A tag-less method for the direct isolation of HUVEC (human umbilical vein endothelial cells) by gravitational field-flow fractionation as a starting point for gene expression analysis

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ABSTRACT

HUVECs (human umbilical vein endothelial cells) are a model for prediction of endothelial dysfunction in various diseases. Cultured cells seem to be a good model for studying molecular processes in endothelial tissue. Nevertheless, they cannot reproduce real physiological status because some biological factors necessarily change in culture conditions. As a consequence, it would be useful to improve preparation of the cellular starting materials from real samples, especially when gene expression studies are further required on the cells. In this work we present a new method for the easy isolation of HUVECs from raw cord blood based on the use of the flow-assisted separative technique, gravitational field-flow fractionation (GrFFF) able to give a relatively pure sample for gene analysis without cell culture steps. The method can be applied to cell populations enzymatically removed from the umbilical vein which can be purified from contaminants cells and used directly for gene expression analysis.

Keywords: gene expression analysis, cell separation, gravitational field-flow fractionation (GrFFF), tag-less cell sorting, human umbilical vein endothelial cells (HUVEC)

1 INTRODUCTION

Huvec (Human umbilical vein endothelial cells) is one of the better model system for investigating on certain patho-physiological conditions of different types of endothelial cells with the aim to predict endothelial dysfunction in adult life. Abnormalities of endothelial cell structure and function may contribute significantly to diseases of blood vessel walls such as thrombosis, atherosclerosis and vasculitis. Since these cells have many relevant roles in these pathologies, its study, and in particular gene expression study, results very important for understanding the patho-physiology of endothelial diseases.

Nowadays the most common approach for its isolation from the human umbilical vein is the enzymatic removal with. a good yield but an heterogeneous cells population composed by Huvec and contaminants such as red blood cells (RBC) and fibroblasts can be obtained [1]. At the moment, these two populations can be negatively selected by in vitro passages. In this way these cells can grow in tissue culture as a homogeneous population and can be used to obtain a reasonable amount of cells able to simulate a model for studying endothelial cells. Nevertheless, they can't reproduce real physiological status because some biological factors necessarily change in culture conditions.

Another approach for cell separation is represented by cell sorter: this method required a starting number of cell superior then that can be isolated from the human umbilical vein.

In the end, MACs separator is another way of cell separator. Its limit is that the marker of Huvec is CD31, the same of the lymphoid cells. So also this method can't be useful in order to obtain a pure sample of the cells of interest. As a consequence, it would be useful to improve preparation of the cellular starting materials from real samples using different techniques, especially when gene expression studies are further required on the cells.

In this work, it is presented a method based on gravitational-field flow fractionation (GrFFF) for the gentle, tag-less isolation of Huvec from raw umbilical cord. GrFFF belongs to field-flow fractionation techniques (FFF) [2,3]. The method can be applied directly to samples obtained after an enzymatic treatment and the fractionation process depends on sample morphology. The selective fractionation of Huvec can be obtained due to the different morphology from contaminant cell; as a consequence, a sample highly enriched in viable Huvec can be obtained in a relative short time, as confirmed by flow cytometry analysis on fractionated cells. Since the high reproducibility of fractionation process, a precise and useful number of selected cells can be obtained by cumulating cells from the injection of different amounts of cell suspension. The sample results suitable for gene expression analysis, a good amount of purified RNA can be prepared.

1.1. Gravitational field-flow fractionation

Among separative techniques, FFF is a family of analytical techniques which is able to sort living cells. Cell

fractionation is achieved through a relatively simple device (an empty capillary cannel) by the combined action of a transporting laminar flow and a field that is applied perpendicularly to the flow. GrFFF is the simplest FFF variant employing Earth's gravity as applied field, and it was able to distinguish morphological and biophysical differences within different cellular (sub)populations. GrFFF-based isolation of human hematopoietic stem cells from patient blood apheresis, and purging of neoplastic from normal lymphocytes have already shown [4]

