Cryopreservation can induce transitory changes in the morphology and differentiation markers of rhabdomyosarcoma cells

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Summary

We investigated whether cryopreservation modifies the biochemical and morphological parameters of differentiation (creatine kinase isoenzymes and desmin and vimentin molecules) in rhabdomyosarcoma cells, used as an experimental model to analyse myogenic differentiation mechanisms. Following cryopreservation of two rhabdomyosarcoma cell lines (A-673 and A-204) using DMSO, the analyses revealed changes in the expression of differentiation markers over the first three days of culture. Increases in the expression of creatine kinase-MM and creatine kinase-MB fractions and in desmin were accompanied by a decrease in vimentin and creatine kinase-BB expression in both rhabdomyosarcoma cell lines. However, no modifications were found in the same period when glycerol was used to cryopreserve the cells. On the fourth day of culture after cryopreservation with DMSO a normal pattern of expression of these molecules was found. Our analyses demonstrate that the cryopreservation procedure may modify the degree of differentiation in rhabdomyosarcoma cells and suggest that this effect may be induced by DMSO. These results are of interest to those working with myogenic cells, in particular those studying differentiation mechanisms.

Key words: Myogenic differentiation - creatine kinase isoenzyme - intermediate filaments - rhabdomyosarcoma cells

Introduction

The polar solvent dimethyl sulfoxide (DMSO) is widely used to cryopreserve cell lines. However, this agent is also a potent stimulator of differentiation in some tumoral cell cultures (Momoi et al., 1986; Morley and Whitfield, 1993) including some rhabdomyosarcoma cell lines, in which DMSO is able to induce an evident differentiating effect (Prados et al., 1993).

Because most rhabdomyosarcoma cells can be induced to differentiate terminally, showing the morphological features of normal muscle differentiation (Erlandson, 1987), these cell lines have been used as an in vitro model to study the myogenic differentiation mechanism and to search for specific markers of diagnostic value (Fernández et al., 1992; Melguizo et al., 1994). Prados et al. (1993) showed that desmin is a good marker of the degree of differentiation in rhabdomyosarcoma cell lines RD, A-673 and A-204. Increased levels of this intermediate filament protein have been related to well-differentiated tumors in muscle cell lines (Molenaar et al., 1985; Sejersen et al., 1993). Moreover, some modifications in the pattern of creatine kinase (CK) expression, which presents three forms (isoenzymes) CK-BB, CK-MB, and CK-MM (Apple and Billadello, 1994; Moss et al., 1989), have been related to changes in the degree of myogenic differentiation (Fernández et al., 1992; Perriard et al., 1989). Recently, another form of this enzyme named macrocreatine kinase (MCK) has been discovered on the mitochondrial membrane of heart, liver and brain cells (Payne and Strauss, 1994).

This paper reports laboratory findings of transitory changes, after cryopreservation, in the

Submitted: February 2, 1998
Accepted: June 22, 1998

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expression profile of CK isoenzyme fractions and desmin (used as markers of myogenic differentation) in rhabdomyosarcoma cell lines A-673 and A-204. This finding may be important in research into myogenic differentiation using rhabdomyosarcoma cell lines.

Materials and Methods

Cell lines and cultures

The rhabdomyosarcoma cell lines used in this study were A-673 and A-204 derived from human embryonic rhabdomyosarcoma (American Type Culture Collection (ATCC), Rockville, MD). Cell line Hs 848, derived from normal striated muscle (ATCC), was used as a control. All cell lines were grown in Dulbecco´s modified Eagle´s medium, supplemented with 4.5 g/l glucose, 20 mM l-glutamine, 3.5 g/l sodium bicarbonate, and 10% fetal bovine serum.

Cryopreservation method

For the cryopreservation procedure, cells were harvested, centrifuged at 600 g for 5 min, and washed twice in PBS.Two solutions were prepared and cooled (4°C) to cryopreserve the cell lines: a solution of 10% glycerol (1.4 mol/l) in 90% fetal calf serum and a solution of 10% DMSO (1.4 mol/l) in 90% fetal calf serum. 1.5 ml of these solutions was added to cooled vials containing 10^6 cells, which were quickly frozen at -20°C for 2 h, and then stored overnight at -80°C in liquid nitrogen.

