Anal Bioanal Chem
DOI 10.1007/s00216-012-6337-4

ORIGINAL PAPER

A tag-less method for direct isolation of human umbilical vein endothelial cells by gravitational field-flow fractionation

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Received: 19 June 2012 / Revised: 28 July 2012 / Accepted: 6 August 2012
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Abstract The analysis of cellular and molecular profiles represents a powerful tool in many biomedical applications to identify the mechanisms underlying the pathological changes. The improvement of cellular starting material and the maintenance of the physiological status in the sample preparation are very useful. Human umbilical vein endothelial cells (HUVEC) are a model for prediction of endothelial dysfunction. HUVEC are enzymatically removed from the umbilical vein by collagenase. This method provides obtaining a good sample yield. However, the obtained cells are often contaminated with blood cells and fibroblasts. Methods based on negative selection by in vitro passages or on the use of defined marker are currently employed to isolate target cells. However, these approaches cannot reproduce physiological status and they require expensive instrumentation. Here we proposed a new method for an easy, tag-less and direct isolation of HUVEC from raw umbilical cord sample based on the gravitational field-flow fractionation (GrFFF). This is a low-cost, fully biocompatible method with low instrumental and training investments for flow-assisted cell fractionation. The method allows obtaining pure cells without cell culture procedures as starting material for further analysis; for example, a proper amount of RNA can be extracted. The approach can be easily integrated into clinical and biomedical procedures.

Keywords Tag-less cell sorting · Gravitational field-flow fractionation (GrFFF) · HUVEC (Human umbilical vein endothelial cells)

Introduction

Cellular and molecular profile analysis has become a routine tool in biomedical research for diagnostic, therapeutic and prognostic applications [1, 2]. Despite the high improvement

Published in the special issue *Analytical Science in Italy* with guest editor Aldo Roda.

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done for molecular analysis and data processing, the sample isolation from clinical specimens is still an open issue. An important challenge is the development of a simple method for cell isolation that would be able to preserve the physiological conditions of cells, thus maintaining all the native information without the introduction of methodological bias.

In this field, human umbilical vein endothelial cells (HUVEC) are one of the best model systems for investigating some physio-pathological conditions of different types of endothelial cells. Endothelial cells (EC) come into contact with all blood components such as nutrient substances including oxygen, biologically active molecules such as hormones, blood cells and drugs. It is well documented that EC play a crucial role in the regulation of blood flow and pressure [3] as well as blood coagulation [4]. Furthermore, EC are involved in inflammation [5], blood vessel formation [6] and are responsible for the bi-directional transmission of biochemical and physical information between the blood and other vascular cells (e.g. smooth muscle cell population) and peri-vascular tissues, respectively. With these features, EC take part in the development of multiple diseases like atherosclerosis, thrombosis, sepsis, and tumor invasion and growth [7].

HUVEC displays a particular potential in predicting endothelial dysfunction in adult life [8]. They play an important role in physiologic haemostasis, blood vessel permeability and in the response of the blood vessel to physiologic and pathologic stimuli. Abnormalities of endothelial cell structure and function may contribute significantly to diseases of blood vessel walls such as thrombosis, atherosclerosis and vasculitis. Their study, in particular gene expression study, is therefore very important for the understanding of the physiopathology of endothelial diseases. As an example, recently an RNA assay was shown able to detect inflamed endothelial cells circulating in whole blood of atherosclerosis patients thus providing proof-of-concept for the diagnosis of pathological inflammation and endothelial dysfunction in atherosclerosis [9]. Nowadays, the most common approach for HUVEC isolation with a good yield from the human umbilical vein is the enzymatic removal by e.g. collagenase A digestion. However, HUVEC often result to be contaminated with fibroblasts, red and white blood cells, platelets and possibly apoptotic bodies. These populations can be negatively selected by in vitro passages [10]. In this way, these cells can grow in culture as a homogeneous population and they can be used to obtain a reasonable amount of cells able to simulate a model for studying endothelial cells. Nevertheless, they cannot reproduce the real physiological status because some biological factors necessarily change in culture conditions. There is evidence for a number of apparent discrepancies between in vivo and in vitro EC. For example, the expression of CD34 (transmembrane molecule on human hematopoietic progenitor cells and on many EC types) is reduced in EC of the HUVEC according to cultivation time [11–13]. Furthermore, Kanda et al. revealed a decrease in the expression of the glucose transporter type 1 (GLUT1) in cultivated bovine peripheral nerve microvascular EC [14]. These cultivation-induced changes lead to the question of whether the energy metabolism in cultured EC is different from that in vivo [7]. In addictions, it is noteworthy that the effects of different drugs, or different growth factors, provide valuable information on the biological response of cells grown in different spatial organizations (2D and 3D organized cells) [15, 16].

