RADIATION RESEARCH

THE NEURAL STEM CELLS RADIOSENSITIVITY

The Valid Method for Consideration of Neural Stem Cells Radiosensitivity: Possible Tool for Estimation of External Influence on Neural Stem Cell

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Summary

We studied radiosenzitivity of neural stem cells (NSCs) by widely used functional test and by method with adherent NSCs. NSCs were given 0-8 Gy, cultivated floating in medium (Group N) or finally adhered to poly-ornitine-coated Petri dishes (Group A). After 6 generation times neurospheres were fixed, stained and their numbers were counted. Finally adherent NSCs showed significantly higher radiosenzitivity than non-adherent NSCs from the dose of 0.5 Gy. Average D0 of group A was 1.85 Gy; average D0 of group N was counted as low as 0.96 Gy.

We assume that the functional test widely used for influences determination to NSCs is not valid for NSCs radiosensitivity measurement. Additionally, NSCs radiosensitivity test should be performed with adherent NSCs.

Key words: Neural stem cells; Radiosensitivity test; Adhesion.

Introduction

Neural stem cells (NSCs) which are selfrenewing cells that generate neurons, astrocytes and oligodendrocytes, reside in the adult hippocampus and via production new dentate gyrus granule neurons (Gage et al., 1998 and Palmer et al., 1997) in all vertebrates including humans (Eriksson et al., 1998). They have a significant importance to baseline hippocampal neurogenesis supporting cognitive functions in adults (Shors et al., 2001). When the level of baseline neurogenesis in various murine strains correlates with hippocampal performance (Kempermann, 2002) and NSCs might be isolated and subsequently cultivated *in vitro*, a valid method for estimation of external influence to NSCs funtions is needed.

Isolated NSC cultivated *in vitro* proliferates and differentiates. Eachone NSC produces a neurosphere. Unfortunatelly, multipotent stem cells such as NSCs have not any suitable differentiation markers, there is not possibility to detect them with high validity. For this stem cells line, the only approach to considerate proliferation ability is to measure number and/or size of NSCs containing neurospheres growing *in vitro*. Suitable cytotoxic external influence for assessment of NSCs altered proliferation is a ionising radiation, when according classic radiobiologic law, firstly published in 1906 by Bergonie and Tribondeau, differentiated cells are more radioresistant than less differentiated cells. Therefore, active stem cells, such as NSCs, are the most radiosensitive cells in organism. Until today, the most radiosensitive cell lines are CD4+ T-lymphocytes with D0 ~ 0.97 Gy and hematopoietic cell line (32D cl 3 clonal line) with D0 ~ 0.97 (Santucci et al., 1994), or 0.89 Gy respectively (Epperly et al., 2003), when D0 is defined as 37 % production new cell colonies compared to non-irradiated samples from the same number of seeded cells. The most relevant results published Snyder et al. (1992), when they measured survival fraction with a D0 of 1.25 Gy of murine neural precursor cells derived from the cerebellum and immortalized with v-myc.

In this study, we sought a NSCs suitable method for estimation of any cytotoxic external and internal influences, herein using ionising radiation.

Material and Methods

NSCs isolation and culture

Neural stem cells were isolated from E20 foetuses of timed pregnant transgenic mice C57Bl/6-TgN (ACTbEGFP)1Osb. Forebrains of E20 mouse foetuses were dissected free of meninges and mechanically dissociated by trituration through a fire polished pipette. Single cell suspensions of neural progenitors were plated into the culture flasks with untreated surfaces (40,000/ cm^2). The culture medium was composed of Dulbecco's Modified Eagle's Medium/Ham's F12 (1:1; Sigma), B27/ N2 Supplements (Gibco), 2 mM L-Glutamine (Sigma), 100 U/ml penicillin and 100 μg/ml streptomycin (Gibco), 10 ng/ml basic fibroblast growth factor (bFGF, PeproTech) and 20 ng/ml epidermal growth factor (EGF, PeproTech). Neural stem cells grew in culture as free-floating neurospheres that were subcultivated two times per week by dissociation into single cell suspension alternatively using Trypsin/EDTA and mechanically. Fresh growth factors were added to the medium every 3rd day. For the experiment, cells from the 6th passage were used.

Experimental setup

NSCs were separately cultivated under two different conditions.

The first group, Group A, was cultivated with NCSs adhered to polyornitine-coated Petri dishes 3.5 cm in diametre with initial cell density 10, 000 cells/1 ml (= 30, 000 cells per dish/9.5 cm^2).

The second group, Group N, was cultivated with NCSs floating in cultivation medium in Petri dishes of the same size (25 cm^2 culture flask) with initial cell density 40,000 cells/1 ml (= 320,000 cells/8 ml/25 cm^2).

Irradiation was performed with ^{60}Co source (Chirana Company, Prague, Czech Republic) with a distance of 50 cm and the dose rate was 1.44 $Gy.min^{-1}$. Samples were irradiated with 0, 0.5, 1, 2, 4, and 8 Gy in single doses and fixed after 6-generation times (6.5 days) in 70% ethanol for 10 min. Group N samples were cytospined prior to fixation.

To enhance NSC adhesion to Petri dishes, specimens in Group A were exposed to 0.3 ml FCS per dish for 1 hour prior discarding medium and subsequently fixed in 70% ethanol for 10 min.