Scanning electron microscopy

Adherent cells on coverslips were fixed for scanning electron microscopy (SEM) with 2.0% glutaraldehyde, dehydrated in graded concentrations of ethanol, and dried using the critical point method. These preparations were coated with platinum and observed with a Hitachi S-800 scanning electron microscope.

Electrophoretic analysis of CK isoenzymes

10 x 10^{10} cells were resuspended in 1 ml PBS and fractionated by up to five rapid cycles of freezing (liquid nitrogen, 15 s) and thawing (room temperature, 1.5 min). The samples were microcentrifuged at 300 g for 3 min, and the liquid suspension fraction containing the molecule of interest was gently pipetted off. A kit specifically designed for CK isoenzymes (Paragon Electrophoretic System, Beckman) was used according to the manufacturer´s instructions (Beckman Instructions 015-556461-L) and the results were evaluated visually under an ultraviolet light source or scanned with a suitable fluorescence densitometer (Appraise, Beckman).

SDS-PAGE and densitometric analysis of intermediate filament proteins

10 x 10^{10} cells were homogenized according to Lewis and Gonzalez (1987) and Lewis et al. (1988) with a buffer containing 1 ml 0.1% Triton X-100 (Sigma), 2 mM Tris, 0.2 mM $CaCl_2$, 0.2 mM ATP, and 0.2 mM 2-mercaptoethanol (2-ME), pH 8.0 (all reagents BioRad Laboratories, Richmond, CA). The homogenate was centrifuged at 13,000 g for 5 min. The supernatant contained cytoplasmic (CP) proteins. The pellet, which contained cytoskeletal (CS) proteins, was washed in the above buffer without Triton X-100, spun at 13,000 g for 5 min and resuspended in 500 µl Laemmli sample buffer without bromophenol blue or 2-ME. This suspension was boiled for 5 min to obtain a supernatant that contained cytoskeletal proteins from cells. Aliquots of the Triton X-100- and SDS-soluble fractions were added to appropriate volumes of Laemmli sample buffer for SDS-PAGE. After polyacrylamide gel electrophoresis of CS and CP subfractions in the presence of SDS (Laemmli, 1970), the gels were transferred to nitrocellulose membranes (BioRad) (Towbin et al., 1979). After a wash with 10 mM TBS (pH 7.4) in 0.05% Tween 20, the membranes were incubated with commercial mAbs against vimentin and desmin (Sigma, St. Louis MO, USA) proteins for 3 h at room temperature. Positivity was detected with horseradish peroxidase-conjugated goat antibody to mouse immunoglobulin (IgM) (Sigma) and developed with 4-chloronaphthol. The bands representing vimentin and desmin were analyzed by densitometry at 550 nm (slit width 4 mm; Beckman Appraise) with the help of an integral computing device. The amounts of protein in the polyacrylamide gels were calculated from the densitometric values as described in Aránega et al. (1991).

