DETERMINING OPTIMAL TRANSPORT CONDITIONS OF IN VITRO CULTURED HUMAN CHONDROCYTES PREPARED FOR AUTOLOGOUS RE-IMPLANTATION USING FLOW CYTOMETRY

G. WOZNIAK*, N. VELIKONJA*, M. KNEŽEVIC*, M. URBAJS*, M. JERAS*, D. RADOŠAVLJEVIĆ† AND P. ROŽMAN*

*Blood Transfusion Centre, Šlajmerjeva 6, Ljubljana, Slovenia
†Educell d.o.o., Teslova 30. 1000 Ljubljana
‡University Medical Centre, Department of Orthopaedic Surgery, Zaloška 9, Ljubljana, Slovenia

1. Introduction

Re-implantation of in vitro cultured chondrocytes is one of the most promising methods for curing deep aseptic lesions of articular cartilage. After cultivation, the cell suspension is submitted to a department of orthopaedic surgery. Transport conditions should guarantee over 90% viability of the shipped cell quantity after 72 hours. Traditionally, trypan blue exclusion test was performed in determining viability and even apoptosis of cultured cells. However, only a limited number of cells can be counted comparing to the size of the sample that can be inspected by flow cytometer analysis. Many flow cytometry studies have focused on the investigation of features of cells that have either died by apoptosis or by necrosis [2, 3]. Non-viable cells can be distinguished from live cells on flow cytometer due to changes in scattering properties and can be excluded from measurements only by proper gating [3]. When cells are cultured in vitro and undergo a severe processing procedure such as trypsinization before measurements, these differences are not clear enough to permit accurate determination of viability. Various fluorescent indicators of membrane integrity have been used for improved dead cell discrimination [1]. Propidium iodide is the most widely used non-vital DNA dye. Like trypan blue, it enters only permeabilised cells. It labels dead cells' DNA, intercalating into nucleic acid molecules. Fluorescein diacetate, on the other hand, as a near neutral molecule diffuses freely into the cell. Once inside, it is converted by intracellular esterases into a fluorescent product that is retained in cells with intact plasma membranes. Both substrates and their products leak rapidly from cells with damaged membranes.

In the assay, we attempted to optimise a procedure for determining viability of cultured chondrocytes intended for autologous transplantations. Double labelling with propidium iodide and fluorescein diacetate was used to differentiate between viable and non-viable cells. The viability was then the criterion for choosing optimal transport medium and temperature for the cell transport. In addition, for any combination of transport factors tested the level of apoptosis was determined using annexin V labelling.

2. Materials and methods

Primary cultures of human articular chondrocytes were established in F12/DMEM supplemented with 15% fetal bovine serum, gentamycin, fungizone and ascorbic acid. The cells were harvested in early passages (3-5) of the culture when grown to approximately 80% confluency. The cells were trypsinized and filtered through 40 μm nylon mesh cell strainers. Transport conditions were simulated in 1.7 ml plastic eppendorf tubes containing 500μl of transport medium with at least 1x 10^6 cells. They were kept at 20-25 or 37 °C for several days. The transport media tested were F12/DMEM free of serum and F12/DMEM supplemented with 10 or 20% FBS. Cell viability was determined after 2, 3 and 6 days of storage.

The reagents were prepared as follows: a 10 mg/ml stock solution of propidium iodide (PI) was prepared in 1x PBS and stored at 4 °C. It was further diluted immediately before use. One mg of fluorescein diacetate (PDA) powder was dissolved in ice-cold acetone. A fresh stock was prepared for each experiment immediately before labelling. For determination of apoptosis, we used a commercially available FITC conjugate of annexin V(Becton Dickinson). For negative control unlabelled reagent was used.

Fluorescent staining of least 1 x 10^5 PBS-washed cells was performed in a 12 x 75 mm-tube. Then 100 μl PBS with the addition of 1 μl of FDA stock solution was added and mixed well. Cells were then incubated and protected from light for 15 min at 37°C. For staining of DNA, cells were added a solution of RNase (17.6 Kunitz units = 200μg/ml) and PI (5 μg/ml) respectively. All samples were kept at 4°C in the dark until analysed by flow cytometer. Finally, 1x10^5 cells were washed in 1xPBS buffer and then resuspended in 100 μl annexin V binding buffer. Labelling with annexin V was performed exactly as instructed by the manufacturer (Becton Dickinson).

Samples were then analysed on a FACScan flow cytometer (Becton Dickinson Immunocytometry Systems). Green FDA and annexin V-FITC fluorescence was collected after a 530/30 nm band-pass filter. Orange emission from PI was filtered through a 585/42 nm band pass filter. Residual spectral overlap was then removed. Photomultiplier tube voltage and spectral compensation were set initially using single FDA or PI stained cells. Settings were then further optimised on samples that were stained with both dyes to compensate for dye-dye interactions. FDA and PI fluorescence were shown on four decade log scales. A minimum of 3000 events were evaluated in each sample. All tests were done at least eight times and the significance of each transport factor studied was determined using Mann-Whitney U-test.

3. Results and discussion

Forward versus side scatter dot plots clearly showed that scatter discrimination between live and dead cells was clear-cut as the overlap of live cells with dead cells was minimal. The gating was more problematic in populations with low viability, where numerous dead and vitaly deteriorated cells caused shading in the lower values of side scatter.