 days, mice were irradiated at 3 or 4 days of age. Cross-sectional histology of the cochlea obtained 24 hr later showed degeneration of the GER epithelium in the organ of Corti (Fig. 3).

Scanning electron microscopic study of the hair cells: In neutron-irradiated 7-day-old mice, protrusions appeared on the apical surface of hair cells in every coil after 6 hr and remained until 72 hr post-irradiation (Fig. 4). The protruding structures were localized adjacent to every row of the outer hair cells. Figure 4C shows the fourth row of the outer hair cells in mice. The sensory cilia of the outer hair cells appeared normal 6 days after neutron exposure (Fig. 4D).

DISCUSSION

This morphological study has shown that the inner ear of newborn mice is susceptible to neutron exposure. The target cell populations were the hair cells and the epithelium at the GER.

The early change caused in the inner ear by monoenergetic neutrons, a high linear energy transfer radiation, was protrusion on the apical surface of hair cells. Radiation-induced protrusions have been reported on the surface of endothelial cells of vessels or of lymphocytes; however,
they were induced by low energy transfer radiation [14, 23]. Polymerization of filamentous actin was associated with the radiation-induced cell structural damage [15]. Acute intoxication or long-term administration of drugs result in morphological changes in the inner ear [20]. For example, aminoglycoside induced production of free radicals in hair cells and the formation of a protrusion-like structure on their apical surface, and caused the degeneration of sensory cilia in guinea pigs [17, 22, 26].

Another change observed in the inner ear following irradiation was the disappearance of the epithelial cells at the GER, which is the remnant of the primordial organ of Corti. During normal maturation of the inner ear, disappearance of the GER structure mediated by caspase-dependent apoptosis starts soon after birth and is complete at 2 weeks of age [11, 17, 24]. In 7-day-old mice, neutron-irradiation led to disappearance of the GER epithelium within 72 hr. Neutrons also induced apoptotic cell death in mice irradiated at 3 or 4 days of age, at which the epithelia had been programmed to die near future. It should be noted that exposure to monoener-

Fig. 1. Cross-section of the organ of Corti at 6, 24 or 72 hr after the neutron-irradiation at 7 days of age (B, D, F) and the age-adjusted control (A, C, E). The pyknotic nuclei were observed at the GER (bracket) at 6 hr after neutron-irradiation (B). Disappearance of the epithelial cells at the GER (bracket) and atrophy of the TM were prominent at 24 hr and 72 hr post neutron-irradiation (D, E). Endothelial cells of the ST became atrophic and decreased in number at 24 and 72 hr 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 endothelial cells of the ST are TUNEL positive in the serial section counterstained with methylgreen (arrow in the insert in B). × 200 (original magnification). GER: great epithelial ridge, OHC: outer hair cells; IHC: inner hair cells; TM: tectorial membrane; ST: scala tympani.
getic neutrons hastened cell death programmed during the process of organ maturation. Radiation exposure in utero induced apoptosis in the predigital regions of embryonic limb buds, where cells were programmed to die within the following 2 days [1]. Susceptibility to radiation in the predigital regions and defects of the digits depended on both the Trp53 status and the radiation dose [29, 30]. These findings suggest that radiation-induced apoptosis in tissues where cells are programmed to disappear during organogenesis is responsible for certain congenital defects or functional impairments.

Since the organ of Corti is fully structured at birth, irradiation at 7 days of age did not induce architectural defects of the inner ear. However, the accelerated disappearance of the GER cells may impact on its function. One possibility is the loss at newborn stages of the GER cell ability to replace damaged hair cells; some lower vertebrates are capable of regenerating sensory hair cells from the GER cells even at adult ages [6, 7, 21]. Another possibility is that supply of nutrients and tropic factors from the GER to hair cells will stop, leading to hair cell damage. The neutron-irradiation increased the deposition of lipofuscin in the epithelium of stria vascularis at 12 months of age. It is likely that radiation exposure accelerates the appearance of physiological hearing impairment.

AKNOWLEDGEMENTS. The authors wish to thank Dr. Y. Harada for useful discussions on the structure of the organ of Corti as assessed by scanning electron microscopy, and the degenerative changes of the stria vascularis with age as assessed by light microscopy. This study was supported by a Grant-in-Aid from the Japan Atomic Energy Research Institute (Y. N., 2004).
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Fig. 4. Outer and inner hair cells of the organ of Corti by SEM. Protrusions (arrow) are observed adjacent to the cilia of hair cells from the 1st to the 3rd row at 72 hr post neutron-irradiation (C) but not in the age-adjusted control (A). One protrusion structure is rupturing (arrow and the insert in C). The sensory cilia look normal at 144 hr post neutron-irradiation (D) and age-adjusted control (B). The 3rd or 2nd coils for A and C, and B or D, respectively. Bar: 5 µm.