Results

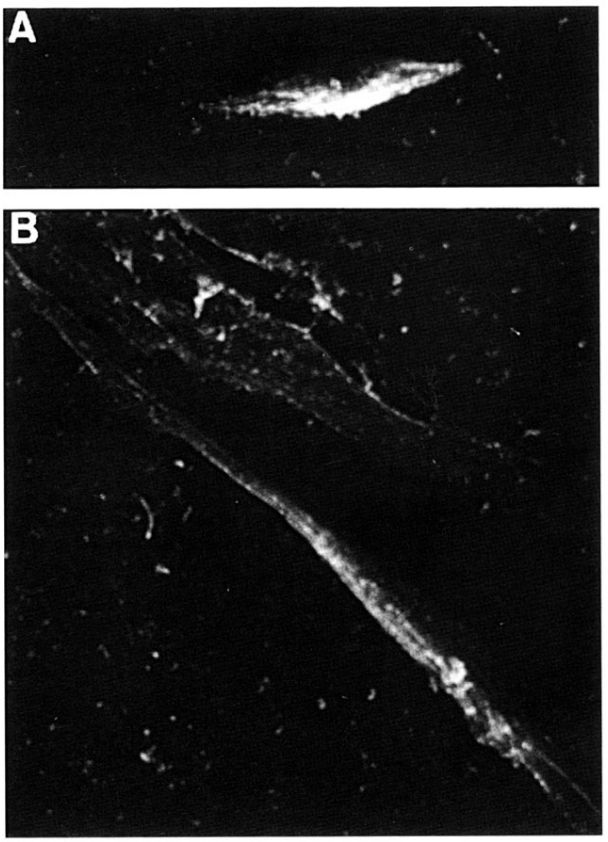
In vitro morphology

Scanning electron microscopy observations on the fourth day after cryopreservation with DMSO showed that A-673 and A-204 cells have the characteristics of undifferentiated cells (Fig. 1A), growing as small, spindle-shaped mononuclear cells and forming confluent aggregates under standard conditions. However, during the first three days after this cryopreservation procedure, most of the populations of both rhabdomyosarcoma cell lines showed an evident increase in the proportion of elongated cells (Fig. 1B), growing in parallel with an increase in cytoplasm, which adhered strongly to the substrate. Some cells developed many branching processes which gave them a characteristic neurite-like appearance (data not shown). Neither of the

rhabdomyosarcoma cell lines showed any changes in their in vitro morphology during the first four days after the cryopreservation procedure with glycerol (data not shown).

Analysis of CK isoenzymes

On the first day of culture after cryopreservation with DMSO, A-673 cell cultures contained 24% CK-MM, 16.2% of CK-MB, and the largest fraction (59.6%) was CK-BB. Line A-204 contained 11.8% of MCK-1, a small fraction (10%) of CK-MM, while the largest fraction found (55.3%) was CK-BB. A large amount (23.2%) of CK-MB was also detected (Table 1). In both cell lines, this modified distribution of CK isoenzymes persisted along the three first days of culture, but on the fourth day the pattern returned to normal proportions (Fernandez et al., 1992). In line Hs 848 cryopreserved with DMSO, and also in all cell lines cryopreserved with glycerol, CK isoenzyme expression did not change substantially during the four days of culture (Table 1).



Modifications of intermediate filaments

In line A-673 and A-204, in the cytoplasmic fraction no significant changes in desmin were observed during the three first days after cryopreservation with DMSO (1.320 µg/µl and 0.650 µg/µl respectively). However, desmin expression decreased on the fourth day after this cryopreservation procedure to reach basal levels (0.930 µg/µl and 0.570 µg/µl respectively). A similar pattern of changes was found for the cytoskeletal fraction. This protein showed little change during the three first days after cryopreservation and decreased on the fourth day (Fig. 2A and B). Analysis of vimentin expression in both A-673 and A-204 cell lines showed that this