All samples were then stained in 10% Giemsa-Romanowski stock solution diluted in destiled water for 5 min. Subsequently, a neurosphere number was counted by microscope IMT-2 (Olympus, Prague, Czech Republic). As a proper neurosphere cell aggregates, showing 3D conformation, were defined, NSC colonies growing in one layer were excluded. The whole experiment was repeated three times.

Data processing

The 2-way t-test with unequal distribution was used for statistical analysis using mean values ± 2x S.E.M.

Results

In non-irradiated samples, 1000 seeded cells generated 13.56 neurospheres in average (group N) and 5.57 neurospheres (group A) respectively.

The D_0 in 3 independent experiments were 1.97, 1.77, and 1.81 Gy with average value of 1.85 Gy in group N and 0.85, 0.83, and 1.19 with average value of 0.92 in group A.

In group N, a significantly lower number of neurospheres was found in samples irradiated by the dose of 2 Gy and higher than in non-irradiated samples.

In group A, a significant difference in a neurosphere number was observed between non-irradiated samples and samples irradiated by the doses of 1 Gy and higher.

Table 1

Selectes experiments of the survival fraction of neurospheres after irradiation ± 2 x S.E.M. Average values of non-irradiated subgroups are equal to100 %.

	0 Gy	0.5 Gy	1 Gy	2 Gy	4 Gy	8 Gy	D_0
Group N	100±18.66	76.61±9.55	54.84±4.17[3]	32.26±3.82[3]	11.69±0.90[3]	3.43±0.69[3]	**1.81**
Group A	100±20.50	46.97±3.54[3, b]	31.65±5.05[3, b]	11.11±2.09[3, b]	1.26±0.52[3, b]	0.42±0.37[3, b]	**0.83**

Probability of value difference to non-irradiated samples: $p < 0.05$ - [1]; $p < 0.01$ - [2]; $p < 0.001$ - [3].
Probability of values difference between Group N and Group A in the same radiation dose: $p < 0.05$ - [a]; $p < 0.01$ - [b]; $p < 0.001$ - [c].

Values of surviving fraction between group A and group N were considerably different from the dose of 0.5 Gy. Results are summarized in Table 1 and Fig. 1.

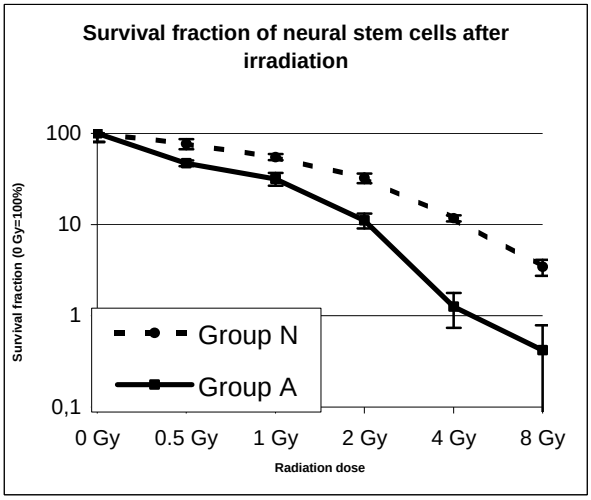

Fig. No. 1: The survival fraction of neurospheres after irradiation with error bars of 2 x S.E.M (95.45 % of confidency interval).

Discussion

Stem cells can be defined as cells with unique abilities to proliferate and differentiate. Proliferation ability is not limited, however under control of still unclear mechanisms. Stem cells are divided into active with actual proliferation activity and passive stem cells so-called quiescent (Quesenberry et al., 2004). Additionally, there are number of results reporting multipotent stem cells conversion into different cell lines. Vescovi et al. (2002), Munoz-Elias et al. (2003) and Shih et al. (2002) observed this wide plasticity potential when e. g. neural stem cells converted into keratinocytes, cardiomyocytes, hepatocytes, and enterocytes. Although the mechanism of stem cells conversion is not still defined, Bjornson et al. (1999) suggested that converting factors are produced by depleted cell populations.

Our experiment with floating NSCs is also called as the functional test serving for determination of external influences (Weiss et al., 1996, and Sakakibara et al., 2002). Experiment with finally adhered NSCs simulates condition for clonnogenity assay of classic cell lines (Abend et al., 2000). When a significant cells number in a neurosphere is dying its adhesivity is altered. In experiment with annexin V detected murine apoptotic neural precursor cells 12 hours after 5 Gy irradiation Limoli et al. (2004) described higher apoptotic induction up to about 37 % compared to control group. Therefore, seriously damaged neuropheres did not be able to adher to Petri dishes surface and they were discarded together with a medium. This reason might explain relative higher neurospheres number with non-adherent NSCs. Therefore, the widely-used functional test (signed as group N) can not be recommended for radiosensitivity assessment due to involving functionally defected NSCs that were not able to adher to Petri dish surface.

From our results follow that sensitivity to ionising radiation of NSCs is similar to radiosensitivity of the most sensitive differentiated cell line, CD4+ T-lymphocytes, with average D0 cca 0.97 Gy (Williams et al., 1994). In other words, our results also confirmed a basic radiobiologic law firstly published in 1906 by Bergonie and Tribondeau (1906).

Further investigations using NSCs will be the objective of our future work.

We assumed that radiosensitivity test of NSCs should be provided with finally adhered NSCs.

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