In order to eliminate contaminants cells, another approach implies that cell population can be treated with selective lysis solutions; however, these approaches can be invasive for the target cells and they are not able to isolate pure cell samples.

Other methods currently used to isolate HUVEC are based on cell sorting. Flow-assisted cell sorting (FACS) is based on flow cytometry and it requires a high starting number of cells. Magnetic-activated cell sorting (MACS) is a proprietary method that allows cells to be easily sorted by the application of a magnetic field. MACS (and also FACS) limitation is that the HUVEC marker CD31 is shared also by lymphoid cells. Therefore, they cannot be used to obtain a pure HUVEC sample. As a consequence, it would be useful to improve preparation of the cellular starting materials from real samples [17, 18].

In this work, we present a new easy approach for the isolation of intact and unmodified cells from clinical specimens based on the tag-less gravitational field-flow fractionation (GrFFF) which belongs to the family of field-flow fractionation techniques (FFF) [19–21].

In GrFFF, cell fractionation is achieved through a relatively simple device (an empty capillary channel) by the combined action of a transporting laminar flow and a field applied perpendicularly to the flow. Among FFF techniques used for cell analysis [22], GrFFF is the simplest FFF variant employing Earth's gravity as applied field and it is able to distinguish morphological and biophysical differences within different cellular (sub)populations. Different types of living cells have been fractionated, such as human hematopoietic stem cells (CD34+) from patient blood apheresis and neoplastic from healthy lymphocytes [23, 24]. A proprietary variant of GrFFF has been also applied to sort human mesenchymal stem cells and obtain fractions with significantly enhanced commitment [25]. Compared to other cell sorting methods, GrFFF shows interesting features: the sample preparation consists in a simple dilution; the mobile phase used for cell fractionation can be chosen of any composition (e.g. cell culture media or physiologic buffers); the ancillary instrumental system required is relatively simple and economic. Due to the "soft" fractionation mechanism, cell viability and native properties are fully preserved after fractionation, and so-sorted cells can be collected and

reused or studied. Moreover, the low cost of the separative device may allow for its disposable use thus avoiding sample cross-contamination among consecutive samples and reducing sterility issues. This work intends to improve the potentialities of GrFFF as cell-sorting technique able to select intact cells direct from clinical specimens in physiological conditions and as a simple method to be integrated with analytical procedures for cell analysis. The new approach is demonstrated towards the tag-less isolation of HUVEC from raw umbilical cord.

Material and methods

Samples

The study was performed at the Department of Mother and Infant Sciences, University of Milan, Foundation IRCCS Ca' Granda Ospedale Maggiore Policlinico. All mothers gave informed consent. Umbilical cords were obtained from pregnancies which underwent elective caesarean section not related to maternal or fetal diseases (breech presentation or maternal request). The inclusion criteria were single, term, normal pregnancies with absence of maternal, placental or fetal pathologies.

Procedures for cell isolation

HUVEC isolation from umbilical cord vein

Endothelial cells were obtained from umbilical cord vein by the following method, as previously described [26].

