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Modulation of In Utero Total Body Irradiation Induced Newborn Mouse Growth Retardation by Maternal Manganese Superoxide Dismutase-Plasmid Liposome (MnSOD-PL) Gene Therapy

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Abstract

To determine the effects of Manganese superoxide dismutase (MnSOD) plasmid liposome (PL) maternal radioprotection on fetal mice, timed pregnant female mice (E14 gestation) were irradiated to 3.0 Gy total body irradiation (TBI) dose, and the number, weight, and growth and development over 6 months after birth of newborn mice was quantitated compared to irradiated controls. Maternal MnSOD-PL treatment at E13 improved pup survival at birth (5.4 ± 0.9 /litter compared to irradiated 3.0 Gy controls 4.9 ± 1.1). There was no statistically significant difference in newborn abnormalities, male to female ratio in newborn litters, or other evidence of teratogenesis in surviving newborn mice from MnSOD-PL treated compared to irradiated controls. However, E13 3Gy irradiated pups from gene therapy treated mothers showed a significant increase in both growth and overall survival over 6 months after birth ($p = 0.0022$).

To determine if transgene product crossed the placenta pregnant E13 mice were injected I.V. with hemagglutinin-epitope-tagged MnSOD (100 μ gm plasmid in 100 μ l liposomes), then 24 hours later fetal mice, placentas, and maternal tissues were removed and tested by both immunohistochemistry and RTPCR for transgene and product. There was no evidence of transgene or product in placenta or any fetal tissue while maternal liver was positive by both assays. The data provide evidence for fetal radioprotection by maternal MnSOD-PL gene therapy before irradiation which is mediated by an indirect bystander effect and is associated with a significant improvement in both survival at birth and growth and development of newborn mice.

Keywords

in utero irradiation; fetal mice; total body irradiation; MnSOD-PL gene therapy

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Introduction

The teratogenic effects of in utero ionizing irradiation on the fetus have been well documented (1-6). Irradiation-induced fetal death and teratogenesis have been demonstrated to increase with exposure at early time of gestation and are dependent upon both species and mouse strain (7-10). Growth retardation and developmental defects have been well documented in the progeny of irradiated mothers (8-11). In clinical radiotherapy, concern for radiation dose to the developing human fetus, in cases where maternal radiotherapy is indicated, include the critical need to calculate internal scatter dose as well as direct irradiation dose (12-14). Concern for irradiation teratogenic effects often enter discussions of termination of pregnancy if at early gestation, or delay in the initiation of radiotherapy to facilitate increased fetal growth, and early pre-term delivery (1, 2, 4). The effects of radiation protector drugs on fetal growth and development are a subject of significant investigation.

We have reported a significant radioprotective effect of systemic intravenous MnSOD-PL administration in adult male and female mice against both the LD 50/30 dose, 9.5 Gy, and a lower 1.0 Gy TBI dose (15). Furthermore, the increased survival after TBI of MnSOD-PL treated mice was not associated with a detectable increase in delayed irradiation effects including life shortening, carcinogenesis or neurological disorders (15).

In the present study, we determined the effect of total body irradiation on mid-gestation (E13) pregnant female C57BL/6HNSd mice with respect to irradiation effects on the fetus. The results demonstrate improvement in newborn mouse survival, as well as growth and development by maternal MnSOD-PL gene therapy. Furthermore, fetal radioprotection was independent of transgene expression in fetal tissues and was mediated through a remote effect of radioprotection.

Results

Total Body Irradiation Sensitivity of E14 Pregnant Female Mice

A dose response curve for irradiation effects on developing fetal mice was first carried out. Mid-gestation pregnant (E14) adult female C57BL/6HNSd mice were irradiated to 0, 1, 3, 5, or 7 Gy total body dose as described in the methods using 3 to 4 litters per dose group (Table 1). At 0 Gy 3 litters with 20 pups were born and all mice survived till weaning. At 1 Gy there were 3 litters with 19 pups alive and 1 dead. Four litters were born at 3 Gy with 9 pups alive and 20 pups dead. At 5 Gy there were 3 litters of 15 pups; all were dead by 48 hours after birth. At 7 Gy 3 litters were born with 19 pups, all were born dead, some in amniotic sacs, 1 fetus had no thorax or abdomen and 1 fetus was small and necrotic. Thus, the high 7 Gy irradiation dose resulted in fetal death in utero, with no live births and maternal mice receiving 5 Gy demonstrated stillborn pups. We, therefore, compared results with MnSOD-PL treated 3 Gy irradiated E14 maternal mice compared to 3 Gy irradiated controls to test the effects of radioprotective gene therapy.

