Microcephaly Due to Low-dose Intrauterine Radiation Exposure Caused by $^{33}$P β Administration to Pregnant Mice

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The effects of low-dose rate/low-dose intrauterine exposure during organogenesis were investigated using pregnant mice and an experimental system employing both 2-D electrophoresis and mass spectrometry. Ras GTPase-activating protein-binding protein 1 and Rho GDP-dissociation inhibitor 1 are known to play vital roles in the morphological development of the nervous system such as synaptic plasticity and elongation of axons. These proteins showed a marked increase in fetal tissues collected 3 days after mothers were administered 3.7 MBq $^{33}$P-ATP. This suggests that active GTPase decreases and inactive GTPase increases resulting in a disruption of neurotransmission. To confirm if these changes were associated with microcephaly, PQBP1 (Polyglutamine binding protein 1) expression was determined by Western blot assay. Administration of 3.7 MBq resulted in a marked inhibition of PQBP1 expression in fetal tissues proving that even low-dose internal exposure was enough to induce changes suggestive of microcephaly. Actual measured values from the maternal injection site confirmed that the total dose over the 3 days after administration is about 50 mGy. This demonstrates that even low-dose intrauterine exposure warrants caution, suggesting that appropriate radiation safety measures such as education pertaining to the risks of exposure is important for pregnant workers.

Key Words: microcephaly, PQBP1 (Polyglutamine binding protein 1), internal exposure, low-dose exposure, CNS (central nervous system)

1. Introduction

When radiation exposure accidents involving the general public occur, the immediate issue becomes whether pregnancies must be artificially terminated to avoid the possibility of anomalies in fetuses that are in the initial stages of pregnancy where they are most sensitive to the effects of radiation. It was reported that a great many people in Europe did terminate their pregnancies immediately after the Chernobyl atomic plant accident, but details on the accuracy of these reports are scarce. The UNSCEAR report in 2000 documented the incidence of anomalies among neonates born in the Belarus area where in-utero exposure was between 8 to 21 mSv. No clear correlation was noted between the incidence of anomalies and those living in these radioactive areas.¹ Most of the anomalies noted in the fetuses were believed to be due to causes other than radiation. WHO specialists also reported that the incidence of congenital anomalies in this region could not be associated with radiation exposure.²

Research in rodents has shown that the effects of radiation exposure will differ depending on the stage of fetal developmental at the time of exposure and can range from fetal death, anomalies, neonatal
death (stillbirth), to failure to thrive. Extrapolating these rodent results to human developmental stages suggests that, if radiation exposure occurs during early implantation (fertilization to day 8), fetal death will occur; however, in most of these cases the fetus is reabsorbed, thus these events often go unrecognized. Furthermore, the fetuses that do survive until birth will have no anomalies. However, if radiation exposure occurs during organogenesis (Day 9 to Day 60 post-fertilization), both anomalies and stillbirths may occur. Anomalies in a wide variety of organs have been reported, but most of these involve external appearance such as missing fingers or a lack of eyes, and very few studies have looked at morphological abnormalities involving the vasculature or neural pathways. In particular, detailed research on internal radiation exposure has hardly ever been reported in the past. In humans, investigational research in atomic bomb survivors has only reported the development of microcephaly. This is because the research in Hiroshima was limited to people who had been exposed to radiation in-utero. This effect was not seen among the Nagasaki bomb survivors. Effects of radiation exposure were noted between weeks 4–15 of pregnancy while the results of exposure were particularly marked between weeks 8 to 15.

Our study was conducted to observe the effects of internal exposure of pregnant mice to low-dose rate and low-dose radiation during organogenesis. Two-dimensional electrophoresis (2DE) and mass spectrometry were used in combination to investigate, in detail, what sort of effects these doses would have on the development of the fetal CNS.

2. Materials and methods

Mice and histochemistry: C57BL/6 were reared in polycarbonate cages in an environmentally controlled room (water temperature: 22±1°C), with a standard 12h light/dark cycle. Injection of mother mice with the 0.37 MBq or 3.7 MBq of 33P-ATP (adenosine 5’-triphosphate) was performed on day 7.5 of pregnancy. Radioisotope was diluted to the appropriate concentrations with normal saline solution immediately before use. To evaluate abnormalities in CNS development, fetuses were removed from the uterus on day 10.5 of pregnancy and examined by two-dimensional electrophoresis and western blotting. Furthermore, laparotomy was performed on 13.5 of pregnancy and the fetuses removed from the uterus were examined histologically. Mice were mated at midnight, and pregnancy day 0.5 was the morning when a vaginal plug was detected.