Briefly, 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 draining. Afterwards 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 g 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 (200 u/ml, Sigma, St. Louis, MO), 200 μl heparin (25,000 UI/5 ml IV 1F, Hospira Italy) and 1 %L-glutamine (Sigma, St. Louis, MO), in order to be counted in a Bürker chamber. The cell suspension was centrifuged again at $463\times g$ for 6 min and suspended in complete culture medium so as to have a concentration of 4.2×10^6 cells/350 μl to allow GrFFF experiments [27].

Primary HUVEC culture

HUVEC obtained from umbilical cord were grown in the 199 medium (Sigma, St. Louis, MO) supplemented with 20 % fetal bovine serum (FBS, Sigma, St. Louis, MO), 3 % penicillin/streptomycin (200 u/ml, Sigma, St. Louis, MO), 200 μl eparin (25,000 UI/5 ml IV 1 F, HOSPIRA Italy) and 1 %L-glutamine (Sigma, St. Louis, MO), at 37 °C in a 5 % CO_2 atmosphere. Medium was changed two times weekly.

Fractionation system

GrFFF device

The fractionation device is a biocompatible, flat channel (GrFFF channel) that can be easily implemented in a fluidic system. It is constituted of two parallel plates made on plastic biocompatible materials (polyvinyl chloride or polycarbonate) separated by a spacer from which the channel volume is removed. Channel dimensions are 4.0 cm in breadth, 30 cm in length and 0.025 cm in thickness with a volume of about 1.3 ml. A peristaltic pump (Miniplus 3, Gilson, Middleton, WI, USA) was employed to generate the mobile-phase flow and sample volume is injected by means of an HPLC syringe into the inlet tube (in PEEK; L=7 cm, I.D.=0.750 mm, O.D.= 1/16"). An UV–vis diode-array detector (ThermoQuest, Austin, TX, USA) was connected to the channel outlet to monitor the elution process, and the online signal at 600 nm generated by eluted analytes (the fractogram) was recorded. A fraction collector was connected to automatically collect cell fractions at given retention times.

All the instruments were placed inside a laminar flow hood to provide sterile working conditions and sterile mobile phase solutions were used to preserve cell sterility during fractionation. In Fig. 1 a schematic view of the fractionation setup is reported.

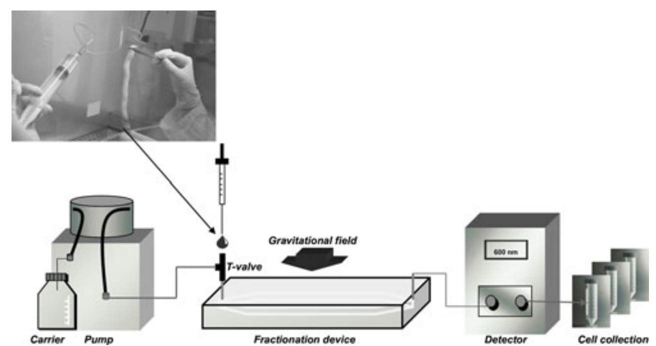

Fig. 1 Schematic view of the fractionation system

GrFFF method

At the beginning of each working day, a procedure for decontamination of the fractionation system was performed by flushing sodium hypochlorite in sterile water at 2 % as active chlorine; then it was washed copiously with sterile, demineralized water. Subsequently, in order to block unspecific interaction sites on the plastic walls, a sterile solution of bovine serum albumin (BSA) 1 % (*w*/*v*) in physiologic phosphate buffer saline (PBS) was flushed for 1 h at 1 ml/min. Finally, the fractionation system was filled with sterile mobile phase consisting of PBS added with 0.1 % (*w*/*v*) BSA, 1 % (*w*/*v*) 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 (12×10^6cells/ml) of cell suspension were injected into the GrFFF system and the flow was activated at a flow rate of 0.25 $\text{ml}\,\text{min}^{-1}$ for 15 s to make the overall sample enter the channel. Subsequently, the flow was interrupted for 2 min to allow sample relaxation, a process necessary to make analytes reach an equilibrium position along the channel thickness as a response of external field. This position depends on native properties of analytes and it results in a characteristic elution velocity [28]. Finally, sample elution was carried out at 1 $\text{ml}\,\text{min}^{-1}$. HUVEC are collected in the fraction eluted in the interval time 7–14 min. Cells collected from repeated runs were pooled and subjected to characterization by means of flow cytometry and to RNA isolation.

