Development of the Irradiation Method for the First Instar Silkworm Larvae Using Locally Targeted Heavy-ion Microbeam

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To carry out the radio-microsurgery study using silkworm, Bombyx mori, we have already developed the specific irradiation systems for eggs and third to fifth instar larvae. In this study, a modified application consisting of the first instar silkworm larvae was further developed using heavy-ion microbeams. This system includes aluminum plates with holes specially designed to fix the first instar silkworm larvae during irradiation, and Mylar films were used to adjust energy deposited for planning radiation doses at certain depth. Using this system, the suppression of abnormal proliferation of epidermal cells in the knob mutant was examined. Following target irradiation of the knob-forming region at the first instar stage with 180-μm-diameter microbeam of 220 MeV carbon (12C) ions, larvae were reared to evaluate the effects of irradiation. The results indicated that the knob formation at the irradiated segment was specially suppressed in 5.9, 56.4, 66.7 and 73.6% of larvae irradiated with 120, 250, 400 and 600 Gy, respectively, but the other knob formations at the non-irradiated segments were not suppressed in either irradiation. Although some larva did not survive undesired non-targeted exposure, our present results indicate that this method would be useful to investigate the irradiation effect on a long developmental period of time. Moreover, our system could also be applied to other species by targeting tissues, or organs during development and metamorphosis in insect and animals.

INTRODUCTION

The silkworm, Bombyx mori is one of ideal experimental animals for study of the biological effects of ionizing radiation and for cell targeting radio-microsurgery due to the substantial information available on its embryology, physiology, and genome sequence.

The ionizing irradiation of the silkworm has long been used as a tool for chromosomal cleavage to generate mutants. The dose response in terms of tissue formation and physiological effects have been also studied. Silkworm larvae exposed to 100 Gy of γ-rays were successful in larval-pupal development with no disorders on external features, but had defects in wing formation. Exposure of silkworm ovary BmN4 cells to 150 Gy of γ-rays did not affect viability. These results indicate that silkworm larvae is much more radioresestant than mammals, and that radiorestance varies among organs.

We have previously reported the effect of heavy-ion radio-microsurgery of the B. mori. It was found that irradiation of the cellular blastoderm stage egg with heavy-ion microbeams (250 μm diameter) caused morphological defects in the resultant embryo. We also have made a fate map of the B. mori egg by examining the interrelation between targeted sites and location of defects arising in the resultant embryos. Targeting of hemopoietic organs with heavy-ion microbeams (2–6 mm diameter) affected the hemopoietic func-
tions of silkworm, e.g., lesions of hemopoietic organs. However, early instar larvae had not been applied for radio-microsurgery using heavy-ion irradiation, because we have not had an adequate preparation for it.

Development of a new method for the first instar larvae using microbeam irradiation is necessary to investigate effects of heavy ions on post-embryonic development or differentiation in holometabolous insect. Recently, we could examine the suppressive effect of irradiation on abnormal proliferation of epidermal cells on knob mutants of *B. mori*. In this paper, we report the technical details of the irradiation method optimized for the first instar larvae of *B. mori* and the performance of our irradiation system with a limit and an error. Moreover, Kotani et al. proposed a biological index for estimating exposure to cosmic irradiation. Our present method would be useful for estimating a standard effect of the index.

**MATERIALS AND METHODS**

**Insects**

A Knobbed (*K*) epidermal mutant strain of the domestic

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**Fig. 1.** A line sketch representing the aluminum plate with small holes employed in the new method and closeup picture of a confined larva. The size of the aluminum plate was 87 mm × 87 mm × 0.8 mm. Many small holes for fixing larvae are present within the plate. The hole size is described in this figure. The closeup picture shows the silhouette of a larva within the hole (observed through the control monitor). The larva confined in the hole was irradiated at knob-forming areas with a 180-μm-diameter microbeam (white circle).
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silkworm, B. mori\textsuperscript{11} was used in all experiments. The larval stage of B. mori is interrupted at four molting stages. The knob character is expressed on the larval dorsal markings. The larvae are easily phenotyped following the third molting and thereafter the knobs become larger from the fourth to fifth instar.\textsuperscript{9} Even at the pupa and adult stages, the K mutant still possesses knobs on the dorsal surface. Larvae were maintained on mulberry leaves at 25°C under a photoperiod consisting of 16-h light and 8-h dark cycles. Larval age is given in days, where day 0 indicates the day when larval ecdysis occurred.

Irradiation with \textsuperscript{12}C ion microbeam

First instar larvae of silkworm were irradiated with 220 MeV \textsuperscript{12}C ion microbeams collimated through a 180-μm-diameter microaperture.\textsuperscript{12,13} These ions were delivered from the azimuthally varying field (AVF) cyclotron located at the Takasaki Ion accelerators for Advanced Radiation Application (TIARA) of the Japan Atomic Energy Agency (IAEA).\textsuperscript{13} The mean value of linear energy transfer (LET) in the tissues exposed was calculated to be 128 keV/μm according to the kinetic energy loss (E\textsubscript{loss}), assuming water equivalence.\textsuperscript{14,15} The following relationship was used to convert particle fluence to dose in Gy: Dose [Gy] = 1.6 × 10\textsuperscript{-9} × LET [keV/μm] × Fluence [particle/cm\textsuperscript{2}].

