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The *in vivo* effect of chelidonine on the stem cell system of planarians

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ABSTRACT

The presence of adult pluripotent stem cells and the amazing regenerative capabilities make planarian flatworms an extraordinary experimental model to assess *in vivo* the effects of substances of both natural and synthetic origin on stem cell dynamics. This study focuses on the effects of chelidonine, an alkaloid obtained from *Chelidonium majus*. The expression levels of molecular markers specific for stem or differentiated cells were compared in chelidonine-treated and control planarians. The use of these markers demonstrates that chelidonine produces *in vivo* a significant anti-proliferative effect on planarian stem cells in a dose-dependent fashion. In response to chelidonine treatment mitotic abnormalities were also observed and the number of cells able to proceed to anaphase/telophase appeared significantly reduced with respect to the controls. Our results support the possibility that chelidonine acts on cell cycle progression by inhibition of tubulin polymerization. These studies provide a basis for preclinical evaluation *in vivo* of the effects of chelidonine on physiologically proliferating stem cells.

1. Introduction

Stem cells are, by definition, undifferentiated cells that have both the ability to self-renew, making identical copies at each division, and to differentiate specialized cells. These cells offer the possibility of a renewable source of replacement cells and tissues, with potential applications in regenerative medicine. However, a number of pathological conditions, such as cancer and psoriasis, are characterized by hyper-proliferative phenomena. Consequently, inhibition of cell proliferation results to be potentially useful in drug discovery to design agents targeted to the treatment of diseases characterized by uncontrolled or abnormal cellular proliferation. In this context, the so-called adult or somatic stem cells (ASCs) have attracted special attention. *In vivo* studies of ASCs are not easy in mammals due to the difficulty of developmental biology studies *in vivo* using vertebrate models. For this reason, most of the knowledge on ASC biology results from *in vitro* studies. Non-mammalian model systems can be alternatively used to assess *in vivo* the effect of substances on these cells.

Planarians are an ideal animal model for *in vivo* analysis of ASCs. Their extraordinary ability to regenerate, as well as to sustain continuous homeostatic turnover of all differentiated cell types, relies on the presence of stem cells that share characteristics with the stem cells of higher organisms, including humans. Planarian regeneration involves remodeling of pre-existing tissues and formation of an outgrowth at a wound region, in which the missing parts are newly formed (the regenerative blastema) (Gentile et al., 2011; Slack, 2011). The discovery that planarian stem cells use evolutionarily conserved mechanisms that are surprisingly similar to the mechanisms we observe in mammalian stem cells, makes these invertebrates suitable to screen the effects of new drugs on stem cells. In addition, the planarian genome shares a large number of genes with the human genome, including sequences involved in various aspects of human biology and disease.

Our work focuses on the analysis of the effects produced *in vivo* by substances of natural origin, particularly a series of compounds contained in *Chelidonium majus*, on planarian stem cells. *C. majus* (greater celandine) is a herbaceous plant of the *Papaveraceae* family that spontaneously grows in temperate areas. As a starting point we studied the effects of chelidonine, an isoquinoline alkaloid derived from *C. majus*. The pharmacological potential of mitotic arrest by chelidonine is considered promising in cancer therapy. In this paper the activity of chelidonine was tested *in vivo* in *Dugesia japonica*, a clonal planarian strain widely used to study drug responsiveness (Kitamura et al., 2003; Nogi and Levin, 2005; Nogi et al., 2009).

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2. Materials and methods

2.1. Animals and treatments

Asexual specimens of *D. japonica* (GI strain) were maintained at 18 °C in autoclaved stream water, fed weekly with chicken liver and starved one week before the experiments. Control groups were represented by planarians soaked in dimethylsulfoxide (DMSO) at different concentrations in the absence of drug and by untreated planarians, maintained in water. As literature data demonstrate that DMSO concentrations >0.1% should be avoided in order to be able to reliably observe any behavioral or toxic effects of hydrophobic drugs in these animals (Pagán et al., 2006; Yuan et al., 2011), we first analyzed the long-term effects of DMSO exposure before using it as a solubility-aiding agent for chelidonine. Thirty planarians were exposed to DMSO in water at different concentrations (0.025%, 0.05%, 0.1%) or maintained in water (control group). The animals were exposed to these experimental conditions for a period of 7 days (long-term DMSO exposure). To assess the effects of DMSO during regeneration, thirty planarians were exposed to the same treatment, but were transected at the prepharyngeal region (Fig. 1) after 1 day of exposure and left to regenerate in different concentrations of DMSO or in water. Chelidonine (Sigma) was freshly prepared in DMSO for each experiment, so that the final concentrations of DMSO in water were 0.025%, 0.05% and 0.1% (corresponding to 5 μM chelidonine, 10 μM chelidonine and 20 μM chelidonine, respectively). Thirty intact worms of similar body size were exposed by soaking to the drug for 3–4 days. In another experimental protocol the animals were transected at the prepharyngeal region after 1 day of treatment and left to regenerate in presence of chelidonine or DMSO (control group). The fragments were sacrificed 3 or 6 days after cutting for successive experiments. In each protocol the solutions (30 ml) were changed daily.