FACS analysis

Either unfractionated or collected cells were separately analysed by flow cytometry to verify the level of enrichment and cell viability obtained upon fractionation. Briefly, cells were firstly incubated in PBS solution enriched with 0.1 % NaN_3 (sodium azide, Sigma, Milano, Italy), 4 % newborn calf serum (NCS, Sigma, Milano, Italy) and 4 % mouse serum (MS, Sigma, Milano, Italy) for 20 min at 4 °C. Then cells were stained at 4 °C for 20 min with 10 μl of 7-amino-actinomycin D (7-AAD, a marker for non viable cells) in order to distinguish viable cells from dead cells and with appropriate monoclonal antibodies: 10 μl of anti-CD31 conjugated with fluorescein isothiocyanate (FITC), 5 μl of 1:20 diluted anti-glycophorin conjugated with phycoerythrin (PE) and 5 μl of anti-CD45 coniugated with PE-Cyanin 7 (PE-Cy7). All antibodies were from Becton Dickinson Biosciences (BD, San José, CA, USA). After washing, samples signals were acquired using a dual-laser equipped FACSCanto I; standard configuration and analysis were performed with FACSDiva software (BD).

The following gating strategy was applied: cells were firstly analysed in a side scatter (SSC) vs. forward scatter (FSC) dot plot, and the percentages of CD31+ (HUVEC and lymphoid cells) and glycophorin+(RBC) cells were defined on a SSC vs. CD31 and on a SSC vs. glycophorin dot plots, respectively. Viability of HUVEC cells was assessed on a SSC vs 7AAD dot plot gated on CD31+ cells only. It was also possible to distinguish "real" HUVEC from lymphoid elements on a CD45-PE-Cy7 vs. CD31-FITC dot plot (pure HUVEC cells show a very low expression of CD45 marker). In addiction we had identified the HUVEC population from the fibroblast contamination through CD31 analysis, an endothelial cells-specific antigen.

RNA isolation and quantification

Four RNA samples were obtained from four different umbilical cords. The extraction of total RNA of each sample was performed from seven different injections. The fractions were washed by phosphate-buffered saline (PBS, Sigma, St. Louis, MO) and sedimented at 463 g for 6 min. After discarding supernatant the pellet was suspended in 1 ml of TriReagent (Sigma, St. Louis, MO), and processed using a standard protocol.

Results and discussion

GrFFF of RBC and cultured HUVEC

For this study, we have isolated HUVEC from human umbilical vein cord by enzymatic digestion as previously described and cultured HUVEC were prepared. We firstly tested the GrFFF fractionation selectivity for HUVEC cells and the contaminants blood cells

Single injections of cultured HUVEC and blood cells confirm that GrFFF is able to selectively elute cells with different morphologies, with high reproducibility.

Human blood cells (containing 5×10^6 RBC) and 3×10^6 HUVEC cells from culture were fractionated under same conditions, following the procedure described above. Representative fractograms are reported in Fig. 2. The HUVEC band shows a retention ratio of 0.176±0.003 and the blood cells band of 0.075±0.002. The blood cells band correspond to the elution of RBC, which represents the most important contaminant; leukocytes and platelets eluted in the void peak, as it was assessed by the injection of samples constituted of these single, blood cell components in the same experimental conditions (data not shown). The difference in retention between HUVEC and red blood cells was due to the different biophysical characteristics of these cells. The fractionation profiles were obtained with high repeatability and good reproducibility (run-to-run %CV of retention times was below 3 %, and day-to-day %CV below 10 %). All injected cells were recovered after fractionation, as evaluated by counting cells before and after the elution process.