First instar larvae were confined in a rectangular chamber (2.95 mm × 0.85 mm × 0.8 mm) consisting of an aluminum plate with holes (Fig. 1). The K epidermal mutants were confined without use of an anesthetic. The size of the chamber allowed for the successful confinement of a larva, such that the larvae (approximately 2.4 mm × 0.8 mm × 0.8 mm) were only capable of restricted movement within the hole of the plate. This was then sandwiched between 100-μm thick films (polypropylene) and irradiated locally with collimated microbeams.\textsuperscript{13} Specimens were irradiated at the fifth larval segment, where the most notable knob forms.\textsuperscript{9} The aperture size used here was large enough to completely encompass the desired target area (Fig. 1 white circle and Fig. 2). In terms of the penetration depth of the beams (range in water: 1200 μm), three sheets of 100-μm-thick Mylar film (polyethylene-terephthalate) were placed on top of the polypropylene film to adjust energy (LET) deposited in the targeted region at 350–500 μm depth (Fig. 2). In planning microbeam irradiation, the Bragg peak of deposited energy in \textsuperscript{12}C ions exists in the downside of larvae (Fig. 2). Within the hole, the shape of the larva and the targeted area were confirmed as determined from the location of three pairs of forelegs, four pairs of prolegs and other characteristics as outlined from its silhouette (Fig. 1). The location to be irradiated was targeted using a personal computer-controlled remote targeting system.\textsuperscript{12,13}

Morphological observations

Following irradiation, larvae were reared on mulberry leaves. All larvae were allowed to develop to maturity, and the morphology of the irradiated sites was observed at each developmental stage under a dissecting microscope. The effects of irradiation were classified by larval appearance. The larvae were classified into three categories as follows: 1) not affected at knobs; 2) partially affected at other regions except knobs; or 3) complete suppression of knob formation.

RESEARCH AND DEVELOPMENT

A variety of mutant strains are available in the B. mori. One of these mutants, “knob”, is an epidermal mutant in silkworm. This mutant has several pairs of protuberances (knobs) at larval marking sites and its characteristics are
expressed following the third molting. It was indicated that the epidermis in the knob region consisted of abnormally proliferated and stratified cells.\(^9,16,17\) We have previously reported the various effects (e.g. the fate of irradiated nuclei or cells) of heavy ions on \(B.\) \textit{mori}.\(^2,4–9\) It is most likely that heavy-ion irradiation could suppress knob differentiation. However, we also found that heavy-ion irradiation at the knob-forming region of third instar larvae hardly affected the knob character at the stage of forth and fifth instar. Thus, the development of a method for the local irradiation of the first instar larvae was necessary to investigate the effects of heavy ions in the early stage.

The limited-area (2–6 mm diameter) targeted irradiation method using heavy-ions has already been established.\(^2,4–9\) However, with these previous methodologies, the larvae were immobilized by taping and then irradiated, a procedure that could not be used to investigate the early developmental stage since larvae at this early stage can easily split away their epidermis. Therefore, we developed the irradiation system optimized for first instar larvae (Figs. 1 and 2, see Materials and methods).

To examine the effects of heavy-ion irradiation on knob formation, larvae irradiated with graded doses (120, 250, 400 and 600 Gy) of \(^{12}\text{C}\) ions were carefully analyzed. As shown in Fig. 3, knobs were generally developed at third, fifth and eighth segments in the mutant, and the knob formation of the irradiated fifth segment was clearly suppressed at either or both sides in the mutants at the fifth instar stage following irradiation with 250, 400 or 600 Gy.

In contrast, the knob formation at the non-irradiated third and eighth segments normally appeared in either irradiation (Fig. 3). The suppression of knob formation was observed at only one side in some larvae. A possible reason that might account for this unilateral knob-suppression phenomenon would be the occurrence of unilateral irradiation resulting from the slight movement of larvae during irradiation. It is conceivable that the ion beam incidence angle to larvae might slightly be changed and the Bragg peak at the end of penetration depth of ions would come to a position of unilateral knob-forming region.

Table 1 summarizes the suppression of knob formation by microbeam irradiation. Knob formation at the irradiated segment was suppressed in a few larvae (5.9%) by irradiation with 120 Gy. When larvae were irradiated with 250, 400 and 600 Gy, knobs disappeared by 56.4, 66.7 and 73.6%, respectively. These results indicate that microbeam irradiation can suppress knob formation of this mutant and that the frequency of knob suppression increased markedly at 250 Gy. The threshold dose for the suppression of knob formation may be considered to fall within the range between 120 Gy and 250 Gy.