The 170 fetuses used in this experiment were fixed for 24h in a 10% formaldehyde solution buffered with 0.15 M sodium phosphate at a pH of 7.3. They were then washed in several changes of the same buffer and embedded in paraffin. Serial sections of 2µm slices were prepared and stained with Mayer’s hematoxylin. The immunohistochemistry protocol was similar to that described previously. Briefly, the section was pre-treated using heat mediated antigen retrieval with sodium citrate buffer for 20 min. The section was then incubated with antibody (ab18207, Abcam of beta III tubulin, ab13847 of caspase 3, ab5176 of histon H3 phosphoS10, ab23345 of TBR2) for 15 min at room temperature and detected using an HRP conjugated compact polymer system. DAB was used as the chromogen. The section was then counterstained with haematoxylin and mounted with DPX. All experiments were approved by the animal ethics committee of the National Cerebral and Cardiovascular Center.

Two-dimensional electrophoresis (2DE) and western blotting: 2DE was performed using 4 complete fetus for each sample. For the western blotting, 2 complete ones were used for each sample. Each sample was first washed in cold saline and then homogenized in the presence of 5 M urea, 2 M thiourea, 2% CHAPS, 2% SB3-10, and 1% DTT. Superna-
tants were collected after centrifugation at 20,000 g for 30 min. The total protein concentrations of the samples were determined using a protein assay kit (Pierce). First-dimensional separation of the proteins was performed on an IPGphor IEF system using immobiline DryStrips pH 4–7 (GE Healthcare Bio- sciences) or Pharmalyte broad range, pH 3–10 (GE Healthcare Life Sciences). The extracts were loaded onto rehydrated immobiline strips and electrophoresed with internal protein markers (Promega). Running conditions were 3,500 volts max for 8 h. Vertical SDS-PAGE was used for the second dimension, using 9–18% acrylamide gradient gels. After 2DE, gels were dyed using a fluorescence staining reagent for the detection of phosphorylated proteins (Pro-Q Diamond phosphoprotein gel stain, Molecular Probes), or all proteins (SYPRO Ruby protein gel stain). Gel images were obtained using a fluorescence scanner, and images were evaluated using ImageMaster Platinum (GE). The signal intensity of all spots was computed using this software. Signal intensity was shown with the value (%Volume value) which was divided by the total of the signal intensity of all spots on gel. The phosphorylation index (i.e., the number of phosphate groups per molecule of protein) was calculated from the two %Volume values as follows: phosphorylation index = (D/S ratio) = (%Vol. value of the Pro-Q Diamond dye spot) / (%Vol. value of the SYPRO Ruby dye spot). Spots satisfying the following two conditions were listed as protein identification candidates: (1) The sum of the % values from two 2DE gels dyed with SYPRO Ruby was 0.1 or more. (2) The relative change in phosphorylation index for a spot in the drug-injected groups was 1.5 or more relative to the saline-injected groups. The gel digests were analyzed by LC/MS/MS with a Waters NanoAcquity HPLC system interfaced to a Thermo- Fisher Orbitrap Velos Pro. Peptides were loaded on a trapping column and eluted over a 75 μm analytical column at 350 nL/min; both columns were packed with Jupiter proteo resin (Phenomenex). The mass spectrometer was operated in data-dependent mode, with MS performed in the Orbitrap at 60,000 FWHM resolution and MS/MS performed in the LTQ. The fifteen most abundant ions were selected for MS/MS.