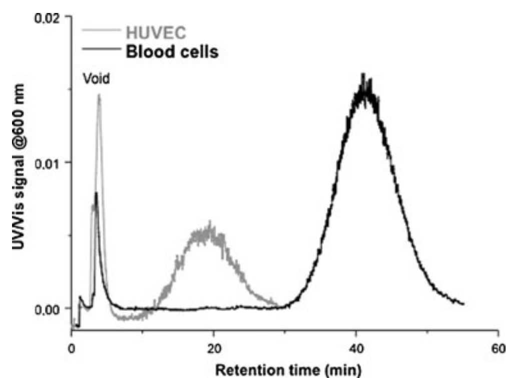

Fig. 2 Fractograms of HUVEC and blood cells. Mobile phase: PBS, 0.1 % (*w*/*v*) BSA, 1 % (*w*/*v*) EDTA and 1 % penicillin/streptomycin. Relaxation time=2 min; elution flow rate=1.0 ml/min. Injected cells, 50 μl of 5×10^6 RBC and 3×10^6 HUVEC culture

GrFFF of cell suspensions from cord blood

When cell suspensions obtained from the enzymatic digestion of umbilical cord samples were injected into the GrFFF system, a typical fractionation profile was obtained as reported in Fig. 3. It shows two main bands: the first at a retention time slightly lower than cultured HUVEC and the second at the retention time characteristic of RBC (see the "GrFFF of RBC and cultured HUVEC" section). The difference on retention times for HUVEC cells was done to the culture procedure that modify cell morphology with respect to fresh HUVEC collected from umbilical cord digestion. The first band was considered as the HUVEC fraction.

Best conditions for mobile phase composition, flow velocity and device preparation were then optimized through the fractionation of 25 umbilical cords in order to maximize cell recovery, viability, fraction purity and throughput. The addition to the PBS employed as mobile phase of 1 % EDTA resulted to be fundamental in order to avoid cells aggregation and improve cell viability and physiological conditions. Flow rate of the mobile phase was adjusted to guarantee cells separation together with short analysis time. Total maintenance of cell viability (results are reported in the "Viability test" section) and total cell recovery after fractionation were achieved. All the fractionated crude samples obtained from enzymatic treatment of cord blood showed the same fractographic profile with differences only in the relative amount of HUVEC and RBC, depending on biological variability.

The first band from 7 to 14 min was collected, and at t= 18 min, the flow rate was increased up to 10 ml/min to make contaminant cells elute quickly. Due to the high reproducibility of the GrFFF fractionation process, three fractions from three different injections of the same sample were pooled, and then characterized by flow cytometry to confirm cell phenotype and viability before using GrFFF to produce the cell fractions for RNA extraction. Because fractions were collected at short retention times, fraction pooling, the total time spent for completing the fractionation step was relatively short.

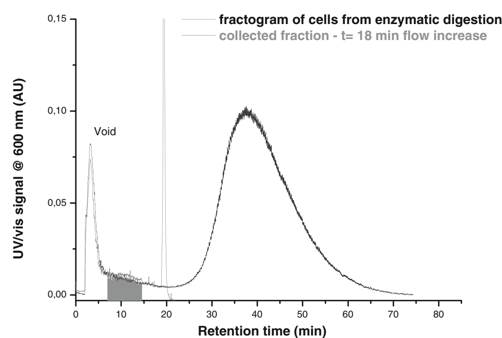

Fig. 3 Representative GrFFF fractograms of cells from umbilical vein after the enzymatic digestion of cord blood. Mobile phase: PBS, 0.1 % (*w*/*v*) BSA, 1 % (*w*/*v*) EDTA and 1 % penicillin/streptomycin. Relaxation time=2 min; elution flow rate=1.0 ml/min. Injected cells, 4×10^6. Collected fraction, (7–15)min. At t=18 min, flow rate increases up to 10 ml/min. *Black line* typical whole fractogram of cells from cord blood. *Grey line* elution of cord blood with collected fraction, t= 18 min flow increase