Sample mixtures from raw sample are injected into the channel inlet through an injection port with a syringe. The sample preparation consisting in a sample dilution is requested and the mobile phase composition can be modulated on a basis of needs; usually, cell culture medium or physiologic buffer can be chosen. The mobile phase flow inside the channel is usually delivered by a peristaltic pump. A detection system with a flow-through cell is connected downstream of the channel outlet for online recording of the signal generated by the eluted analyte (the fractogram). Most commonly used detection systems are the ultraviolet–visible spectrophotometric detectors for LC. A fraction collector can be positioned downstream of the detection system to collect fractionated analytes. Due to the "soft" separation principle cell viability and native properties are fully preserved after fractionation, and so-sorted cells can be collected and reused or studied. Due to the biocompatibility and simple use, the GrFFF separative device can be easily integrated within assessed procedures into clinical laboratories for specific cell preparations. Moreover, the low cost of the separative device may allow for its disposable use with the advantages to avoid carry-over among consecutive samples.

2 GrFFF APPROACH TO HUVEC ISOLATION

The study was performed at the Department of Mother and Infant Sciences, University of Milan, Foundation IRCCS Ca' Granda Ospedale Maggiore Policlinico and all patients gave informed consent. Twenty-five umbilical cords were obtained from pregnancies which underwent to elective caesarean section not related to fetal growth (breech presentation or maternal request). The inclusion criteria were absence of maternal, placental, or fetal pathologies.

2.1. Cells suspension

Endothelial cells were obtained from umbilical cord vein by the following method.

The umbilical cord was severed from the placenta soon after birth, placed in a sterile container and held at 4°C until processing. Under sterile conditions, the two ends of umbilical cord were cut off and the vein was perfused with about 100 mL of physiological buffer to wash out the blood and allow to drain. Then it was infused with 0,1% collagenase A (Roche, Milan, Italy). The umbilical cord, suspended by its ends, was placed in a water bath containing physiological buffer and incubated at 37°C for 8 min. After incubation, the collagenase solution containing the endothelial cells was flushed from the umbilical cord by perfusion with about 30 mL of sterile phosphate-buffered saline (PBS, Sigma, St. Louis, MO). The effluent was collected in a sterile 50 mL conical centrifuge tube and sedimented at 463 xg for 15 min. The pellet was suspended in 1 mL of medium 199 (Sigma, St. Louis, MO) supplemented with 20% fetal bovine serum (FBS, Sigma, St. Louis, MO), 3% penicillin/streptomycin (200u/mL, Sigma, St. Louis, MO), 200µl eparin (25000 UI/5mL IV 1F, Hospira Italy) and 1% L-glutamine (Sigma, St. Louis, MO), in order to be counted at Burker chamber. The cell suspension was centrifuged again at 463 xg for 6 min and suspended in complete culture medium so as to have a concentration of 4x106 cells/350 µl to allow GrFFF experiments [5].

2.2. GrFFF fractionation of cell suspension

The fractionation device was filled with sterile mobile phase consisting of PBS added with 0.1 % (w/v) BSA, 1% EDTA and 1% penicillin-streptomycin. The cell mixture obtained after enzymatic treatment was resuspended in 350 µl of fresh culture medium just before the injection. Aliquots of 50 µl at a concentration of $4x10^6$ cells/mL were injected into the GrFFF system, the flow was activated at a flow rate of 0.25 mL min-1 for 15 s to make the overall sample enter the channel. Subsequently, flow was interrupted for 2 minutes to allow sample relaxation. Finally, sample elution was carried out at 1 mL min-1. Huvec are collected in the fraction eluted in the interval time (7-14 minutes).

Cells collected at the same elution time values from repeated runs were pooled and subjected to characterization by means a flow cytometry and to RNA isolation .

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A typical fractionation profile obtained for this cell sample is reported in Figure 1 where two resolved bands are present. By means of the injection of single cell types, it was possible to confirm that the retention times of the two main population are characteristic for Huvec and RBC respectively.

