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Adult stem cell plasticity: Neoblast repopulation in non-lethally irradiated planarians

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ABSTRACT

Planarians are a model system for studying adult stem cells, as they possess the neoblasts, a population of pluripotent adult stem cells able to give rise to both somatic and germ cells. Although over the last years several efforts have been made to shed light on neoblast biology, only recent evidence indicate that this population of cells is heterogeneous. In this study we irradiated planarians with different non-lethal X-ray doses (1–5 Gy) and we identified subpopulations of neoblasts with diverse levels of tolerance to X-rays. We demonstrated that a dramatic reduction of neoblasts occurred soon after non-lethal irradiations and that de-novo proliferation of some radioresistant cells re-established the primary neoblast number. In particular, a strong proliferation activity occurred at the ventral side of irradiated animals close to the nervous system. The produced cells migrated towards the dorsal parenchyma and, together with some dorsal radioresistant cells, reconstituted the entire neoblast population demonstrating the extreme plasticity of this adult stem cell system.

Introduction

The ability to remain in an undifferentiated state and the capability, on cue, to undergo an asymmetric division and generate a cell with a stem cell fate and a daughter cell that differentiates, are unique for stem cells in multicellular organisms. Embryonic stem (ES) cells are pluripotent and so capable of giving rise to cells that make up every organ in the adult. Mammalian ES properties became progressively restricted during development and, in the adult, stem cells are generally multipotent or unipotent and are most often used for the maintenance or repair of the tissue in which they reside. This tissue-specificity has now been questioned by evidence suggesting that, under certain conditions, adult stem cells may possess potential for tissue regeneration (Loebinger and Janes, 2007; Zech et al., 2007). More *in vivo* studies are necessary to understand adult stem cell biology. Unfortunately, researches on adult stem cells have not been pursued vigorously as adult stem cells are generally few in number and there are not many animal systems that enable *in vivo* studies of these cells. Freshwater planarians (Platyhelminthes) provide an example of one of these suitable model systems. Indeed, these organisms possess a population of adult pluripotent stem cells, the neoblasts, able to give rise to both somatic and germ cells. This advantage, coupled with the successful application of molecular, cellular and genomic approaches, as well as the possibility to perform loss of function studies by the RNA interference (RNAi) technique, makes planarians a sound model system for *in vivo* investigations on adult stem cell biology (Saló, 2006; Sánchez Alvarado, 2007; Rossi et al., 2008). Neoblasts are small cells (5–10 μm of diameter), with a high nucleus/cytoplasm ratio, scattered throughout the parenchyma and absent in the most anterior part of the head and the pharynx. Neoblasts also accumulated in clusters along the midline and the dorso-lateral parenchyma (Salvetti et al., 2000; Orii et al., 2005). Neoblasts are involved in regeneration giving rise to regenerative blastema as well as in tissue homeostasis, and they are the only proliferating cells in asexual planarians (Rossi et al., 2008). For this reason, neoblasts are destroyed a few days after treatment with lethal doses of X-rays, while differentiated cells are unaffected over the same time period and with the same doses (Wolff and Dubois, 1948). Dissimilar to what was observed in planarians, it has been recently demonstrated that in *Macrostomum lignano* high X-ray doses (40 and 80 Gy) resulted in a lack of somatic stem cell proliferation, while S-phase cells were still visible in the gonads (Pfister et al., 2007).

Neoblast population is composed of distinct subpopulations of cells, as demonstrated by the identification of a *Dugesia japonica* homologue of the *Drosophila Piwi* gene (*DjPiwi-1*) that is exclusively expressed in a group of neoblasts distributed along the dorsal body midline (Rossi et al., 2006). Moreover, the recent identification of a *nanos*-related gene (*Djnos*) in *D. japonica* allowed to identify a second subpopulation of

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neoblasts (Sato et al., 2006). *nanos*-positive cells are located along the dorso-lateral parenchyma (the presumptive testes forming regions) and in two ventral spots (the presumptive ovary forming regions), which are considered to be the germline stem cells (Sato et al., 2006; Handberg-Thorsager and Saló, 2007; Wang et al., 2007). Heterogeneity among neoblasts is also confirmed by the existence of two different subpopulations of irradiation-sensitive neoblast-like cells (X1 and X2) identified by X-ray treatments and FACS analysis (Reddien et al., 2005b; Hayashi et al., 2006). These groups of neoblasts diverge in both expression of proliferation markers and in ultrastructural features and they have been classified as type A (concentrated in the X1 fraction) and type B (concentrated in the X2 fraction) cells (Higuchi et al., 2007). So, the term neoblasts is not referred to a single population of stem cells but to a compound of different subpopulations of stem cells each of which expresses particular molecular markers and, consequently, could have different biological properties.

Here, we utilize X-ray treatments to investigate *in vivo* the planarian adult stem cell system and we provide further evidence that neoblasts are heterogeneous. We also demonstrate that the early reduction of the neoblast number that occurs after non-lethal X-ray irradiation is followed by the activation of cell proliferation that allows the reconstitution of the complete neoblast system.

Materials and methods

Animals

Planarians utilized in this work belong to the species *D. japonica*, asexual strain GI (Orii et al., 1993). Animals were kept in autoclaved stream water at 18 °C and starved at least for 2 weeks before being used in the experiments. Regenerating fragments were obtained by transection between auricles and pharynx or tail and pharynx.