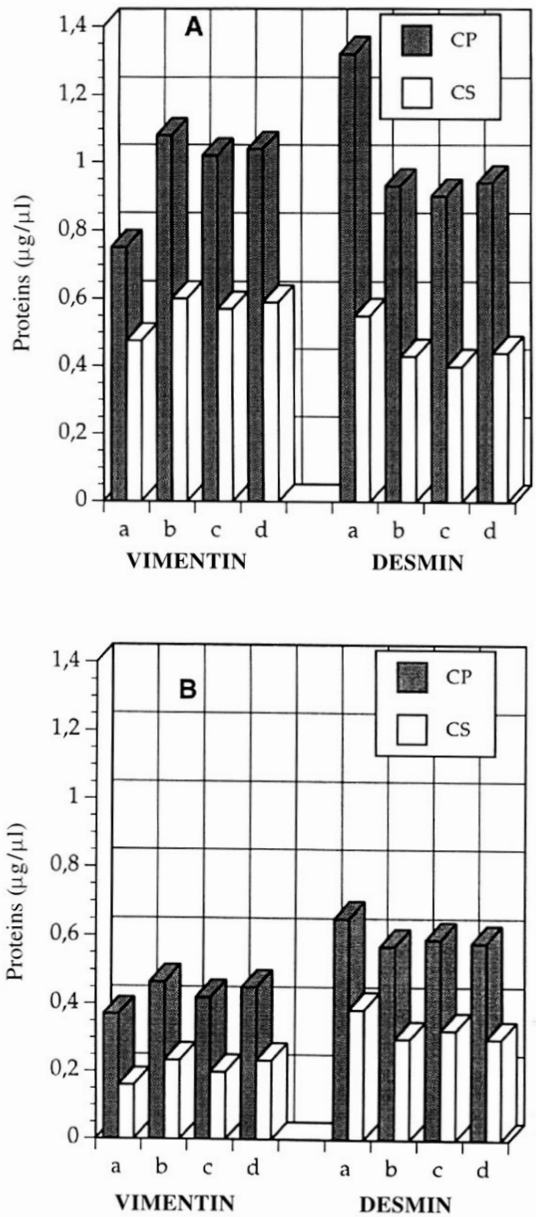
Fig. 1.– Representative scanning electron micrographic image of a human rhabdomyosarcoma cell line showing morphological changes caused by cryopreservation. **A**, Scanning electron micrographic image of a small spindle-shaped mononuclear A-673 cell under standard growth conditions on the fourth day after cryopreservation (x 500). **B**, Scanning electron micrographic image of an elongated A-673 cell under standard growth conditions on the second day after cryopreservation (x 500).

Table 1.– Electrophoretic analysis of creatine kinase isoenzymes in A-673, A-204 and Hs 848 cell lines on the first and fourth days (basal levels) after cryopreservation.

			CK-MM	CK-MB	CK-BB	MCK1
A-673	DMSO	a	24.0±2.4	16.2±3.2	59.6±2.9	--
		b	12.0±3.2	2.1±1.5	85.9±3.1	--
	Glycerol	a	12.5±2,3	2.2±2.6	84.5±2.4	--
		b	14.3±3.0	1.9±1.3	83.0±3.0	--
A-204	DMSO	a	10.0±1.8	23.2±3.1	55.3±2.6	11.8±3.4
		b	17.6±2.8	1.7±1.5	74.4±2.8	6.3±2.1
	Glycerol	a	15.8±1.9	1.5±1.2	75.1±2.8	6.4±2.4
		b	16.5±3.4	1.5±1.0	75.6±2.4	6.4±2.4
Hs 848	DMSO	a	95.2±3.5	4.8±3.0	--	--
		b	98.0±3.1	1.0±1.0	--	--
	Glycerol	a	97.4±2.4	2.5±1.3	--	--
		b	95.5±2.4	3.6±1.2	--	--

All data are means ± SEM of four measurements. The significance of the differences was determined by comparison of the means (Student's t test) between the first and fourth day of culture. First day (a) and fourth day (b) after cryopreservation

protein remained unchanged in the cytoplasmic fraction along the first three days after cryopreservation (0.750 µg/µl and 0.370 µg/µl respectively). This protein increased and reached basal levels on the fourth day after cryopreservation (1.080 µg/µl and 0.465 µg/µl respectively). The effects of the cryopreservation method with DMSO on vimentin expression in the cytoskeletal fraction were similar to those observed for the cytoplasmic fraction (Fig. 2A and B). Desmin and vimentin expression in the cytoplasmic and cytoskeletal fractions in both A-673 and A-204 cell lines did not show significant changes during the first four days after the cryopreservation procedure with glycerol. Studies on CK isoenzyme distribution and desmin and vimentin expression carried out at different times of culture after cryopreservation with DMSO and glycerol (5, 10 and 20 days) revealed no changes in relation with the fourth day after cryopreservation (data not shown).