MnSOD-PL Radioprotective Gene Therapy Improves Fetal Mouse Survival, Growth, and Development

Groups of timed pregnant female mice (E13) next received (100 µg MnSOD plasmid in 100 µl liposomes) intravenously as published (15) and were irradiated 24 hours later along with noninjected control E14 pregnant mice to 0, or 3 Gy. The total number of pups was counted at birth, at day 2, and again at weaning, and the number of pups per litter was also scored (Table 2, Figure 1). Mice that were not irradiated showed no deleterious effect of MnSOD-PL treatment (Table 2, Fig. 1). The data showed a significant decrease in number of pups/litter from the pregnant mice irradiated to 3 Gy compared to control unirradiated mice. There was also a significant decrease in the weight of newborn pups from 3 Gy irradiated pregnant mice receiving MnSOD-PL compared to unirradiated mice (Table 2). There was a significant decrease in the weight at weaning of pups from 3 Gy irradiated E14 pregnant mice compared to unirradiated controls ($p = .0024$) (Table 2). At 6 months after birth, there was a significant decrease in weight in all pups from irradiated mothers (Table 1, Figure 1). However, pups from MnSOD-PL treated then 3 Gy irradiated E14 pregnant mice had a significant increase in survival over 220 days compared to those from 3 Gy irradiated controls ($p = 0.0022$) (Figure 2).

MnSOD-PL Radioprotective Gene Product Does Not Detectably Cross the Placenta

To determine whether fetal radioprotective gene therapy was associated with detectable transfer of the MnSOD-Plasmid transgene or its product across the placenta, groups of E13 pregnant female mice received intravenous hemagglutinin (HA) epitope-tagged MnSOD-PL (21) and 24 hours later were euthanized according to IACUC institutional procedures. All tissues were Isolated and Tested Separately by Both In Situ Immunohistochemistry for Detection of Hemmagglutinin epitope tag (16), and by RTPCR (17-21) for detection of MnSOD transgene transcript according to published methods. Individual fetal mice with associated umbilical cord and placenta were removed, and sagittal sections prepared for immunohistochemistry. RTPCR was carried out in each fetal sample as well as in maternal tissues.

Examination of ten individual fetal mice from three different HA-MnSOD-PL injected mothers demonstrated no detectable hemagglutinin in placenta or in any fetal tissue (Figure 3). In contrast high levels of Ha-MnSOD-PL transgene product were detected in the maternal liver (Figure 3). RTPCR analysis of maternal and fetal tissues demonstrated a high level MnSOD-transgene in maternal liver (Figure 3). In Figure 3E, results are presented for a representative tissue from one of 10 per group including: maternal liver, liver, heart, lungs, spleen, intestine, kidney (lanes 2 – 8 respectively) and fetal whole body tissues (lanes 9-13). We also tested 5 separate placentas from each of 5 pups, removed one day after HA-MnSOD-PL injection of the mother (lanes 16-18). Examination of maternal bone marrow, skeletal muscle, and lung, showed no detectable MnSOD-transgene by RTPCR at the 24 hour time point. These results are consistent with previous publications of transgene clearance in adult mice over 24 – 48 hours after I.V. injection of MnSOD-PL and are associated with a decrease in detectable levels of MnSOD gene product over 48 – 72 hours (18).

