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Short Communication

Flow cytometric cell cycle analysis of cultured brown bear fibroblast cells

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Abstract

The aim of this study was to assess by flow cytometry the cell cycle of brown bear fibroblast cells cultured under different growth conditions. Skin biopsies were taken in Cantabria (Spain) from a live, anaesthetized brown bear. DNA analysis was performed by flow cytometry following cell DNA staining with propidium iodide. Serum starvation increased ($P < 0.01$) the percentage of G0/G1 phase cells (92.7 ± 0.86) as compared to cycling cells (39.7 ± 0.86) or cells cultured to confluency (87.3 ± 0.86). DMSO included for 48 h in the culture significantly increased ($P < 0.01$) the percentage of G0/G1 phase of the cell cycle at all concentrations used and decreased percentages of S phase in a dose-dependent fashion. Roscovitine increased the G0/G1 phase of the cell cycle ($P < 0.01$) at 15 μM concentration. Interestingly, the G2/M stage significantly increased at 30 and 50 μM compared to the control and 15 μM ($P < 0.02$). The cell cycle of brown bear adult fibroblast cells can be successfully synchronized under a variety of culture conditions.

Keywords: Brown bear; Cell cycle; Flow cytometry; DMSO; Roscovitine

1. Introduction

Assisted reproductive technologies have been used to help in the preservation of endangered or threatened animals such as the African wild cat (Gomez et al., 2003) and giant panda (Han et al., 2003; Spindler et al., 2004; Hori et al., 2006). Although nuclear transfer raises controversial questions in its applications to wildlife conservation (Holt et al., 2004), the potential of this technology as a valuable tool for aiding in the conservation of some endangered and threatened animals should not be ignored. In Spain, the Cantabric brown bear (*Ursus arctos pyrenaicus*) is at risk of extinction with a population estimated at only 100 animals. Efforts have been made to protect the declining environmental conditions and to preserve semen, cells and somatic tissues.

When populations or sub-population are at risk of extinction, nuclear transfer may be a valuable approach for species restoration (Gomez et al., 2006). Somatic cell nuclear transfer (SCNT) has been successfully applied in domestic and laboratory animals and in wild animals (Gomez et al., 2003; Loi et al., 2001; Williams et al., 2006). The control of cell cycle stage of donor cells is a relevant factor in the development of SCNT embryos. Differences in DNA content of donor nuclei vary according to the phase of the cell cycle and may affect the interaction with the recipient cytoplasts. Researchers have used different approaches to synchronize the cell cycle of the donor cells, among them, cell confluency-contact inhibition

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(Hinrichs et al., 2006) and serum starvation (Li et al., 2003). In addition, chemical inhibitors have been used such as roscovitine (Gibbons et al., 2002), dimethyl sulfoxide (DMSO) (Hashem et al., 2007), butyrolactone I (Kues et al., 2000), aphidicolin (Collas et al., 1992), demecolcine (Li et al., 2005), Hoechst 33342 (Kühholzer and Prather, 2001), mimosine (Vacková et al., 2003) or colchicine (Lai et al., 2001) that result in cell cycle arrest at specific points. However, no work has been done on the control of the cell cycle stages in brown bear. The aim of this study was to assess by flow cytometry the cell cycle of brown bear fibroblast cells cultured under a variety of cell cycle-arresting treatments.

2. Materials and methods

2.1. Establishment and culture of fibroblast cells

Skin biopsies were taken in Cantabria (Spain) from a live, anaesthetized brown bear (*Ursus arctos*). A procedure to obtain culture and cryopreserve skin-derived fibroblasts from brown bears has been described (Caamaño et al., 2005). Briefly, two skin biopsies were taken from the inner thigh. Biopsies were manually cut into small pieces, mixed together and enzymatically digested with collagenase Type IV (300 units/mL) (Sigma C5138) for 14 h at 38 °C. Disaggregated cells were centrifuged at 1600 rpm for 10 min and the pellet was diluted with D-MEM (Sigma D5671) containing 10% Fetal Bovine Serum (FBS). Cells were placed in a 25 cm^2 flask for culture under 5% CO_2 in air and high humidity at 38 °C. Confluent fibroblast monolayer was obtained after five days in culture. Two to four passages were performed using 75 cm^2 flasks before freezing fibroblasts in D-MEM containing 10% DMSO and 10% FBS.