Cytofluorimetric analysis of fractionated cells

Flow cytometric analysis was performed on collected fraction in order to verify purity. Following the scheme described in the "GrFFF of cell suspensions from cord blood" section, we analysed six fractions collected after GrFFF of six cord blood samples. Typical flow cytometry dot plots of unfractionated and fractionated cells are reported in Fig. 4a, b. From these graphs, it is evident an enrichment of CD31+ cells (HUVEC), corresponding to region *R2*, and a depletion of glycophorin+cells (RBC) corresponding to region *R1* in the fractionated sample. The CD31+ cells were further characterized in terms of viability and purity: the presence of viable cells corresponding to region *R3* (as it will be further discussed in "Viability test" section) and the purity of HUVEC cells corresponding to region *R4* confirmed the ability of the GrFFF fractionation method to select pure viable target cells.

Results of fraction purity and cell viability are summarized in Fig. 5 where the percentage of cells vs selected markers was reported. Data are expressed as mean±SD. A value of $P<0.05$ was considered to be statistically significant. Firstly, samples can be divided into two main groups which are different for initial RBC contamination and HUVEC percentage due to their biological variability: in group A (three samples), there are samples starting with an RBCs contamination of 60±8 % and an estimated HUVEC percentage of 40±5 %; in group B (three samples), there are

Fig. 4 **a** Typical flow cytometric dot plots for unfractionated. Region 1 (*upper quadrant* of glycphorin-PE vs SSC) represents red blood cells; region 2 (*upper quadrant* of CD31-PE vs SSC) represents cells positive for CD31 and it is considered to estimate HUVEC enrichment; region 3 (*lower quadrant* of 7AAD-PE vs SSC) represents viable cells; region 4 (*upper* and *lower left side quadrant* of CD31-FITC vs CD45-PE-Cy7) represents cells positive for CD31 and negative for CD45 and it is considered to estimate cell purity. **b** Typical flow cytometric dot plots for fractionated samples. For the region explanation, see Fig. 4a caption

samples starting with higher RBC contamination (95±5 %) and with only a 2.0±0.5 % of estimated HUVEC.

After GrFFF, an enrichment in HUVEC was obtained in both cases with a final sample purity limited by the initial sample contamination. For samples with a lower contamination (group A), the GrFFF process yielded a (2.4±0.4)-fold increase for CD31 expression and a high sample purity (final HUVEC percentage of 90±5 %). The system was able

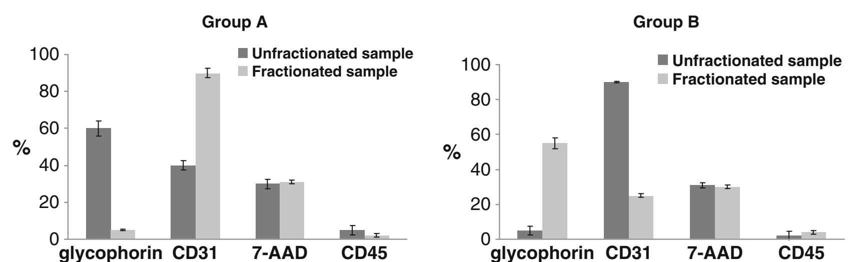

Fig. 5 FC characterization of unfractionated and fractionated cells. Results expressed as mean±SD for $N=3$

to give a fraction suitable for direct RNA extraction for gene expression analysis. In samples with a higher initial contamination (group B), higher increase of CD31 expression corresponding to (10±2)-fold increase was reached, though with a lower final purity (final HUVEC percentage of 25± 2 %). Nonetheless, even if a better enrichment was obtained for a sample with higher initial RBC contamination (group B) because cell amount in the starting material was lower than 10 %, direct processing of cord blood was not able to give a proper amount of HUVEC for direct RNA extraction. Even after fractionation, for all samples the estimated HUVEC presented low CD45 expression, which allows excluding lymphoid elements.