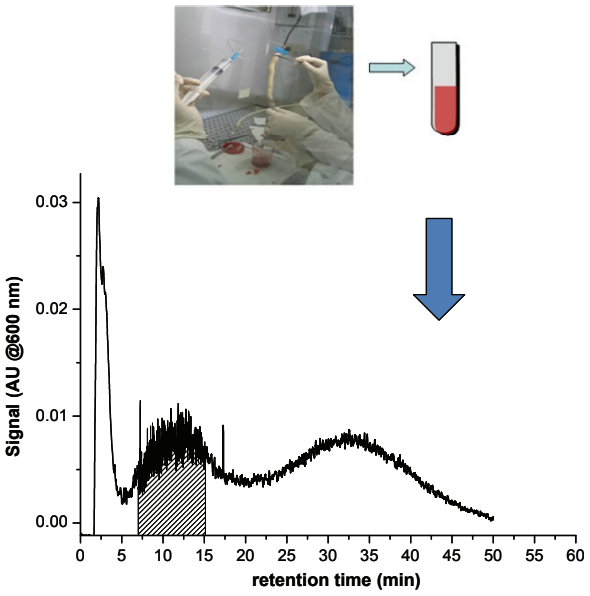

Figure1. Enzymatic digestion of a umbilical cord to obtain cell suspension. GrFFF fractogram of injected cell suspension with fraction collected (7-15 minutes).

The fraction from 7 to 15 minutes was collected and due to the high reproducibility of the GrFFF fractionation process (run-to-run %CV of retention times below 3% and day-to-day %CV below 10%), three and seven fractions from subsequent injections were added for flow cytometry (FC) characterization and RNA extraction respectively.
The FC analysis show that after the fractionation process a cell fraction with a well defined morphology and an high enrichment on cells CD31+ was obtained. A mean enrichment in CD31+ cells of 90%. with an high depletion on red blood cells was obtained. A representative result of FC analysis on collected fraction is reported in Figure 2.

In order to investigate if GrFFF technique makes the cell suffer or their vitality change we observed three different cell cultures: control cell population of not-fractionated Huvec, total cell population before fractionating and cell fractions. As usual we obtained the typical behaviour: adhesion in less than 48h and cells demonstrated the typical growth pattern that led to a sub-confluent culture in 5-7 days.

We used TriReagent to extract total Huvec RNA from seven fractions collected. We generated 4 RNA samples from 4 different umbilical cords. After precipitating total RNA for about a week we obtained 5340,5 ± 492.5 ng. We guess the yield after one month precipitation would be higher. Although there were some variations in the amount of RNA recovered among individuals, all samples provided sufficient RNA for subsequent microarray analysis. Nowadays new sensible techniques can afford an array to be done by 3000ng of total RNA. The quality of isolated RNA was acceptable from all samples generated.

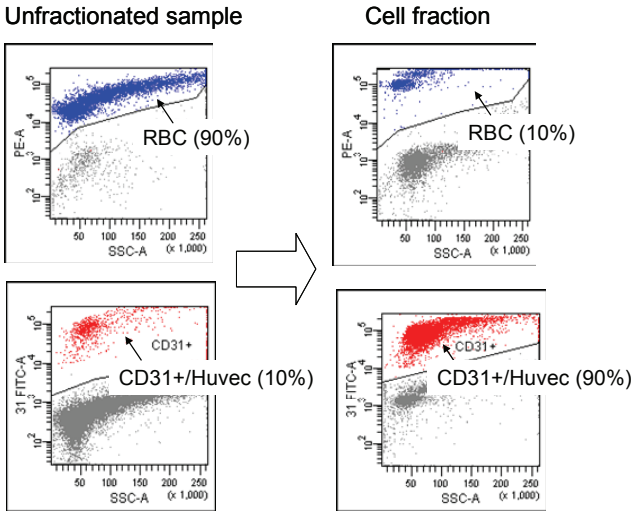

Figure 2. Flow cytometric analysis of unfractionated sample and collected fraction. Glycophorin-PE (RBC marker) *vs* SS (side scatter) dot plot and CD31-FITC (Huvec marker) *vs* SS (side scatter) dot plot.

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4 CONCLUSIONS

These results indicate promising use of GrFFF as a method to highly enrich Huvec population from raw clinical specimens. Obtaining a good amount of purified cells could avoid cell cultures and be useful for further studies such as gene expression analysis. The developed GrFFF method allowed obtaining high enrichment levels of viable Huvec in specific fraction and it showed to fulfil major requirements of analytical performance, such as selectivity and reproducibility of the fractionation process and high sample recovery.
The method and relevant technology can be easily implemented in biomedical laboratories with low instrumental and training investments.

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