2.2. Flow cytometric analysis

DNA content and cell cycle analysis were performed by flow cytometry. Cell suspensions and DNA staining with propidium iodide were performed following an optimized method based on the Vindelöv technique (Vindelov et al., 1983). Samples were analyzed in a Cytomics FC-500 cytometer (Beckman Coulter). A total of 20,000 cells per sample were collected by using a 488 nm excitation and a 605–635 nm bandpass filter. Cells were gated on forward light scatter versus side light scatter such that only cells without debris were assayed. The DNA histogram analysis was performed prior to manual elimination of aggregates by Modfit LT 3.0 software (Verity Software House), and the percentages of cells existing within the various phases of the cell cycle were automatically calculated by the program with the same algorithm in all the samples.

2.3. Cell treatments

In experiment 1, thawed fibroblast cells (second passage) were seeded in three 25 cm^2 flasks (8.5×10^5 cells/flask). After 24 h in culture, fibroblast cells were exposed to one of three treatments: (1) cells cultured to 70–80% confluency (cycling cells), (2) cells cultured to 100% confluency and then cultured for an additional five days (contact inhibition) or (3) cells cultured in serum-starved conditions for five days.

Fibroblast cells were exposed to cell cycle inhibitors and dose–response experiments were performed for each of the chemicals, DMSO and roscovitine. In experiment 2, cells were seeded in four 75 cm^2 flasks at a concentration of 1.25×10^6 cells/flask and cultured for 24 h. The culture medium was removed and replaced with medium containing DMSO (Sigma D5879) at 0%, 1%, 2% or 3% for 48 h. In experiment 3, thawed fibroblast cells were seeded in four 75 cm^2 flasks at a concentration of 1×10^6 cells/flask and cultured for 24 h. The culture medium was removed and replaced with culture medium containing roscovitine (Sigma R7772) at a concentration of 0, 15, 30, or 50 μM and cultured for another 24 h. In all the above experiments, cells were cultured under the same conditions (5% CO_2 in air and high humidity at 38 °C).

2.4. Statistical analyses

The above experiments were replicated three times. In each experiment, cells from each replicate were analyzed by flow cytometry on separate occasions. On each occasion, two samples of each treatment were analyzed ($n = 6$). Statistical analysis was performed by using the GLM procedure of SAS. Differences between treatments were determined by using LSM and were considered significant when $P < 0.05$.

3. Results

In experiment 1, the percentages of G0/G1 and S phase cells differed under different growth conditions. Serum starvation for five days increased ($P < 0.01$) the percentage of G0/G1 phase cells as compared to cycling cells or cells cultured to confluency. Cells cultured to 70–80% confluency contained higher ($P < 0.01$) percentages of S and G2 + M cells compared to cells cultured to confluency or serum starved, respectively (Table 1 and Fig. 1).

Cell cycle inhibitors, DMSO and roscovitine, were added to the cultures in an attempt to synchronize adult fibroblast cells in G0/G1 phase of the cell cycle. In experiment 2, DMSO included for 48 h in culture significantly increased ($P < 0.01$) the percentage of cells in G0/G1 phase at all concentrations used. However, only DMSO at 3% showed an

Table 1
Percentages (±SD) of brown bear fibroblasts existing in the various phases of the cell cycle after treatment with different growth conditions

Treatment	Cell cycle phase		
	G0/G1	S	G2/M
70% Confluency	39.6 ± 3.4^a	35.1 ± 2.3^a	25.2 ± 4.4^a
100% Confluency (contact inhibition)	87.2 ± 1.0^b	8.3 ± 2.1^b	4.4 ± 1.1^b
Serum-starved	92.7 ± 0.9^c	1.7 ± 0.4^c	5.5 ± 0.8^b

[a,b,c] Values within a column having unlike superscripts are different ($P < 0.01$).