X-ray irradiation

Intact planarians were exposed to 1, 2, 3, 5, 10, 15 and 30 Gray (Gy) single-dose of hard X-rays (200 kVp, 12 mA, 1 Gy/min) using a Stabilipan 250/1 instrument with a 2 mm aluminum (Al) filter (Siemens, Gorla-Siama, Milan, Italy) equipped with a Radiation Monitor 9010 dosimeter (Radcal Corporation, Monrovia, CA, USA). The animals were sacrificed at: a) 1, 2, 3, 4, 7, 8, 9, 10, 11, 14, 23, 27 or 40 days after irradiation for *in situ* hybridization experiments; b) 4, 7, 11 or 14 days after irradiation for immunohistochemistry; c) 11 days after irradiation for TEM analysis; d) 1, 2, 3, 4, 5, 6, 7, 9, 14, 25 or 30 days after irradiation for BrdU staining and mitosis count; e) 1, 3, 7, 12 days after irradiation for FACS analysis. For regeneration experiments, animals were transected 60 h after a treatment with 5 Gy of X-rays and sacrificed 15, 21 or 28 days after the transection for whole mount *in situ* hybridization. In some experiments, planarians were treated with 5 Gy of X-rays, kept in stream water for 30 days and irradiated again with an additional X-ray dose of 5 Gy. After this second radiation treatment, animals were sacrificed 2, 4, 7 or 14 days for whole mount *in situ* hybridization. Untreated animals with correspondent time of starvation and/or regeneration were utilized as controls.

BrdU labelling and detection

X-ray-irradiated or untreated (control) planarians were injected with a 10 mM BrdU solution using the Nanoject Microinjectior (Drummond). For detection of BrdU on macerates, 6 h after injection planarians were dissociated in 250 µl of a glycerol/acetic acid/distilled water solution (1:1:13) for 20 h at 4 °C. Twenty µl of cell suspension was placed on a microscope slide and dried for a couple of hours. Slides were immediately re-hydrated with two washes of PBS plus 0.5% Triton X-100 (PBST 0.5) for 5 min each, treated with 1 N HCl-PBST 0.5 solution for 30 min and rinsed three times in PBST 0.5 for 5 min each. After treatment with 20 µM proteinase K in PBS plus 0.1% Triton X-100 (PBST 0.1), slides were washed in PBST 0.1 for 1 min and then blocked in 10% non-fat dry milk (Bio-Rad) in PBST 0.5 for 10 min. After blocking, slides were rinsed in PBST 0.1 for 1 min and then incubated with 1:50 dilution of anti-BrdU antibody (Abcam) in 1% dry milk in PBST 0.1 for 30 min. Following 3 washes of 10 min each in PBST 0.5 slides were rinsed in PBST 0.1 and incubated in 1% dry milk in PBST 0.1 solution containing 1:200 dilution of fluorescein isothiocynate-conjugated anti-mouse antibody (Molecular Probes) for 30 min. Following two washes in PBST 0.5 and one in PBS, slides were mounted for microscope analysis. Slides were scored with a Zeiss Axioplan photomicroscope to count the BrdU-positive nuclei. About 8×10^4 nuclei were examined for each preparation and only nuclei with fine grained bright florescence through the nuclear matrix and bright perinuclear chromatin staining were taken as positive. Ten µl of cell suspension was used in a hemocytometer to count the number of total cells. The relative number of BrdU-positive nuclei was calculated dividing the absolute number of positive nuclei by the number of total cells. Three independent samples were analyzed for each experimental condition and two different counting were carried out for each sample.

For immunofluorescent detection of BrdU-positive cells on tissues sections, non-irradiated animals and 5 Gy-treated planarians at the 4th and 7th day after the X-ray treatment were injected as described above. Twenty-two hours following microinjection, specimens were fixed in relaxant solution (Kobayashi et al., 1998) for 17–24 h at 4 °C and then included in paraffin. After rehydratation in ethanol series, sections were treated with 5 µg/ml of Proteinase K (Sigma) for 5-15 min at 37 °C and then incubated in 1 N HCl in PBST for 5 min at 55 °C. After washing in PBST, samples were firstly incubated in 10% goat serum for 30 min at room temperature and then incubated with 1/100 anti-BrdU monoclonal antibody (BD Pharmigen) in 10% goat serum for 1 h. After washing, samples were incubated with 1:100 dilution of HRP conjugated anti-mouse antibody and the signal was amplified by using Tyramide Signal Amplification Kit according to the manufacturer instructions (Molecular Probes).

Mitosis analysis on cell macerates

X-ray-irradiated (5 Gy) and non-irradiated (control) planarians were treated in 3‰ colchicine in stream water for 6 h, dissociated in 250 µl of a glycerol/acetic acid/distilled water solution (1:1:13) for 20 h at 4 °C and then stained with 20 µg/ml Hoechst No.33342. Twenty µl of cell suspension was placed on a microscope slide, dried for a couple of hours and then mounted for microscope analysis. Slides were scored with a Zeiss Axioplan photomicroscope to count the number of mitotic figures. About 8×10^4 nuclei were examined for each preparation. Ten µl of cell suspension was used in a hemocytometer to count the number of total cells. The mitotic index was calculated dividing the number of mitosis by the number of total cells in which mitosis were counted. Three independent samples were analyzed for each experimental condition and two different counting were carried out for each sample.

In situ hybridization

Whole mount *in situ* hybridization was performed as described in Rossi et al. (2007). Section *in situ* hybridization was performed according to Kobayashi et al. (1999).

DNA templates for *DjPiwi-1* was prepared as described in Rossi et al. (2006); *DjMCM2* was prepared according to Salvetti et al. (2000); *DjPum* was prepared according to Salvetti et al. (2005); *CIP29* Gi 32900868 was prepared according to Rossi et al. (2007). *Djnos*