

Effects of low-dose heavy ions on embryonic development in mice and on melanocyte differentiation in the epidermis and hair bulb

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The effects of prenatal low-dose irradiation with heavy ions on embryonic development in mice and on melanocyte differentiation are not well understood. We performed whole-body irradiation of pregnant C57BL/10J mice at embryonic Day 9 (E9) with a single dose of γ -rays, silicon, argon or iron ions. The number of living embryos and embryonic body weight at E18 decreased after exposure to heavy ions at high doses. Malformations such as small eyes and limb anomalies were observed in heavy-ion-treated embryos, but not in γ -ray-treated embryos. The frequency of abnormally curved tails was increased by exposure to γ -rays and argon and iron ions even at a dose of 0.1 Gy ($P < 0.05$). In contrast, a dose-dependent decrease in the number of epidermal melanoblasts/melanocytes and hair bulb melanocytes was observed after 0.1 Gy irradiation with γ -rays or heavy ions ($P < 0.01$). The decrease in the number of dorsal hair bulb melanocytes, dorsal and ventral epidermal melanoblasts/melanocytes and ventral hair bulb melanocytes was not necessarily correlated with the linear energy transfer of the radiation tested. Moreover, the effects of heavy ions were larger on the ventral skin than on the dorsal skin, indicating that the sensitivity of melanocytes to heavy ions differs between the dorsal and ventral skin. Taken together, these results suggest that the effects of the low-dose heavy ions differ between cell types and tissues, and the effects on the prenatal development of mice and melanocyte development are not necessarily greater than those of γ -rays.

Keywords: heavy ion; anomaly; melanoblast; melanocyte; epidermis; hair bulb

INTRODUCTION

Ionizing radiations such as X-rays [1–4] and γ -rays [5, 6] inhibit normal animal development and the proliferation and differentiation of various cell types. However, the effects of high-linear energy transfer (LET) radiation on the prenatal and postnatal development of animals have not been fully studied. We previously reported that the effects of prenatal irradiation with carbon [7], silicon, argon and iron ions [8] on postnatal development in mice were greater than those of prenatal irradiation with γ -rays. However, the effects of prenatal low-dose irradiation with γ -rays or heavy ions on embryonic development in mice have not been well studied.

The skin is the first tissue affected by radiation exposure. Thus, it is very important to understand the mechanism of action of ionizing radiation in protecting the skin from radiation-induced damage. The skin consists of multiple cell types including epithelial tissue cells such as keratinocytes and sebaceous gland cells, connective tissue cells such as fibroblasts and endothelial cells, immune system cells such as T cells, leukocytes and Langerhans cells and ectodermally derived cells such as melanocytes and Merkel cells [9]. Pigment-producing cells, melanocytes, are derived from the neural crest, a pluripotent embryonic cell population [10]. Neural crest cells migrate from the dorsal to the ventral side around embryonic Day 9 (E9) [10].

Melanoblasts, the precursors of melanocytes, proliferate and migrate throughout the skin over the entire body [11]. The proliferation, differentiation and migration of melanocytes are regulated autonomously or by the tissue environment, particularly keratinocytes [12–14]. Most epidermal melanocytes migrate into the hair bulb and the pigment-accumulating organelles, the melanosomes [9], are transported to surrounding keratinocytes to produce pigmented hairs [15]. Melanocytes are a very good model for investigating the effects of heavy ions on the proliferation and differentiation of animal cells owing to their high sensitivity to radiation [7, 8]. Ionizing radiations, such as X-rays and γ -rays, affect mouse melanoblasts during the early stages of development. Affected mice possess patches of pigmentless white hair (white spots) in the mid-ventrum [16–18]. Low doses of heavy ions of carbon, silicon, argon and iron ions induce white spots in the mid-ventrum much more frequently than γ -rays [7, 8]. However, the mechanism by which low-dose heavy ions induce ventral white spots is unknown.

We focused on the effects of prenatal irradiation with low doses of γ -rays and silicon, argon and iron ions on embryonic development in mice as well as on embryonic melanocyte development by scoring the changes in pregnancy, litter size, average number of living embryos and body weight, the frequency of developmental anomalies and the number of epidermal melanoblasts/melanocytes and hair bulb melanocytes in the dorsal and ventral skin. Our study was designed to answer these questions about how low doses of heavy ions affect embryonic development in animals.

MATERIALS AND METHODS

Mice

C57BL/10J (nonagouti black) mice were given water and a commercial diet (OA-2; Clea Japan, Tokyo, Japan) *ad libitum* and maintained at $24 \pm 1^\circ\text{C}$ with 40–60% relative humidity and 12 h of fluorescent light/day. Female mice were placed with males and checked for vaginal plugs to confirm copulation. The time of vaginal plug formation was counted as Day 0 of pregnancy. This study was approved by the ethics committee of the National Institute of Radiological Sciences (NIRS) in accordance with the guidelines of the National Institutes of Health.

Radiation

On E9 unanesthetized pregnant females were placed in a box with walls of thickness 10 mm (γ -rays) or 20 mm (silicon, argon and iron ions) made of acrylic resin and were given a single dose of whole-body irradiation with acute γ -rays (^{60}Co , dose rate, approximately 0.3 Gy/min) or silicon, argon or iron ions (dose rate, approximately 0.3–0.4 Gy/min) with total doses of 0.1, 0.25, 0.5 or 0.75 Gy.