Discussion

The present data demonstrate that intravenous MnSOD-PL administration to pregnant female mice at mid-gestations results in significant radioprotection of fetal mice that are subsequently exposed to 3 Gy total body irradiation. Previous studies have documented the radioprotective capacity of MnSOD-PL transgene product in adult male and female mice (15, 16, 18-23). In prior studies, both male and female mice receiving MnSOD-PL 24 hours prior to TBI demonstrated significant improvement in survival from the 9.5 Gy, LD 50/30 dose or a lower 1.0 Gy dose of total body irradiation (15). The known late irradiation induced life shortening in survival after TBI was not exacerbated in either male or female mice and male mice showed a surprising improvement in late irradiation induced life shortening (15). There was no increase in detectable tumors, histopathologic evidence of neurodegenerative disease in the increased number of survivors in either 9.5 Gy or 1.0 Gy, groups that received MnSOD-PL prior to irradiation (15).

The method of MnSOD-PL administration is indeed advantageous over other treatment modalities in that transgene product is produced continuously in the recipient animal for 48 – 72 hours after one transgene administration (30-38). For radioprotection, the MnSOD enzyme is not only required at the time of irradiation, but also for 24 – 48 hours after irradiation to provide continuous protection not only from the first wave of apoptosis of normal cells by irradiation, but by the secondary and tertiary waves that are induced by cytokine release, including TGF β , TNF α , and IL-1, from irradiated tissues (30). The timing of administration of transgene, 24 hours prior to irradiation allows for transgene incorporation, mRNA transcription, and message translation (32). Continual production of transgene product in vivo for 48 to 72 hours after initial expression is the known result of a single administration (30, 37). The transgene product expression, therefore, is protracted and the advantage of gene therapy is to continually provide a “depot” of MnSOD-protein in cells and tissues where targeting the mitochondria can occur continually. In contrast, administration of MnSOD protein has been shown to be ineffective even in multiple administrations (36).

In the present studies, pregnant female mice were treated to a dose of 3 Gy TBI that was associated with early post partum newborn death (30-38). Pregnant female mice receiving 3.0 Gy demonstrated a significant decrease in the number of live births, and newborn mice showed decreased growth and development compared to non-irradiated controls. Irradiation induced decrease in growth and development of fetal mice has been documented previously (8-10). In the present studies, MnSOD-PL administration 24 hours prior to 3.0 Gy irradiation did not significantly change the number of pups/litter or weight at weaning. There was also no change in weight at six months after birth. However, there was an increase in overall survival of pups from pregnant mice that received MnSOD-PL 24 hour before 3 Gy total body irradiation compared to pups from pregnant mice irradiated to 3 Gy only. These results are consistent with a radioprotective effect of MnSOD-PL gene therapy on the mid gestation mouse fetus (16, 18-23). The mechanism of irradiated newborn mouse defects may be through delayed growth of the newborn mice, placental defects – maternal or fetal, or both mechanisms.

The teratogenic effects of ionizing irradiation are well documented (1-6). In rodent models, as well as larger mammals, total body irradiation of pregnant females has been demonstrated to result in fetal death, growth abnormalities, and multi-generational transmission of genetic mutations as well as chromosome abnormalities. In murine model systems, teratogenic abnormalities are highest, early in the developmental process, and decreases with gestational age of the fetus (24). We saw no effect of 3 Gy or MnSOD-PL on teratogenesis in E13 mice. Studies on earlier gestation fetal mice may be required to detect such differences.

The fetal protective effects of MnSOD-PL were not associated with detectable MnSOD gene product in the fetal tissues by measure of HA-MnSOD, epitope-tagged gene product, or transgene transcript in fetal tissues as measured by RTPCR. The results indicate that the fetal radioprotective effect was mediated by a remote effect of radioprotection. Ionizing irradiation increases inflammatory cytokines in irradiated tissues, as well as levels of inducible nitric oxide synthase (20, 23, 26-27). These small molecules that are transmitted across the placenta from maternal tissues may have been reduced in MnSOD-PL treated mice, and this reduced level may have decreased secondary cytokine elevations in the fetus. However, since fetal tissues were irradiated and the same inflammatory proteins would have been expected to be elevated as part of the inflammatory response to irradiation in utero it is likely that the transmission of a protective mediator(s) was delivered across the placenta as has been demonstrated in other systems (25). Further studies will be required to document the mechanism of indirect protection of the fetus by maternal MnSOD-PL.