Viability test

The possibility to preclude stress and maintain good vitality of the cells along the whole procedure is significant factor. Viability of selected cells was evaluated by means of cytofluorimetric analysis and culture of the isolated cells.

Freshly trypsinized and enzymatic-digested cells were resuspended in the GrFFF mobile phase. Trypan blue was added to a part of the cell suspension at a final concentration of 0.1 %. Blue-stained cells were considered non-viable cells and cell density was adjusted to 10^6 cells/ml. This cell suspension constituted the total HUVEC population. A part of this cell suspension was used for GrFFF and the other part was taken as control sample of the cell population. After GrFFF, control cells and fractionated cells were centrifuged, and cell pellets were re-suspended in PBS for cytofluorimetric analysis. Cell viability was not affected by the fractionation process as assessed by flow cytometry using 7AAD; as described in Fig. 4 and quantified in Fig. 5. The expression level of 7-AAD marker of non-viable cells was comparable for fractionated and unfractionated cells; levels of 30 % of non-viable cells were obtained, mainly due to the enzymatic digestion process used for the cells extraction from unbelical vein.

Results also show, both for the fractionated and the unfractionated cells, an adhesion in less than 48 h, and a typical growth pattern that led to a sub-confluent culture in 5–7 days. A total cell recovery after fractionation process was estimated by flow cytometry.

Extraction of RNA yield

HUVEC (42×10^4) were obtained from seven collected fractions and TriReagent was used to extract total RNA. Four RNA samples were obtained from four different umbilical cords giving 5,340±493 ng of total RNA. This result is consistent with a total target cell recovery as assessed by flow cytometry.

Although some variations in the amount of RNA recovered among individuals were observed, all fractionated samples provided sufficient RNA for a direct subsequent gene analysis, such as Real-Time PCR but also for microarray or Deep Sequencing analysis.

Conclusions

Molecular expression pattern obtained from clinical specimens represents a valuable diagnostic tool to study and identify mechanisms involved in many important diseases. However, current methods used to isolate cells from which to extract the RNA present some serious drawbacks. Among them, the requirement of a high number of cells, low recovery, high-cost instrumentation and invasiveness of the approaches, which do not guarantee maintenance of the cell physiological properties, for example differentiation, adhesion, cell shape, mitochondria contents, energy metabolism, inflammatory markers, antigen and protein expression [7, 15, 18, 29].

Our results demonstrate that HUVEC can be isolated from raw clinical samples with a GrFFF-based method. From the GrFFF system, a proper amount of HUVEC can be collected in less than 45 min with high purity of fractionated cell suspensions. The relevant technology can be easily implemented in biomedical laboratories with low instrumental and training investments.

From these results, GrFFF proves as an elective approach for cell isolation in physiologic conditions from complex matrices for further specific applications; such as the study of physiological and pathological samples through the gene expression analysis of the direct fractionated materials from raw samples. The use of an array of GrFFF devices together with the automation of the fractionation procedure will allow increasing the enrichment productivity and possibly avoiding fraction pooling, reducing the process time.

Acknowledgements We are grateful to Chiara Bolego for her precious help and suggestions in HUVEC culture and their isolation from umbilical cord sample.

Competing interest Barbara Roda, Andrea Zattoni, and Pierluigi Reschiglian are associates of the academic spinoff company byFlow Srl (Bologna, Italy). The company mission includes know-how transfer, development, and application of novel technologies and methodologies for the analysis and characterization of samples of nanobiotechnological interest.

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