Ions were accelerated up to 490 (Si) or 500 (Ar, Fe) MeV/nucleon with the heavy ion medical accelerator in Chiba (HIMAC) at NIRS, as described previously [19, 20]. Mice were irradiated at the plateau region of the Bragg curve of each ion beam. Under these conditions, the dose and LET were similar throughout the whole body thickness; 1% for Si, 2% for Ar and 10% for Fe. The dose average LET for Si, Ar and Fe was estimated at 57.1 ± 0.5 (standard deviation, calculated from theoretical Bragg curve fitted to data measured on each experimental day), 100 and 220 keV/ μm (estimated using slimCODE), respectively, at the sample (embryo) position. Irradiation was performed at the same time in the morning. The control mice were concurrent with each radiation group. The controls from each different irradiation (γ -rays and Si, Ar and Fe ions) were pooled because no statistically significant differences were observed among them.

Embryonic development in mice

The number of pregnant females at E18 per female with a vaginal plug (frequency of pregnancies), the average numbers of living embryos and the proportion of living embryos were scored. Average body weight was measured at E18.

Histochemical analysis

Skin was removed from the dorsal and ventral regions of the embryonic trunk at E18 and fixed with 16% neutral formalin in phosphate buffer (pH 7.0) for 16–24 h at 2°C . Tyrosinase-containing differentiated melanocytes were detected as follows. Fixed tissue samples were washed with distilled water and incubated with 0.1% L-3,4-dihydroxyphenylalanine (L-dopa; Wako Pure Chemical Industry, Osaka, Japan) solution in phosphate buffer (pH 7.4) for 16–24 h at 37°C [21]. They were then fixed with 10% neutral formalin for 16–24 h at room temperature, washed with distilled water, transferred through a graded series of ethanols into xylol and embedded in paraffin. Serial 10- μm sections were deparaffinized and counterstained with eosin. For the combined dopa–premelanin reaction (combined dopa–ammoniacal silver nitrate staining), deparaffinized sections of dopa-treated tissue were incubated in 10% ammoniacal silver nitrate (Wako) solution for 8 min at 58°C [21–23]. Ammoniacal silver nitrate staining was used for the light microscopic detection of melanoblasts with only unmelanized stage I and II melanosomes as well as differentiated melanocytes and metallic silver particles being deposited with a high degree of selectivity [24, 25]. The dopa reaction reveals only tyrosinase-containing differentiated melanocytes. Specimens were counterstained with eosin. The number of melanocytes (cells positive to the dopa reaction) and the number of stage I and II melanosome-containing melanoblasts plus melanocytes (cells positive to the combined dopa–premelanin reaction)

were counted for ten consecutive sections and expressed per 0.1 mm² of the epidermis. The number of melanoblasts was calculated by subtracting the number of cells positive to the dopa reaction from the number of cells positive to the combined dopa–premelanin reaction. Melanoblasts were also detected by immunostaining for tyrosinase-related protein-1 (TRP-1) and TRP-2 (dopachrome tautomerase) [26]. A melanoblast was defined as an unpigmented cell containing stage I and/or II melanosomes and no tyrosinase activity.

Whole-mount skin preparations

Dorsal and ventral skin samples were incubated with 0.1% L-dopa solution, fixed with 10% neutral formalin for 16–24 h at room temperature, washed with distilled water and transferred through a graded series of ethanols into xylol. The specimens were mounted in Canada balsam on microscope slides under cover slips with the epidermis upwards [27]. Whole-mount preparations were examined under a light microscope and the number of melanocytes within the hair bulb was scored.

Statistical analysis

The statistical significance of differences in the frequency of pregnancies, living embryos and external malformations was determined using the nonparametric Mann–Whitney *U*-test. The statistical significance of differences in the average number of living embryos, average body weight, number of epidermal melanoblasts/melanocytes and number of hair bulb melanocytes was determined using Student's *t*-test (two-tailed) for comparison of groups of unequal size.

RESULTS

Effects of irradiation on embryonic development in mice

We observed no change in the frequency of pregnancy due to irradiation; however, the proportions of living embryos and their average number in mice treated with iron ions at the dose of 0.75 Gy were significantly different from those in control mice (Table 1). The average body weight of living embryos in mice treated with 0.75 Gy γ -rays, 0.5 and 0.75 Gy of silicon or iron ions and all doses of argon ions was significantly different from that in the controls (Table 1), indicating that higher doses of iron ions markedly inhibited normal development and that all types of radiation tested reduced embryonic body weight.

We found that γ -rays and heavy ions induced several types of externally visible malformation including curved tail, small eyes, hemorrhage, abnormal limbs, tongue and tooth malformations and anophthalmia (Fig. 1). Hemorrhage was observed even in control mice at a high frequency

(17.5%) and the frequency of hemorrhage was not dependent on the dose and LET of radiation (data not shown). Limb defects included small or short limbs, reduced number of digits and total absence of digits. The frequencies of external malformations differed between tissues. Malformations such as small eyes and limb anomalies were observed in heavy-ion-treated embryos but not in γ -ray-treated embryos (Table 2). Curved tails were most frequent in embryos exposed to γ -rays and heavy ions. Curved tails were observed even with 0.1 Gy of γ -rays ($P < 0.001$), argon ions ($P < 0.001$) and iron ions ($P < 0.05$, Table 2). The 0.25-Gy dose generally induced a lower frequency of curved tails than the 0.1-Gy dose and was not different from the control for three (Si, Ar and Fe) of the four types of radiation. Furthermore, in the case of silicon ions, the effect was observed only at the highest dose (Table 2). Thus, sensitivity to γ -rays or heavy ions differed between tissues.