The present data may be useful in clinical radiotherapy where normal tissue radioprotection by localized MnSOD-PL gene therapy is being evaluated in clinical trials with respect to safe use in pregnant women (17). The development of radiation counter measures particularly those applicable to a large populations, also requires consideration of the effect of a radiation protector or radiation mitigator drug on the developing fetus (28). The present evidence suggests that MnSOD-PL administration to pregnant females should not be deleterious to the fetus and may be indirectly protective by a beneficial remote effect.

Materials and Methods

Pregnant Mice

Timed pregnant E13 female C57BL/6HNSd mice were derived according to published methods (29). Three 6 – 8 week old mice were placed in the cage with 1 comparably aged male mouse and females observed daily for detection of the vaginal plug according to published methods (29). Once vaginal plugs were detected, females were placed 1 per cage and held for 13 days. The 13 day mid-gestational time point was chosen as teratogenic effects of irradiation in this gestational period have been well documented. Mice received intravenously MnSOD-PL (100 µg plasmid DNA) on E-13. Subgroups of mice were irradiated on E14. All pups were followed for litter size, pup weight at weaning and six months after birth, and pup survival for 200 days after birth.

Total Body Irradiation

Mice were irradiated to varying doses using a Cesium Gamma cell irradiator dose rate 70 Gy/minute (15). Mice were irradiated according to published procedures (15).

MnSOD-PL Radioprotective Gene Therapy

The method for preparation of MnSOD-Plasmid Liposomes has been published previously. Briefly, the small pNVL3-MnSOD-plasmid was prepared according to published methods, and mixed with lipofectmine liposomes according to published methods (20). Pregnant female mice received 100 microgram of plasmid at 100 microliter of liposome intravenously 24 hours prior to total body irradiation.

Epitope-Hemagglutinin-Tagged MnSOD-Plasmid Liposomes

Epitope-tagged MnSOD construct has been described previously (16). Cells transfected with HA-MnSOD-PL have been demonstrated to produce epitope labeled MnSOD which has biological radioprotective capacity identical to that of native plasmid liposomes, but has detectable hemagglutinin by in situ immunohistochemistry scoring.

Tissue sections from explanted embryos, placenta, and adult female mouse organs, were prepared for immunohistochemical staining according to published methods. Five sections from each adult mouse organ and ten sections from each explanted fetus and attached umbilical cord and placenta were analyzed for percent of hemagglutinin positive cells reporting at least 1000 cells per slide.

Reverse Transcriptase Polymerase Chain Reaction

The methods for RTPCR, quantitating MnSOD transgene transcript has been published previously (17). A DNA ladder utilizing MnSOD-plasmid as cDNA was used as positive control. Under these conditions, a lowest concentration of blank plasmid was detectable by adding positive control plasmid cDNA to tissue preparation of control mouse liver. Organs from adult female tissues, as well as fetal tissues were prepared for RTPCR according to published methods.

Statistics

Student T-test was used to compare different treatment groups as previously described (20). A Log-rank (Mantel-Cox) test was performed to compare survival of mice for 200 days after birth (15).

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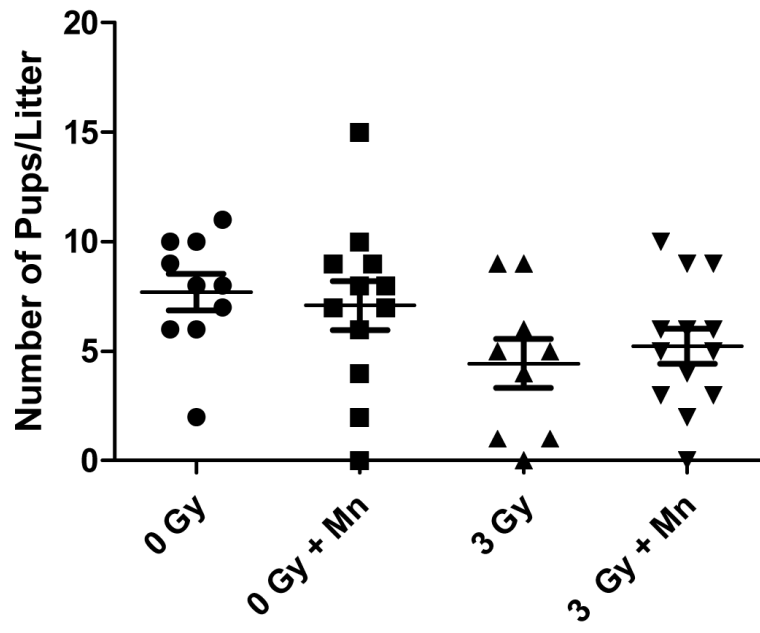