Discussion

During normal myogenesis, the concentrations of CK-MM and CK-MB isoenzymes increase steadily, such that these forms gradually replace the BB fraction, which is more characteristic of embryonic tissue (Perriard et al., 1989; Apple and Billadello, 1994). Desmin expression increases and vimentin expression decreases as differentiation progresses in rhabdomyosarcoma cells (Carter et al., 1989; Coindre et al., 1989, Fernández et al., 1991; Wijnaendts et al., 1994). Desmin, vimentin and CK-BB have thus been considered good markers of myogenic differentiation (Dias et al., 1987; Fernández et al., 1992).

In rhabdomyosarcomas, a higher proportion of well-differentiated tumor cells stain for desmin, whereas most poorly-differentiated tumors are negative for it (Dias et al., 1987). Increased vimentin expression has been reported in primitive rhabdomyosarcomas, and loss of expression has been considered evidence of differentation in this type of tumor (Melguizo et al., 1994; Molenaar et al., 1985). The inverse relationship between desmin and vimentin expression in cell lines A-673 and A-204 during the first three days of culture can also be interpreted as evidence of progressive differentiation. However, these modifications are transitory since CK concentrations return to basal values on day four of culture (Fernández et al., 1992), and desmin expression declines while vimentin expression increases, indicating a process of dedifferentiation. Our findings suggest that the rhabdomyosarcoma cell lines analyzed here may spontaneously modulate their degree of differentiation after cryopreservation. In line Hs 848 we observed no changes in the expression of these CK isoenzymes during the four days of culture immediately after cryopreservation. This lack of modulation may have been because this line originates from normal striated muscle cells, which are already highly differentiated.

DMSO, a polar solvent used in the cryopreservation procedure of cell lines, is also a specific cell differentiation agent (Morley and Whitfield, 1993; Melguizo et al., 1994). This agent has been shown to induce progressive reorganization of the cytoskeleton, which was fully deve-

Fig. 2.– Changes in intermediate filament protein expression in the cytoplasmic (CP) and cytoskeletal (CS) fractions of rhabdomyosarcoma cell lines A-673 (**A**) and A-204 (**B**) after cryopreservation. Vimentin and desmin were quantified in µg/µl from the densitometric values according to the formula: amount of protein in the sample used for SDS-PAGE (1µg/µl) x densitometric value of the protein-containing band, divided by 100. The significance of the differences was determined by comparison of the means (Student's t test) between the first and fourth day of culture. First (a) and fourth day (b) after cryopreservation with DMSO; First (c) and fourth day (d) after cryopreservation with glycerol.

loped after four days of continuous exposure (Lampugnani et al., 1987). The reason for the changes in the expression of differentiation markers in rhabdomyosarcoma lines after cryopreservation is unknown, although they may be related to the presence of DMSO in the freezing medium because cryopreservation with glycerol, used in our experiments as a control, did not modify the expression of differentiation markers. In fact, protein expression in both A-673 and A-204 rhabdomyosarcoma cell lines was significantly altered by short treatment with DMSO without cryopreservation (Melguizo et al., 1994). These experiments showed that this agent increased desmin and decreased vimentin expression, clearly indicating that it induces changes typical of differentiation in rhabdomyosarcoma cells. In this regard, Prados et al. (1993) have described modifications in the expression of different proteins such as actin, a-actinin and tropomyosin in rhabdomyosarcoma cell lines after exposure of cells to DMSO for only 3 h, supporting the hypothesis that this agent may be responsible for the cell differentiation observed after cryopreservation with DMSO.

In conclusion, we have found that cryopreservation may modify the degree of differentiation in rhabdomyosarcoma cell lines during the first three days of culture after thawing. This effect may be induced by the DMSO in the culture medium, and may alter the results of experiments with these cells during this period of culture.

Acknowledgments

We thank Ms Carmen Amézcua for her competent technical assistance, and Ms Karen Shashok for translating parts of the original manuscript into English. This work was supported by the Fondo de Investigaciones Sanitarias de la Seguridad Social (FIS) through Project no. 95/0974. The award of a grant (97/5436) from the FIS to J. A. M. is acknowledged.

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