Effects on the development of epidermal melanoblasts/melanocytes

The dopa (Fig. 2A, C) and combined dopa–premelanin (Fig. 2B, D) reactions revealed cells positive to the dopa reaction (melanocytes) and to the combined dopa–premelanin reaction (melanoblasts plus melanocytes) in the dorsal skin of the unirradiated control (Fig. 2A, B) and irradiated (Fig. 2C, D) embryos at E18. In the control skin, many melanocytes (Fig. 2A) with well-developed dendrites as well as numerous melanoblasts plus melanocytes (Fig. 2B) were observed in the epidermis, dermis and hair follicles. However, irradiation with silicon ions (Fig. 2C, D) markedly decreased the melanocyte (Fig. 2C) and melanoblast/melanocyte (Fig. 2D) populations in the epidermis, dermis and hair follicles. Similar results were obtained following exposure to γ -rays or to argon or iron ions (data not shown). Deposition of dopa–melanin (Fig. 2C) and metallic silver particles (Fig. 2D) was also markedly reduced. As shown in Fig. 2C and D, skin thickness and hair follicle development were also markedly reduced in silicon-irradiated embryos. Similar observations were obtained with argon and iron ions in comparison with control embryos and γ -ray irradiation (data not shown). The number of epidermal melanocytes and melanoblasts in the irradiated skin decreased as the dose increased.

In the ventral skin, the number of melanoblasts and melanocytes in the epidermis, dermis and hair follicles was much lower than in the dorsal skin. γ -rays and heavy ions decreased the number of melanoblasts and melanocytes in the epidermis, dermis and hair follicles in the ventral skin with a greater decrease than in the dorsal skin (data not shown).

The number of epidermal melanocytes (Fig. 3A, C) and melanoblasts (Fig. 3B, D) per 0.1 mm² epidermis in irradiated dorsal (Fig. 3A, B) and ventral (Fig. 3C, D) skin

Table 1. Effects of γ -rays and heavy ions on the embryonic development of mice

| Radiation | Dose of irradiated mice (Gy) | Number | Pregnancies ^a | Number of embryos | Number of living embryos | Average Number of living embryos ^b | Body weight (g) ^c |
|-----------|------------------------------|--------|--------------------------|-------------------|--------------------------|---|------------------------------|
| Control | 0 | 45 | 29 (64.4%) | 148 | 137 (92.6%) | 4.72 \pm 0.42 | 1.14 \pm 0.01 |
| γ | 0.1 | 12 | 10 (83.3%) | 55 | 51 (92.7%) | 5.10 \pm 0.90 | 1.14 \pm 0.02 |
| | 0.25 | 12 | 6 (50.0%) | 35 | 31 (88.6%) | 5.17 \pm 1.01 | 1.13 \pm 0.02 |
| | 0.5 | 12 | 10 (83.3%) | 57 | 47 (82.5%) | 4.70 \pm 0.67 | 1.14 \pm 0.02 |
| | 0.75 | 12 | 10 (83.3%) | 65 | 62 (95.4%) | 6.20 \pm 0.61 | 1.06 \pm 0.02** |
| | Si | 0.1 | 15 | 11 (73.3%) | 48 | 45 (93.8%) | 4.09 \pm 0.81 |
| Si | 0.25 | 14 | 12 (85.7%) | 51 | 51 (100%) | 4.25 \pm 0.92 | 1.13 \pm 0.02 |
| | 0.5 | 12 | 9 (75.0%) | 55 | 55 (100%) | 6.11 \pm 0.95 | 1.02 \pm 0.02*** |
| | 0.75 | 16 | 11 (68.8%) | 48 | 39 (81.3%) | 3.55 \pm 0.77 | 0.98 \pm 0.02*** |
| Ar | 0.1 | 10 | 8 (80.0%) | 30 | 30 (100%) | 3.75 \pm 0.82 | 1.08 \pm 0.03* |
| | 0.25 | 7 | 5 (71.4%) | 30 | 30 (100%) | 6.00 \pm 0.84 | 1.00 \pm 0.02*** |
| | 0.5 | 15 | 5 (33.3%) | 17 | 16 (94.1%) | 3.20 \pm 1.02 | 1.00 \pm 0.03*** |
| | 0.75 | 7 | 7 (100%) | 41 | 22 (53.7%) | 3.67 \pm 0.92 | 0.84 \pm 0.02*** |
| Fe | 0.1 | 13 | 8 (61.5%) | 43 | 41 (95.3%) | 5.13 \pm 0.91 | 1.13 \pm 0.02 |
| | 0.25 | 11 | 8 (72.7%) | 43 | 38 (88.4%) | 4.75 \pm 1.05 | 1.10 \pm 0.02 |
| | 0.5 | 13 | 9 (69.2%) | 44 | 40 (90.9%) | 4.44 \pm 0.84 | 0.99 \pm 0.02*** |
| | 0.75 | 15 | 9 (60.0%) | 50 | 16 (32.0%)* | 1.78 \pm 0.52*** | 0.83 \pm 0.03*** |

^aThe proportion of females pregnant at 18 days to females with vaginal plug.

^bThe proportion of living embryos at 18 days in gestation to embryos observed in the uterus of mother at 18 days in gestation was shown as average litter size with standard error of the mean (SEM).

^cBody weight of 18-day-old embryos was shown as average weight with SEM.

Significantly different from control (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).



Fig. 1. An embryo at E18 exposed to iron ions at the dose of 0.75 Gy at E9. Various externally visible malformations such as curved tail (long arrow), hemorrhage (short arrows) and abnormal limb (arrow head) are observed.

was dramatically decreased. Even in embryos exposed to γ -rays or heavy ions at the dose of 0.1 Gy, the number of epidermal melanocytes and melanoblasts was markedly decreased (Fig. 3) and decreased further as the dose was increased. In the dorsal skin, the greatest decrease in the number of melanocytes was observed in argon-irradiated embryos (Fig. 3A, C). However, there was no marked difference in the number of melanocytes between embryos exposed to γ -rays or to silicon or iron ions. In contrast, no difference in the number of dorsal melanoblasts was observed following irradiation with γ -rays or silicon, argon or iron ions (Fig. 3B), whereas ventral melanoblasts were much more sensitive to irradiation with heavy ions than with γ -rays (Fig. 3D, $P < 0.05$). Thus, γ -rays and heavy ions may decrease the number of melanoblasts and melanocytes in the embryonic epidermis and have a larger effect on the ventral skin than on the dorsal skin. However, it is also possible that the very small number of ventral melanocytes in the epidermis may be a confounder when comparing control and irradiated groups.