The same samples used for 2DE were used for western blotting. First, equal quantities of each sample were mixed with SDS sample buffer (125 mM Tris-HCl, 4% SDS, 10% sucrose, 2% DTT) and heated for 5 min at 95°C. Each sample was then run on 4% SDS-PAGE gels using standard procedures. Subsequently, the proteins were transferred onto a PVDF membrane (GE Healthcare Bio-Sciences) using a blotting unit (GE Healthcare Bio-Sciences) with blotting buffer (25 mM Tris-HCl, 200 mM glycine, 10% methanol, 0.02% SDS). Blots were incubated with primary antibodies against PQBP1 (ab100797), APC4 (ab72149) and GAPDH (ab8245) for 16h at 4°C and with the corresponding secondary antibody (GE NA934, anti-rabbit IgG, HRP-linked whole Ab Donkey, or GE931, anti-mouse IgG, HRP-linked whole Ab Sheep) for 1h at RT. Finally, the PVDF film was used to expose the X-ray film.

Statistical analysis: At least 3 runs were performed for western blotting and data were expressed as the mean±S.E. Deviations between the saline group and drug administration group were analyzed with the Chi-squared test where a p value <0.01 was considered statistically significant.

3. Results and discussion

Mass spectrometry results from pregnant animals administered intraperitoneal doses of 3.7 MBq were compared to those from the control group. It was possible to identify 4 protein types that increased markedly in fetal tissue after radiation exposure (Fig. 1-(1)). These were: (1) Ras GTPase-activating protein–binding protein 1, (2) Creatine kinase B-type, (3) Rho GDP-dissociation inhibitor 1, and (4) Thioredoxin domain-containing protein 12.
Increased GTPase-activated protein (GAP) suggested enhanced hydrolysis from GTP to GDP, resulting in decreased amounts of activated GTPase. Moreover, if this was accompanied by increases in guanine nucleotide dissociation inhibitor (GDI), GDP dissociation was inhibited with an increased prevalence of inactivated GTPase. Past reports show that low molecular weight G proteins regulate various functions including neuron morphology and signal transmission between neurons. In particular, the Ras family has been associated with synaptic plasticity, i.e., the ability to change neuronal pathways depending on memory and learning stimuli, and the Rho family is known to play a major role in morphological development such as the lengthening of neuronal axons or dendrites. Based on our results showing a decrease in activated GTPase but an increase in inactive GTPase, it is easy to surmise that functional signal transduction in the nervous system will be disrupted after administration of 3.7 MBq. Creatinine kinase is an enzyme that produces creatine and ATP from creatinine phosphate and ADP. It is a dimer protein comprising 2 subunit forms called Brain (B) and Muscle (M). Type B in particular, increases along with abnormalities of the nervous system. These protein content changes suggest that abnormalities of the nervous system have occurred due to internal radiation exposure. Thioredoxin is an
antioxidant enzyme molecule with an SH group that expresses both oxidative and reductive activity. It is a multifunctional protein involved in intracellular signal transmission and provides protection against oxidative stress/reactive oxygen. Increases in this protein suggest that the effects of radicals (indirect effects) resulting from radiation are also increased. This shows that the effects of intrauterine exposure are mediated through radicals.

Based on changes in the above-mentioned proteins, we hypothesized that radiation exposure no doubt influenced fetal CNS. Based on this hypothesis, we prepared tissue specimens from 13.5-day-old fetuses to observe the effects of radiation on brain development. First, a TBR2 (neural-progenitor marker) antibody was used to determine immunochromatic assay data. Compared to the control group, fetuses exposed in-utero by administrating a 0.37 MBq intraperitoneal dose to pregnant mothers showed an increase in TRB2-positive cells (brown staining in Fig. 2) in the SVZ layer (subventricular zone) of the fetal brain (Fig. 2). However, in the 3.7 MBq dose group, a marked decrease in TRB2-positive cells was noted. The increases in TRB2 in the 0.37 MBq group data were believed to be due to a stimulatory effect of low dose radiation (hormesis effect). Staining with an antibody to caspase-3 (apoptosis marker) showed that although hardly any cell death was observed in the control group, many apoptosis-positive cells (brown staining in Fig. 2) were observed in the VZ to SVZ areas of the 3.7 MBq dose group. Immunochromatic assay images using antibodies to β-III-tubulin (Tuj1; neuron marker) showed that compared to the 0.37 MBq or control group, the 3.7 MBq dose group had a marked increase in positive cells (brown staining in Fig. 2). In other words, higher radiation doses led to greater CNS toxicity.