Many larvae died (43.4% in mean) after \(^{12}\text{C}\) ion irradiation. The lethality rate was relatively constant in all experimental groups (120 Gy to 600 Gy). Since only 13.2% of sham-irradiated larvae died, the high fatality must be accounted for by the irradiation. Most of died larvae did so at molting, metamorphosis and adult eclosion. In some irradiated silkworms, the esophagus or midgut appeared weak following shock. It is well known that insect midguts include...
stem cells,^{18–20} and that stem cells are vulnerable to ionizing radiation.\(^\text{21}\) Indeed, the midgut of dead larvae was found to break off during molting. Moreover, when the wing disc of larva (located directly above the midgut) was irradiated with 120 Gy, 69% of the larvae died (personal communication).

In this study, \(^{12}\)C ions struck certain parts of the midgut. However, when irradiated at the wing disc, the ions pierce through the intestine. On the other hand, epidermal cells of silkworms are considered to be extremely resistant to heavy ions.\(^\text{22}\) So that the high mortality of silkworms observed must be due to high irradiation doses to the midgut. As shown in Fig. 2, if the irradiation area shifted from its intended location, the midgut would also be simultaneously irradiated with relatively high doses.

An optimized irradiation system had been established to irradiate all the transformation stages in the silkworm (Table 2). In the present study, we have further developed the new irradiation method for the first instar larvae of \(B. \) mori. We have completed the irradiation method for all the stages of silkworm, except for adult. Use of this targeted irradiation method would provide another approach towards investigating the effect of irradiation on post-embryonic development. Especially, this investigation can extend over a long developmental period of time following irradiation of an organ (e.g. wing disc, silk gland, brain, testis or ovary) using first instar silkworm larvae. The issue concerning low survivability derived from irradiation on unwilling exposure needs to be addressed. Adjusting the position of the Bragg peak at the depth-direction of the target might resolve this issue. We are currently attempting to suppress knob formation using 260 MeV neon \((^{20}\text{Ne})\) ions, because its range is shorter (700 \(\mu\)m in water) and undesired irradiation to another organs would be lesser than \(^{12}\)C ions.

### Table 1. Suppression of knob formation by microbeam irradiation

| Dose (Gy) | Total No. of moths formed | No. of knobs suppressed | No. of affected by irradiation | No. of unaffected knobs | Rate unaffected by irradiation (%) | Rate affected by irradiation (%) | Rate knob suppressed (%) |
|-----------|---------------------------|-------------------------|-------------------------------|-------------------------|----------------------------------|-------------------------------|-------------------------|
| Control   | 600                       | 72                      | 53                            | 66                      | 8.3                              | 91.7                          | 73.6                    |
| 120       | 250                       | 55                      | 31                            | 40                      | 27.3                             | 72.7                          | 56.4                    |
| 250       | 400                       | 66                      | 44                            | 51                      | 22.7                             | 77.3                          | 66.7                    |
| 600       | 600                       | 72                      | 53                            | 66                      | 8.3                              | 91.7                          | 73.6                    |

### Table 2. Methods for heavy-ion local irradiation to silkworm

| Developmental stages irradiated | Egg\(^\text{8}\) | First to second instar larva | Third instar larva to pupa\(^\text{23–25}\) |
|--------------------------------|----------------|-----------------------------|---------------------------------------------|
| Immobilizing method            | –              | Confined to a hole of an alminum plate sandwiched between thick films | Fixed by taping on an acrylic resin plate with holes |
| Available heavy-ion beam size (diameter) | 20–500 \(\mu\)m | 20–500 \(\mu\)m | 2–6 mm |

UV laser microbeam has been used for local targeted irradiation, as well as the present heavy-ion microbeam. However, UV lasers heat the target area of high doses, thereby denaturating proteins and causing necrosis in target cells.\(^\text{22}\) This type of UV laser-induced epidermal cell death at the prospectively knob forming region may cause fatal bleeding. In addition, UV has another limitation by the scatter of the light in the target. Heavy-ion microbeam irradiation could overcome the shortcomings of UV lasers, because heavy ions do not denaturate proteins.

Our present method may not be limited to the silkworm. At present, radiation response of mammalian cells is mainly characterized by DNA damages. Radiation-induced DNA lesions such as double-strand breaks (DSBs) are properly repaired by several proteins that form nuclear foci at the sites of DNA damage.\(^\text{23}\)\(^\text{24}\)\(^\text{25}\) Especially, a single iron (Fe) ion traversing the cell nucleus has been known to induce a cluster of DSBs along the ion track.\(^\text{24}\) Radiation responses, such as cell cycle perturbation, induction of signal transduction pathways, changes in gene expression and cell behavior are followed. Recently, nonirradiated lymphoma cells are influenced to the irradiated neighbor cells termed the “bystander effect” has been demonstrated.\(^\text{25}\) The finding of the bystander effects may be important for the radiotherapy.\(^\text{26,27}\) These biological responses are produced \textit{in vivo} (in individuals), and, essentially, we should consider the effects of ionizing radiation as a risk for individual. Our presented method would provide a technical advance in locally targeted irradiation for tissue, organ and individual.

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