Table 2. Frequency of external malformations in 18-day-old embryos exposed to γ -rays and heavy ions

| Radiations | Dose (Gy) | Number of embryos | Curved tail | Small eyes | Abnormal limbs | Other anomalies |
|------------|-----------|-------------------|---------------|--------------|----------------|-----------------|
| Control | 0 | 137 | 4 (2.9%) | 0 (0%) | 0 (0%) | 2 (1.5%) |
| γ | 0.1 | 55 | 10 (19.6%)** | 0 (0%) | 0 (0%) | 0 (0%) |
| | 0.25 | 31 | 13 (41.9%***) | 0 (0%) | 0 (0%) | 0 (0%) |
| | 0.5 | 47 | 14 (29.8%***) | 0 (0%) | 0 (0%) | 0 (0%) |
| | 0.75 | 62 | 29 (46.8%***) | 0 (0%) | 0 (0%) | 1 (1.6%) |
| Si | 0.1 | 45 | 0 (0%) | 0 (0%) | 0 (0%) | 1 (2.2%) |
| | 0.25 | 51 | 0 (0%) | 2 (3.9%) | 0 (0%) | 0 (0%) |
| | 0.5 | 55 | 2 (3.6%) | 1 (1.8%) | 1 (1.8%) | 0 (0%) |
| | 0.75 | 39 | 13 (33.3%***) | 0 (0%) | 2 (5.1%) | 0 (0%) |
| Ar | 0.1 | 30 | 8 (26.7%***) | 1 (3.3%) | 0 (0%) | 1 (3.3%) |
| | 0.25 | 30 | 3 (10%) | 0 (0%) | 0 (0%) | 0 (0%) |
| | 0.5 | 16 | 8 (50%***) | 1 (6.3%) | 0 (0%) | 0 (0%) |
| | 0.75 | 22 | 18 (81.8%***) | 2 (9.1%)* | 0 (0%) | 4 (18.2%)* |
| Fe | 0.1 | 41 | 5 (12.2%)* | 0 (0%) | 0 (0%) | 2 (4.9%) |
| | 0.25 | 38 | 4 (10.5%) | 2 (5.3%)* | 0 (0%) | 0 (0%) |
| | 0.5 | 40 | 10 (25%***) | 5 (12.5%***) | 1 (2.5%) | 0 (0%) |
| | 0.75 | 16 | 11 (68.8%***) | 8 (50%***) | 1 (6.3%) | 5 (31.3%)* |

The number of anomalies was scored in each embryo at E18. Other anomalies include tongue and tooth malformations, anophthalmia, etc. Significantly different from control (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

Effects on the development of hair bulb melanocytes

The dopa reaction revealed well-developed hair follicles with many hair bulb melanocytes in addition to developing hair follicles in the dorsal skin of the control (Fig. 2A). However, in the dorsal skin of silicon ion-exposed embryos (Fig. 2C), the development of melanocytes was delayed and the number of hair follicles was decreased. Similar results were obtained for dorsal skin exposed to γ -rays or argon or iron ions (data not shown). Skin development in the ventral region of embryos exposed to γ -rays or silicon, argon or iron ions was more delayed than in the dorsal region (data not shown). Analysis of L-dopa reactivity in whole-mount preparations of dorsal skin of control embryos showed that the hair bulbs of large hair follicles contained many melanocytes with well-developed dendrites in control dorsal skin (Fig. 4A). Hair bulb melanocytes as well as their dendrites were observed by adjusting the focus to the cell body or dendrites of melanocytes in whole-mount preparations. In addition, numerous smaller developing hair follicles were also observed (Fig. 4A). In contrast, the dorsal skin of embryos exposed to silicon ions (Fig. 4B) contained fewer large or developing hair follicles than control skin. Similar results were obtained for the dorsal skin exposed to γ -rays or to argon or iron ions (data

not shown). Moreover, the number of melanocytes in hair bulbs of the dorsal skin was greatly decreased by exposure to γ -rays or heavy ions (Fig. 5A, $P < 0.001$). The order of the effects was γ -rays < silicon < argon \approx iron ions, suggesting that inhibition of the development of hair bulb melanocytes in the dorsal skin is not necessarily proportional to LET. In contrast, the number of hair bulb melanocytes in the ventral skin was decreased much more than that in the dorsal skin, with no differences between radiation species (Fig. 5B). Even in embryos exposed to only 0.1 Gy γ -rays or heavy ions, the number of hair bulb melanocytes was significantly decreased ($P < 0.001$), and decreased further as the dose increased. These results suggest that γ -rays and heavy ions inhibit the development of embryonic hair bulb melanocytes in the ventral skin more than in the dorsal skin. It is also possible that the extremely small number of hair bulb melanocytes in the ventral skin may be a confounder when comparing between control and irradiated groups and among the various radiation species.