Next, immunochromatic assays using antibodies to histone H3 phosphorylated at Ser10 (PH3; marker of mitotic cells) were used to study nerve progenitor cell growth. Results showed that in the unexposed control group, PH3 positive cells formed a single
line in the VZ layer. However, in the 3.7 MBq dose group, PH3 positive cells were found scattered throughout the VZ and SVZ layers and even extending outside those areas (Fig. 2). It is possible to speculate that brain structures were being destroyed.

Furthermore, a Western blot assay was used to investigate PQBP1 (Polyglutamine binding protein 1) expression in order to confirm that the changes associated with microcephaly as reported among atomic bomb survivors at Hiroshima were reflected in our data on nervous system abnormalities derived from these tissue specimens. More marked inhibition of PQBP1 expression was noted in the 3.7 MBq administration group showing that changes similar to microcephaly occurs even with low-dose rate or low-dose intrauterine exposure (Fig. 1-(2)). However, expression of APC4 (ubiquitin ligase complex subunit) indirectly bound to PQBP1, did not differ from control.

This leads us to the question of just how much radiation the fetus was exposed to under these experimental conditions. A 3.7 MBq dose of 33P-ATP was injected into the peritoneum of pregnant mice on day 7.5 of pregnancy. Fetuses were surgically extracted at 24, 48 and 72 h after maternal intraperitoneal injection and whole fetal radiation doses were measured with a liquid scintillation counter (Table 1). Levels were 0.19 mGy at 24 h after administration and this level was maintained up to 72 h after injection. Thus the cumulative dose over 3 days was 0.56 mGy and the distribution rate to the placenta was ≤1%. After 72 h, there was a slight decrease in radiation due to decay with 0.15 mGy at 96 h, and 0.13 mGy at 120 h. The cumulative dose over 5 days was approximately 1 mGy. This exposure was much lower than previously-reported from external exposure data, meaning that the CNS effects presented here occurred at much lower external exposure doses than those reported in the past. We must however take into account the effect of β rays released from the maternal peritoneal injection site and thus it will be a higher total dose than the level calculated above. Actual measured values from a 3.7 MBq dose at the maternal injection site showed a total dose over the 3 days after administration of about 50 mGy. It may be difficult to measure the β radiation based on fetal external radiation exposure in the amniotic fluid, and so fetal intrauterine exposure will be impossible to calculate accurately.

Nearly 7 years have passed since the Fukushima nuclear plant accident (2011) and the annual environmental radioactivity exposure of those living near the nuclear plant is said to be around 50 mSv. The lack of historical data on low-dose rate and low-dose

| hours after injection | radiation doses of injection sites | radiation doses of fetuses |
|-----------------------|-----------------------------------|----------------------------|
|                       | dpm (×100,000) | mGy/day | dpm (×100,000) | mGy/day |
| ~24                   | 89388 (8)        | 19      | 924 (10)       | 0.19   |
| 24~48                 | 88265 (5)        | 18      | 897 (5)        | 0.19   |
| 48~72                 | 88201 (5)        | 18      | 829 (8)        | 0.18   |
| 72~92                 | 83311 (3)        | 17      | 701 (4)        | 0.15   |
| 92~120                | 73213 (3)        | 15      | 621 (3)        | 0.13   |

Figures in parentheses are number of mice/fetuses used.
radiation exposure makes it difficult to determine whether these levels are actually dangerous for humans. However, reports of people living in the Ramsar region of the Middle East have shown them to be without any health issues despite environmental radiation levels far higher than those in Fukushima. This suggests that the areas around Fukushima should be safe. Recent findings of occupational exposure in pregnant workers have estimated that the mean total dose over the entire pregnancy ranged from 0.08 mSv to a maximum of 0.89 mSv. A vital finding from the current study is that in our experiment on the effects of low-dose rates and low-dose exposure using a 3.7 MBq $\beta$ radiation source, administration of this dose during organogenesis caused major damage to the fetal CNS system. This demonstrates that even low dose intrauterine exposure requires caution, suggesting that appropriate radiation safety measures such as education pertaining to the risks of exposure is important for pregnant workers.