Figure 1. E13 pregnant mice (10-13/group) were injected with MnSOD-PL (MN) and irradiated on E14 along with non injection control mice to either 0 or 3 Gy. Pups were counted at the time of weaning and the number of pups per litter was determined. Pregnant female mice receiving 3 Gy TBI with or without MnSOD-PL had significantly decreased litter sizes compared to 0 Gy mice ($p = 0.0291$ or 0.0482). MnSOD-PL increased litter size in the 3Gy irradiated groups ($p = 0.0799$).

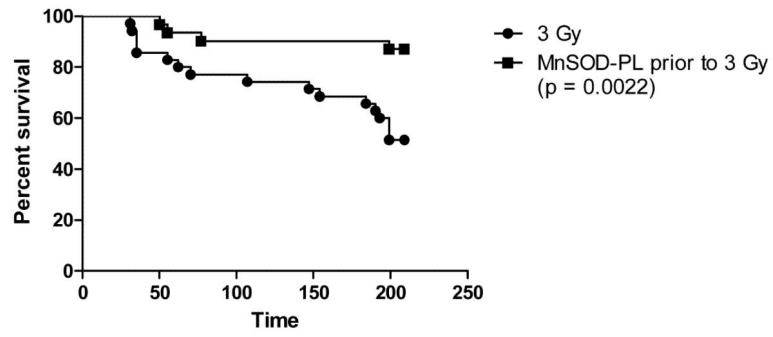


Figure 2. Pups from MnSOD-PL + 3 Gy or 3 Gy pregnant mice were followed for over 200 days after birth. Pups from pregnant females that were treated with MnSOD-PL before 3 Gy TBI showed increased survival compared to pups from non injected 3 Gy irradiation pregnant mice ($p = 0.0022$).