Skin site differences in the effects of γ -rays and heavy ions

Table 3 summarizes the skin site difference in the effects of γ -rays and heavy ions at the two doses of 0.1 and 0.75 Gy. The decrease in the number of cells (% of control) in hair

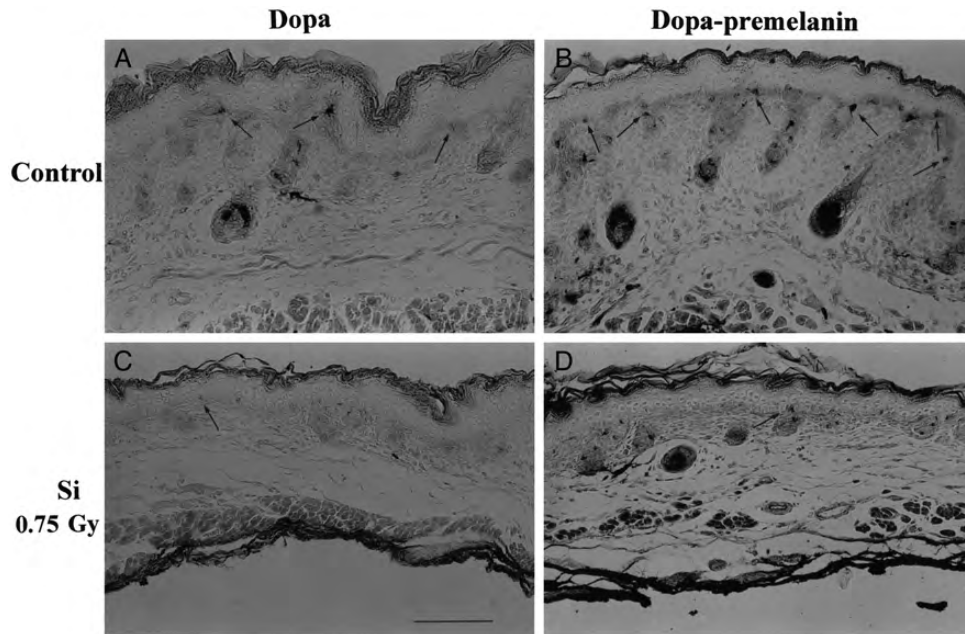


Fig. 2. Histochemical sections of the dorsal skin of unirradiated control and irradiated mice at E18. Cells positive to the dopa reaction (melanocytes, **A, C**) as well as to the combined dopa–premelanin reaction (melanoblasts plus melanocytes, **B, D**) are shown. In control skin, melanocytes (**A**) with well-developed dendrites as well as melanoblasts/melanocytes (**B**) are observed in the epidermis (arrows), dermis and hair follicles. However, in the skin irradiated with silicon ions (0.75 Gy), the numbers of melanocytes (**C**; arrow) as well as melanoblasts plus melanocytes (**D**; arrows) are markedly decreased. Moreover, the deposition of dopa–melanin (**C**) as well as metallic silver particles (**D**) is decreased compared with control skin. Scale bar, 100 μm .

bulb melanocytes and epidermal melanocytes was greater than that in epidermal melanoblasts. Moreover, the decrease in the number of epidermal melanocytes and hair bulb melanocytes in the ventral skin was higher than in the dorsal skin, argon ions having the greatest inhibitory effects. These results suggest that the differentiation of melanocytes from melanoblasts in the ventral skin is readily influenced by γ -rays and heavy ions compared with that in the dorsal skin.

DISCUSSION

In the present study, we used γ -rays and heavy ions to induce several types of external malformation; the frequencies of which differed between tissues. Although the basis of the differing organ radio-sensitivities cannot be completely explained at present, the radio-sensitivity of different cell types may differ between γ -rays and heavy ions. Our present data suggest that embryonic development in animals is not necessarily dependent on LET and ion species, although it is reported that the postnatal development of animals is dependent on LET and heavy ion species [3].

Exposure to 0.1 Gy of argon or iron ions or γ -rays induced tail defects, whereas 0.1 Gy of silicon ions did not. Silicon ions may have different effects on the development of different tissues and organs; for example, cartilage

development may be much less sensitive to silicon ions than other tissues. Such specificity is partly supported by our observation that exposure to 0.75 Gy of silicon ions induced tail defects in only a minority of embryos (33.3%) in comparison with argon (81.8%) and iron (68.8%) ions. Similarly, the development of the lens and retina may be much less sensitive to silicon ions than to argon or iron ions.

At low doses of high-LET radiation, the particle flux to which a cell is exposed is usually very small. In the present study using 0.1 Gy of silicon, argon and iron ions at 57, 100 and 220 keV/ μm , each cell received an average of 1.1, 0.6 and 0.3 particles per 100 μm^2 , respectively, which is approximately the area of the cell surface. Thus, numerous cells will not have been hit by these particles, suggesting that the direct biological effects of radioactive particle exposure, such as cell death, should not be evident at doses as low as 0.1 Gy. Taken together, this suggests that the effects of low-dose exposure to heavy ions may be fundamentally different from those of low-dose exposure to γ -rays; however, we did not observe any considerable difference between the effects of low-dose γ -rays and heavy ions on general embryonic development or the frequency of external malformations.