Fetal death is known to occur both before implantation or immediately after implantation at very low doses of 0.1 Gy ($\beta$ radiation 0.1 Gy = 0.1 Sv) based on results from animal studies. External irradiation of rodent fetuses on day 8 has already been studied extensively. A dose of 0.25 Gy produces deformed fetuses (anomalies), and such anomalies increase as the dose is increased. However, hardly anything is known about the effects of low-dose rate and low-dose internal exposure on nervous system abnormalities. Since CNS cells are also rapidly dividing at this stage of fetal development, they are highly susceptible to radiation. Intrauterine exposure of atomic bomb survivors was associated with not only anomalies, but also mental retardation and decreases in IQ. When predicting the effects of radiation exposure on the nervous system, the relationship between the stage of brain development and the stage of pregnancy is extremely important. After fertilization, normal brain development can be divided into 4 stages: Stage 1 (Weeks 0–7), Precursor neurons and neuroglia appear and begin rapid mitosis; Stage 2 (Weeks 8–15), Neuron counts increase dramatically and neurons migrate to the places where they are supposed to develop and lose their mitotic ability; Stage 3 (Weeks 16–25), Differentiation accelerates with increases in synapse formation, and cellular organization of the brain is completed; Stage 4 (Week 26 onward), Cellular organization of the brain, differentiation, synapse formation continues in the cerebrum and cerebellum. In the cohort of 1598 patients exposed in-utero to atomic radiation, 30 children were reported to have developed severe mental retardation and all of these cases were identified before age 17. Expression of mental retardation is most apparent when exposure occurs during Stage 2 of brain development and the severity increases with dose. Reports have shown that MRI testing revealed that structural anomalies of the brain were clearly associated with mental retardation. In our study, exposure to a dose of 50 mSv resulted in a marked decrease in PQBP1 protein. Based on previously published articles from other researchers, mutation of the PQBP1 gene is associated with mental retardation in addition to the microcephalia. Further developments from our study may allow us to elucidate mechanisms involved in the mental retardation induced by radiation.

Three mechanisms are believed to play a vital role in the development of microcephaly. These include an increase in the efficiency of neural stem cell differentiation (neural stem cells are therefore depleted), promotion of cell death in neural stem cells, and disruption of differentiated neuron cell movement. Recent results on PQBP1 have shown that if their function is stopped by revising the genes responsible for this function, although microcephaly will occur, the previously described mechanism does not apply, and instead, cell cycles during brain formation in fetuses are abnormally extended. Genetic abnor-
malities of PQBP1 have been referred to by various names including Renpenning syndrome, Sutherland-Haan syndrome, and Hamel syndrome. These show that in addition to clinical symptoms of intellectual disability, a high incidence of microcephaly-related complications is another characteristic, and its incidence is extremely high even among genetic causes of intellectual disabilities. As can be discerned from the results of this study, in-utero radiation exposure led to a marked decrease in PQBP1. Consequently, this extends the cell cycles causing a decrease in the frequency of mitosis leading to a decrease in the production of neurons by reducing the number of mitoses in neural stem cells. There are also reports that PQBP1 may be associated with neurodegeneration. Interestingly, in radiation sensitivity tests using fibroblasts from patients with neurodegenerative diseases such as Huntington’s disease, the cells appear to become highly sensitive to radiation after disease onset. In other words, they become susceptible to damage by even a small dose of radiation. Inhibition of PQBP1 expression is not only due to induction of CNS toxicity, but may also be implicated in changes in radiation sensitivity.

Declaration of Conflicting Interests

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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要旨

33P β線源の妊娠マウスへの投与によって起こった
低線量胎内被ばく誘発小頭症

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本実験は、器官形成期にある妊娠マウスを使って、低線量胎内被ばくの影響を、2次元電気泳動と質量分析を組み合わせた実験系から調べた。その結果、Ras GTPase-activating protein–binding protein 1とRho GDP-dissociation inhibitor 1が、3.7 MBq 33P-ATPの母親への投与により、胎児組織で著しく増加することがわかった。この時の、母親の注射部位周辺で、投与後3日間における総線量は約50mGyであった。さらにこの生化学的変化が小頭症に関連するものかを確かめるために、PQBP1 (Polyglutamine binding protein 1) 発現をウエスタンブロット法で調べてみたところ、3.7 MBq投与により、胎児組織でPQBP1発現が抑制されており、低線量の内部被ばくでも小頭症が疑える変化が誘発されたことがわかった。