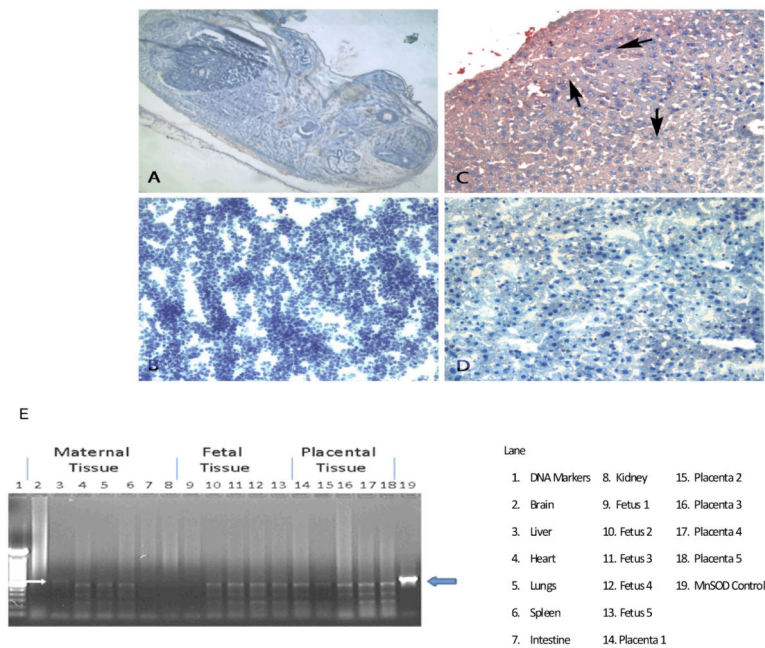


Figure 3. Immunostaining for Hemagglutinin positive HA-MnSOD gene product in fetal and maternal tissues and RTPCR showing maternal liver positive for transgene on mRNA. A) Whole fetus (E13) negative tissues – 2 x; B) Fetal liver (E13) – negative for HA-MnSOD (20 x); C) HA-MnSOD-PL injected maternal liver – (20 x) (arrows show HA-MnSOD positive areas); D) Control uninjected maternal liver – (20 x), and E) RTPCR of maternal and fetal tissues for detection of human MnSOD transgene mRNA. Tissues were prepared as described in the Materials and Methods Section. Maternal liver positive (black arrows) all other tissues are negative. E13 pregnant females were injected intravenously with Epitope-Hemagglutinin-Tagged MnSOD-PL (100 μ g in 100 μ l) and sacrificed 24 hr later. Fetal mice, placenta, and various tissues from the mother were removed and frozen in liquid nitrogen or in OCT. Samples frozen in OCT were sectioned, stained for hemagglutinin and examined microscopically. No staining was detected in the fetal mice (n = 10) or placenta (n = 10) (2 x); or in fetal mouse liver (20 x), or control uninjected maternal liver (D) (20 x). In contrast Hemagglutinin positive areas were detected in Ha-MnSOD treated maternal liver (C) (20 x) (red color, black arrows). RTPCR was performed on RNA extracted from maternal mouse tissues and fetal mice using primers specific for the human MnSOD transgene. Only maternal liver showed detectable expression of human MnSOD transgene.

Table 1
Maternal Total Body Irradiation Dose Dependent Fetal Mouse Death

| Dose of Irradiation (Gy) | Number of Litters | Number of Pups Born | Number of Pups Born Dead | Number of Pups Alive at Weaning |
|--------------------------|-------------------|---------------------|--------------------------|---------------------------------|
| 0 | 3 | 20 | 0 | 20 |
| 1 | 3 | 19 | 1 | 18 (p = 0.4918) |
| 3 | 4 | 29 | 20 | 9 (p = 0.0084) |
| 5 | 3 | 15 | 15 | 0 (p = 0.0011) |
| 7 | 3 | 19 | 19 | 0 (p = 0.0011) |

C57BL/6NHsd E14 time pregnant mice were irradiated to doses of 0, 1, 3, 5 or 7 Gy whole body irradiation. The number of pups born alive or dead were determined. (p values indicate significant lethality compared to nonirradiated controls.)

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Table 2
MnSOD-PL Treatment of Pregnant Females Protects Fetal Mice from Total Body Irradiation

| Group | Number of Pups/Litter at Weaning | Weight at Weaning | Weight at 6 Months After Birth |
|-----------------|----------------------------------|----------------------------|--------------------------------|
| 0 Gy | 7.7 ± 0.8 | 11.7 ± 0.3 | 25.8 ± 0.5 |
| 0 Gy ± MnSOD-PL | 6.7 ± 1.3 | 11.6 ± 0.2 | 30.4 ± 0.8 (p < 0.0001) |
| 3 Gy | 4.4 ± 1.1 (p = 0.0291) | 10.1 ± 0.5 (p = 0.0024) | 20.4 ± 0.7 (p < 0.0001) |
| 3 Gy ± MnSOD-PL | 5.2 ± 0.9 (p = 0.0482) | 10.6 ± 0.4 (p = 0.0202) | 20.8 ± 0.8 (p = 0.006) |

C57BL/6NHsd timed pregnant mice (E13) were injected intravenously with MnSOD-PL (100 µg plasmid DNA in 100 µl liposomes) then were irradiated 24 hrs later (E14) along with control (E14) mice to 0 or 3 Gy. At the time of weaning the number of mice per litter and weight were determined. Newborn mice were followed for 6 months then were weighed again. Statistical differences compare each irradiated group to unirradiated control mice 0 Gy.