The number of epidermal melanoblasts/melanocytes and hair bulb melanocytes in the dorsal and ventral regions

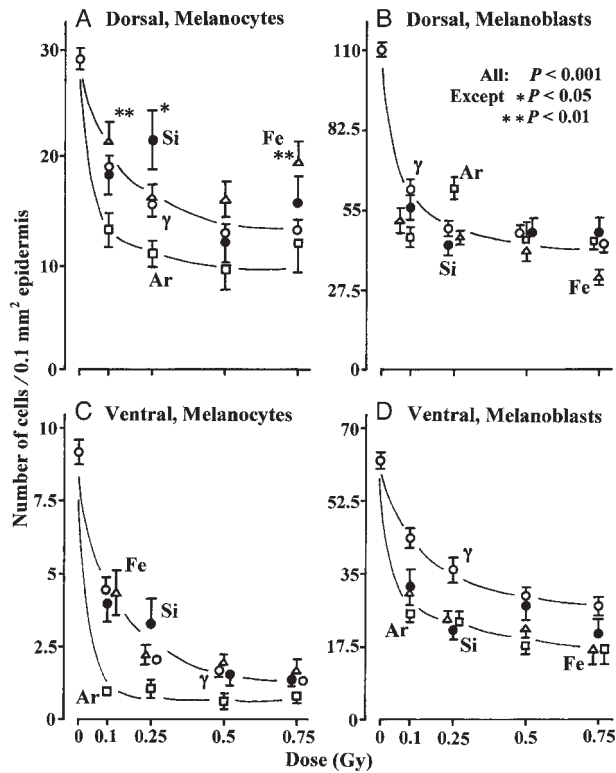


Fig. 3. Effects of γ -rays and heavy ions on the number of epidermal melanocytes (A, C) and melanoblasts (B, D) in the dorsal (A, B) and ventral (C, D) skins of mouse embryos at E18. Pregnant females were given a single exposure to different doses of γ -rays (white circles), silicon (black circles), argon (squares) and iron (triangles) ions at E9. Melanocytes (cells positive to the dopa reaction; A, C) and melanoblasts plus melanocytes (cells positive to the combined dopa–premelanin reaction) were counted. The number of melanoblasts (B, D) was calculated by subtracting the number of melanocytes from the number of melanoblasts plus melanocytes. The number of melanocytes and melanoblasts per 0.1 mm² epidermis in the dorsal and ventral skins is dramatically decreased even with 0.1 Gy-treated embryos, and gradually decreased as the dose was increased. The effects of γ -rays and heavy ions on the number of epidermal melanocytes and melanoblasts in the ventral skin are greater than in the dorsal skin. The scale of the y axis is different between the dorsal and ventral skins.

was markedly decreased even at 0.1 Gy of irradiation ($P < 0.001$) and gradually decreased as the dose was increased. The decrease in the number of dorsal hair bulb melanocytes as well as the number of dorsal and ventral epidermal melanoblasts/melanocytes and ventral hair bulb melanocytes did not necessarily correlate with LET of the radiation tested. These results suggest that prenatal exposure to low doses of heavy ions did not have a greater effect on embryonic melanocyte development than exposure to γ -rays. This low dose (0.1 Gy) is below the previously established threshold dose (0.25 or 0.5 Gy) for inhibition of the

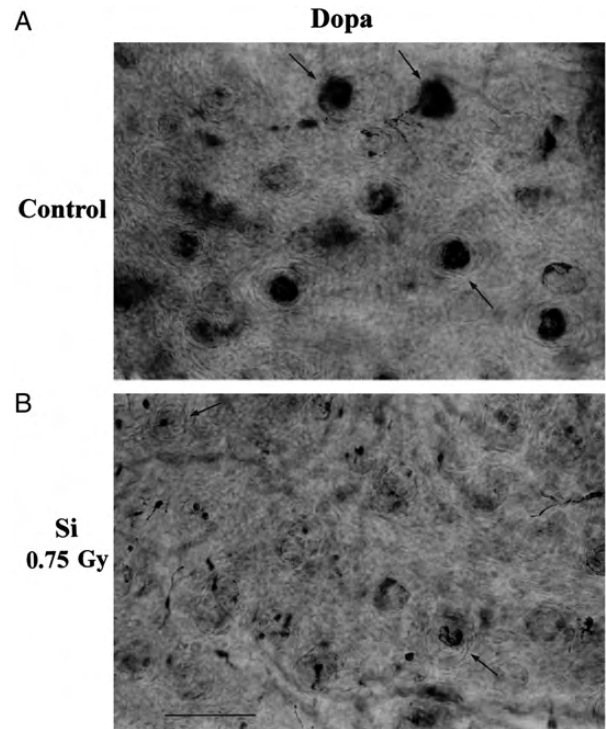


Fig. 4. Whole-mount preparations of the dorsal skins in control and irradiated mice at E18. The skins were treated with dopa solution. Melanocytes are observed in the hair bulbs of the dorsal skin in control and irradiated (silicon ions, 0.75 Gy) embryos. In control skin (A), numerous dopa-positive melanocytes with well-developed dendrites are observed in the hair bulbs (arrows). However, in the dorsal skin exposed to silicon ions (B), hair bulb melanocytes (arrows) are decreased in number. Scale bar, 100 μ m.

development of embryonic animal cells [5], suggesting that our experimental system is useful for determining the minimum dose of ionizing radiation required for the inhibition of embryonic development of melanoblasts and melanocytes.

The effects of prenatal irradiation with low doses of heavy ions were compared between embryonic development and melanocyte development in mouse embryos. Tail development and skin melanocytes had similar sensitivity to irradiation; 0.1 Gy led to a significant frequency of curved tails and decreased the numbers of epidermal melanoblasts/melanocytes and hair bulb melanocytes, and these abnormalities increased as the dose was increased. Although these defects were dose-dependent, they were not necessarily LET-dependent. However, low-dose heavy ions were not effective in inducing eye or limb defects. These results suggest that the effects of low doses of heavy ions differ between different cell types and tissues.