2.2. Preparation of dissociated cells

Dissociated planarian cells were prepared according to Baguñà and Romero (1981) and stained with Hoechst 33342 DNA dye. A total of 10 μl of cell suspension was used to calculate the number of metaphase cells *versus* anaphase/telophase cells. The number of mitotic figures was normalized to the number of total cells and the values are mean ± standard deviation (s.d.) of six independent samples, analyzed in duplicate for each experiment, assuming as 100% the value of control planarians.

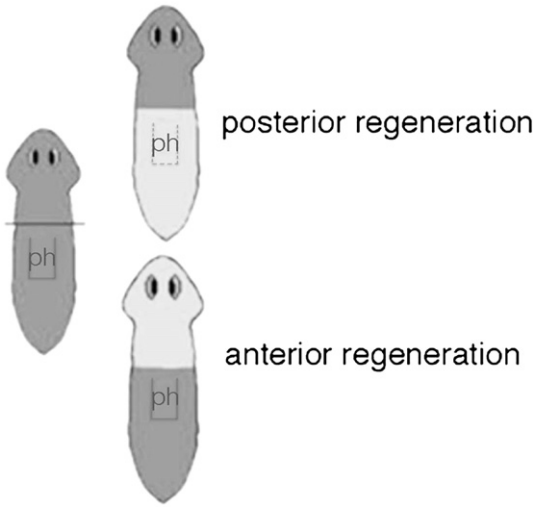

Fig. 1. Schematic representation of amputation procedure of a planarian. Black line indicates the amputation level. Complete regeneration of the missing body parts occurs in about 10 days at 18 °C. Regenerated body parts are shown in light gray. ph: pharynx.

2.3. RNA isolation, reverse transcription and real time RT-PCR

Total RNA was isolated using the Nucleospin RNAII kit (Macherey-Nagel), according to the manufacturer's instructions. Before cDNA synthesis, each extraction was tested for the absence of genomic DNA. cDNA was synthesized using GoTaq® 2-step RT-qPCR system (Promega). For each experiment nine different RNA extractions were performed with three independent experimental groups. Each RNA sample was obtained from an experimental group including 6 different regenerating fragments (3 head fragments + 3 tail fragments). Real time RT-PCR was performed at least three times for each examined gene, using three replicates for each cDNA. SYBR Green chemistry-based RT-PCR was carried out on a Rotor-Gene 6000 Real time-PCR (Corbett Research). Details of the procedures are given in Table S1, according to the MIQE guidelines (Bustin et al., 2009). Preliminary experiments were performed to evaluate the transcriptional stability of candidate endogenous reference genes (Yuwen et al., 2011). Under our experimental conditions (0.025%, 0.05%, 0.1% DMSO), the stability of two planarian endogenous reference genes, *D. japonica elongation factor-2* (*DjEF2*) and *D. japonica β-actin* (*DjACTB*) was analyzed using NormFinder software. *DjEF2* was the most stable gene and for this reason it was selected as reference for comparative gene expression analysis. Expression levels of *DjEF2* and *DjACTB* reference genes after DMSO treatments were analyzed by real time RT-PCR (Fig. S1).

2.4. Statistics

All values are presented as mean ± (s.d.) of at least three different experiments in triplicate. Data tabulation and descriptive statistics were performed with Microsoft Excel. The statistical analysis was performed by means of non-parametric ANOVA Kruskal–Wallis test and Student's *t*-test (data not shown). According to Shapiro–Wilk test, the distribution of the logarithmically transformed data was not significantly different from that of a normal distribution. In addition, homoscedasticity analysis, determined with Hartley's F max test, revealed no differences in variability between the groups (in all samples the F max calculated was lower than the critical value at 0.05). For mitosis analysis, statistical significance was determined using the two-tailed unpaired Student's *t*-test. A value of $P<0.05$ was considered statistically significant.

2.5. In situ hybridization

Whole mount *in situ* hybridization was performed according to Umesono et al. (1997) with minor modifications (Nogi and Levin, 2005). DIG-labeled antisense riboprobe was synthesized by RNA polymerase (Roche), employing linearized *Djmcm2* (cDNA: region 168 to 761 bp) in pGEM-T Easy vector (Promega) as the template.

3. Results

3.1. Effects of chelidonine on tissue homeostasis and regeneration

Under our experimental conditions, 20 μM chelidonine treatment resulted in abnormal phenotypes. On the contrary, no effects on mortality or gross abnormalities were observed in DMSO-treated planarians that appeared similar to the control group maintained in water. Most of the 20 μM chelidonine-treated planarians exhibited lesions and ventral curling (Fig. 2A–D). This phenotype, although consistent with a stress effect, strongly resembles the stereotypical stem cell-defective phenotype observed when stem cells are selectively eliminated by γ-irradiation or when animals are subjected to RNAi for