The present results suggest that 0.1 Gy of γ -rays and of heavy ions can induce cell death in neural crest cells or melanocyte stem cells in the embryonic skin. Krebs *et al.* [28] used electron microscopy to analyze retina cells of 4-week-old rats after exposure to 450 MeV/nucleon iron

ions where LET was 195 keV/ μm (a lower LET than in our experiments). They reported that iron ions caused pyknosis in retina cells at 2.5 Gy but not at 0.1 Gy. Although the discrepancy between their results and the findings reported here cannot be explained at present, it may involve cell and/or species differences (rat adult cells vs. mouse embryonic cells) as well as differences in the types of ions and the LET of the radiation used. In addition, the so-called

bystander effect or the interaction between melanoblasts/melanocytes and keratinocytes or fibroblasts may be relevant here. The mouse epidermis consists mostly of keratinocytes (approximately 98%) and a few melanoblasts/melanocytes (approximately 2%), and the dermis is composed predominantly of fibroblasts, in addition to melanoblasts/melanocytes, endothelial cells and blood cells [9]. Hair follicles are made up of melanoblasts/melanocytes, keratinocytes and fibroblasts. Keratinocytes and fibroblasts surrounding melanoblasts/melanocytes may be hit by heavy ion particles even at a dose of 0.1 Gy because numerous keratinocytes and fibroblasts exist with melanoblasts/melanocytes. Irradiation of embryos with heavy ions may lead to cell death or inhibition of keratinocyte or fibroblast proliferation. The loss of or a decrease in the keratinocyte or fibroblast population may result in the loss of or a decrease in the growth factors or cytokines that they secreted [29–32], resulting in the inhibition of melanocyte proliferation and differentiation, and ultimately a decrease in the number of melanoblasts and melanocytes. Thus, the bystander effect or the cellular interaction between melanoblasts/melanocytes and keratinocytes or fibroblasts may be another factor that controls the number of melanoblasts and melanocytes. Wu *et al.* [33] reported that the death of rat keratinocytes caused by iron ions may be due to cell cycle arrest, and they used DNA microarray analysis to show that numerous cell cycle-related genes were upregulated. It is also possible that γ -rays and heavy ions inhibit the differentiation of melanocytes from melanoblasts. This possibility is consistent with our finding that the decrease in the number of epidermal melanocytes in the ventral skin was greater than that of epidermal melanoblasts in the ventral skin in irradiated embryos. According to this hypothesis, γ -rays and heavy ions may directly or indirectly inhibit the differentiation of melanocytes through keratinocyte-derived

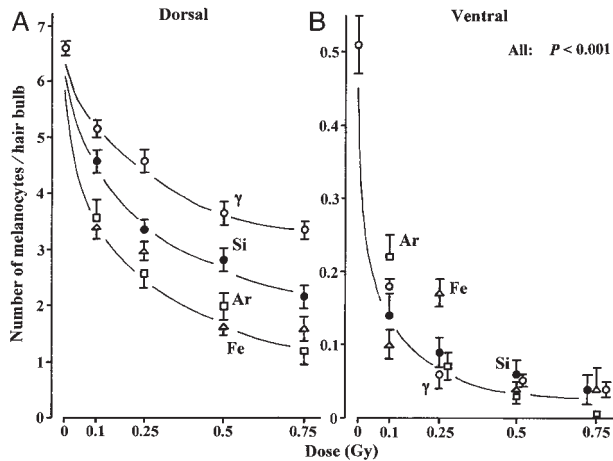


Fig. 5. Effects of γ -rays and heavy ions on the number of hair bulb melanocytes in the dorsal (A) and ventral (B) skins of mice at E18. Pregnant females were given a single exposure to different doses of γ -rays (white circles), silicon (black circles), argon (squares) and iron (triangles) ions at E9. The number of hair bulb melanocytes in the dorsal (A) and ventral (B) skin is dramatically decreased even with 0.1 Gy-treated embryos ($P < 0.001$), and gradually decreased as the dose was increased. The effects of γ -rays and heavy ions on the number of hair bulb melanocytes in the ventral skin are greater than in the dorsal skin. The scale of the y axis is different between the dorsal and ventral skins.

Table 3. Skin site difference in the number of epidermal melanoblasts/melanocytes and hair bulb melanocytes exposed to γ -rays and heavy ions at the doses of 0.1 and 0.75 Gy

| Dose (Gy) | Radiations | Dorsal epidermal melanoblasts | Ventral epidermal melanoblasts | Dorsal epidermal melanocytes | Ventral epidermal melanocytes | Dorsal hair bulb melanocytes | Ventral hair bulb melanocytes |
|-----------|------------|-------------------------------|--------------------------------|------------------------------|-------------------------------|------------------------------|-------------------------------|
| 0.1 | γ | 56.5 \pm 2.7 | 70.2 \pm 4.1** | 65.5 \pm 3.5 | 48.0 \pm 4.8** | 78.1 \pm 2.6 | 35.3 \pm 2.0*** |
| | Si | 50.8 \pm 4.3 | 51.5 \pm 6.6 | 62.9 \pm 6.7 | 43.4 \pm 6.7* | 69.3 \pm 3.5 | 27.5 \pm 5.9*** |
| | Ar | 41.5 \pm 3.3 | 40.8 \pm 3.4 | 45.2 \pm 5.2 | 10.2 \pm 1.6*** | 54.2 \pm 5.0 | 43.1 \pm 5.9 |
| | Fe | 46.6 \pm 4.2 | 48.9 \pm 5.1 | 73.8 \pm 6.0 | 46.9 \pm 8.4* | 51.4 \pm 3.0 | 19.6 \pm 3.9*** |
| 0.75 | γ | 39.3 \pm 2.4 | 44.0 \pm 3.4 | 44.8 \pm 3.1 | 14.9 \pm 2.0*** | 51.1 \pm 2.1 | 7.8 \pm 2.0*** |
| | Si | 43.0 \pm 4.5 | 33.2 \pm 6.0 | 53.7 \pm 8.5 | 15.4 \pm 3.0*** | 32.8 \pm 3.0 | 7.8 \pm 3.9*** |
| | Ar | 40.3 \pm 2.6 | 26.9 \pm 5.7* | 40.6 \pm 9.5 | 8.8 \pm 3.0** | 18.1 \pm 3.4 | 1.0 \pm 1.0*** |
| | Fe | 28.7 \pm 2.2 | 27.2 \pm 4.9 | 67.1 \pm 6.5 | 17.9 \pm 4.3*** | 24.0 \pm 3.3 | 7.8 \pm 5.9* |

Each value is the percentage of control \pm SEM. Statistically significant differences in the number of cells were observed in the ventral skin compared with that in the dorsal skin (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

melanogen [29–32] (inhibition of the activities of tyrosinase, TRP-1 and TRP-2 as well as of melanosome formation and maturation) [34]. In addition to the tissue environment, melanocyte proliferation and differentiation are regulated by numerous factors including endocrine hormones [21, 34–36]. It is possible that γ -rays or heavy ions reduce the production and release of endocrine hormones.

We found no good correlation between LET and the decrease in the number of melanoblasts or melanocytes. Our previous study showed that exposure to γ -rays or heavy ions resulted in white spots in the mid-ventrum of exposed offspring in an LET-dependent manner [8]; heavy ions were more effective than γ -rays. Relative biological effectiveness (RBE), estimated from the frequency of ventral spots in 22-day-old C57BL/10J mice, was 2.3 for silicon, 3.1 for argon and 4.5 for iron ions [8]. Epidermal melanoblasts/melanocytes and hair bulb melanocytes at E18 may be sensitive to radiation irrespective of LET, in which case the decrease in the melanoblast and melanocyte populations would not correlate with LET. In contrast, the absence of melanoblasts and melanocytes in the hair bulbs in the white spots of 22-day-old mice reflects the history of the hair bulb melanoblasts/melanocytes (cell death, inhibition of cell proliferation and differentiation or cell migration) from E18 to postnatal Day 22. Thus, during this period the lack of newly growing hair follicles and the absence of migrating melanoblasts and melanocytes from the dorsal epidermis results in ventral white spots. The ventral white spots may reflect the behavior of epidermal melanoblasts/melanocytes during this period, and this process is assumed to be dependent on LET.

The white spots were generally restricted to the mid-ventrum and tail tip, which are the last tissues to be populated by migratory neural crest cells, starting around E9 [8]. It is possible that γ -rays or heavy ions induce cell death in the neural crest cells or melanocyte stem cells, thereby reducing the number of viable melanoblasts/melanocytes that can migrate and colonize hair follicles, resulting in white spots. Alternatively, damage produced by γ -rays or heavy ions even at the low dose of 0.1 Gy may inhibit melanoblast proliferation and reduce the number of viable melanoblasts/melanocytes that can colonize hair follicles. In the present study, the number of melanoblasts and melanocytes in the epidermis and hair follicles was significantly decreased by irradiation with γ -rays or heavy ions even at 0.1 Gy. During the migration of neural crest cells or melanocyte stem cells from the dorsal to the ventral region, damaged cells may gradually stop proliferating and the ventral hair follicles lacking migrating melanoblasts develop into white spots at the mid-ventrum. Moreover, we cannot exclude the possibility that irradiation inhibited the differentiation of melanoblasts from neural crest cells and/or the differentiation of melanocytes from melanoblasts.

The ventral skin contains fewer number of epidermal melanoblasts/melanocytes and hair bulb melanocytes than

the dorsal skin [35]. This may be partly because of the gradual decrease in the number of neural crest cells during migration from the dorsal to the ventral region [10, 11]. In the present study, the decrease in the number of epidermal melanocytes and hair bulb melanocytes after irradiation with γ -rays or heavy ions was much more evident in the ventral skin than in the dorsal skin. If the sensitivity of melanocytes in the ventral skin was similar to that in the dorsal skin, the decrease in melanocytes in the ventral skin would have been a similar level to that seen in the dorsal skin. Thus, it is possible that the ventral skin melanocytes were more sensitive to radiation than the dorsal skin melanocytes. However, the differences in the response of melanoblasts and melanocytes between the ventral and dorsal skin were not clear enough to justify any conclusions because the melanoblast and melanocyte populations in the ventral skin are too small to be compared with those in the dorsal skin. Moreover, we cannot exclude the possibility that the effects of irradiation involve the migration of melanoblasts and melanocytes from dorsum to ventrum.

Argon ions reduced the number of melanocytes in both the dorsal and ventral region more efficiently than other radiation types. It is possible that argon ions greatly inhibited melanocyte differentiation from melanoblasts through reduction in the activities of melanin-related enzymes (tyrosinase, TRP-1 and TRP-2) rather than melanoblast cell death [9].

The present study differs from a previous study with respect to the effects of low-dose heavy ions on embryonic development in animals in that the effects of 0.1 Gy γ -rays or heavy ions on the development of tail and melanocytes in the present study were greater than the effects on survival to postnatal Day 22 and the frequency of ventral white spots [8]. Our experimental system is likely to contribute to the elucidation of how low-dose heavy ions affect embryonic development in animals.

In conclusion, the overall development of mouse embryos and the appearance of developmental abnormalities in the eyes and limbs were not necessarily dependent on the dose and LET of heavy ions, and irradiation with heavy ions decreased the numbers of epidermal melanoblasts/melanocytes and hair bulb melanocytes. We suggest that heavy ions may cause cell death in the tail, eyes and limbs as well as in melanoblasts/melanocytes, or inhibit melanoblast/melanocyte proliferation and differentiation directly or indirectly through the tissue environment even at the low dose of 0.1 Gy.

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