In vitro Three Dimensional Scaffold-free Construct of Human Adipose-derived Stem Cells in Coculture with Endothelial Cells and Fibroblasts

GRIGORE BEREA[1,2], GHEORGHE GH. BALAN[1]*, VASILE SANDRU[3], PAUL DAN SIRBU[1]

[1] Gr T Popa University of Medicine and Pharmacy, Faculty of Medicine, 16, Universitatii Str., 700115, Iasi, Romania

[2] Regional Clinical Hospital Neamt, Department of Orthopaedics, 1 Traian Blvd., 610137 Piatra Neamt, Romania

[3] Clinical Emergency Hospitals of Bucharest, Gastroenterology Research Department, Bucharest, 10 Calea Floreasca, 014452 Bucharest, Romania

Complex interactions between stem cells, vascular cells and fibroblasts represent the substrate of building microenvironment-embedded 3D structures that can be grafted or added to bone substitute scaffolds in tissue engineering or clinical bone repair. Human Adipose-derived Stem Cells (hASCs), human umbilical vein endothelial cells (HUVECs) and normal dermal human fibroblasts (NDHF) can be mixed together in three dimensional scaffold free constructs and their behaviour will emphasize their potential use as seeding points in bone tissue engineering. Various combinations of the aforementioned cell lines were compared to single cell line culture in terms of size, viability and cell proliferation. At 5 weeks, viability dropped for single cell line spheroids while addition of NDHF to hASC maintained the viability at the same level at 5 weeks Fibroblasts addition to the 3D construct of stem cells and endothelial cells improves viability and reduces proliferation as a marker of cell differentiation toward osteogenic line.

Keywords: microenvironment-embedded 3D structures, tissue engineering, Stem and endothelial cells, viability and cell proliferation, ascorbic acid, hydrocortisone, Na-pyruvate L-Glutamine

One of the orthopaedic major challenges is to deal with bone defects repair. Bone substitutes mixed with primary patient-derived cells are extensively used in basic research and clinic [1]. Bone defects can be treated using cell-seeded scaffolds in which Adipose-derived Stem Cells (ASCs) play an important role; these cells have been applied in for bone defect treatment in both animals and humans showing induced osteogenesis, mainly in vivo [2-5]. Even if the mechanism of osteogenic differentiation was described mainly in vivo, the interactions between ASC, endothelial cells and other cells in the microenvironment are still subject for extended debates. Also, the behaviour of mesenchymal stem cells (hMSC) in three dimensional co-cultures with human umbilical vein endothelial cells (HUVECs) was previously described [6]. However, this study focused on hMSC and HUVECs and compared their behaviour and osteogenesis in specific conditioned media. Other studies [7-9] emphasizes the fibroblast role in prevascularization of tissue-engineered constructs. Most literature approaches regarding complex interactions between stem cells, vascular cells and fibroblasts are either using hMSC as stem cells, mouse fibroblasts or 2D constructs.

The present study is focused on generation and proliferation/viability of three dimensional constructs using adipose derived stem cells (hASC), human umbilical vein endothelial cells (HUVECs) and normal dermal human fibroblasts. All three dimensional constructs were scaffold free in order to evaluate long term viability and potential use for polymer scaffold engraftment prior to grafting in in vivo experiments. hASC choice for stem cells was due to the ease of harvest (liposuction), use, culture and availability.

Experimental part

Cell lines and culture methods

The experimental design included hASC, HUVEC and normal dermal human fibroblasts purchased from Lonza. Culture media was also purchased from Lonza for each specific cell line. At arrival, cryovials with cell lines were thawed and dispensed in 175cm^2 sterile cell culture ready flasks containing 35 mL warm cell-specific media. At 16h media was replaced with fresh one and flasks were incubated for 48h. Then each flask with the three cell lines was splitted; cells were detached by trypsin EDTA (4mL for 3 min in a 175cm^2 cell culture flask), while trypsin inactivation was completed by 10 mL RPMI with 10% serum. No antibiotic was used for any of the cell lines. After 2 passages, cell was detached and spheroids were generated using the classical method previously described by Ivascu et al., [10]. Briefly, after cell detachment, viability assessment by trypan blue and count, cells were dispersed in cooled endothelial growth media (EGM) containing 1% matrigel, at 4°C. Media with cells was dispensed in 96-well plates (Costar) precoated with a layer of agarose [11-13]. Spheroids can be formed by plating 1.5% agarose (molten at 90°C) to form a thin coating over well surface. Agar coating is cell repellent and cells are stitching together, also stimulated by the presence of 1% matrigel in cell-containing media. The forced-floating method using agar is easy to reproduce, inexpensive and time-saving [13]. Each well of the 96-well plate was filled by 200µL EGM with cells. After dispensing 200µL media with cells in each well, according to the conditions described below, plates were spinned down at 300g for 5 min at 4°C. Plates were then incubated at 37°C 5% CO_2 and 99.6% humidity. Spheroids were formed in the next 24 hours and regularly observed in a inverted Nikon microscope with phase contrast and fluorescence capabilities. All measurements were performed in Fiji ImageJ open source platform [14], using also custom made macros to speed up image processing protocol.

The experimental procedures were realized in accordance with the mandatory principles of the Ethical Committee of the Grigore T. Popa University of Medicine and Pharmacy Iasi [15, 16].

* email: balan.geo@yahoo.com

Live/dead assay and proliferation assays

Dead cells were detected by ethidium homodimer reagent (ThermoScientific) at a concentration of 2µM, following the procedure described by the manufacturer. Total spheroid size was evaluated by staining with Hoechst 33342 (ThermoScientific, solution 10mg/mL diluted 1/5000) and area measurement by ImageJ.

CellTiter-Glo Luminescent Cell Viability Assay (Promega) was used to assess ATP levels in the cells applied and proliferated on the samples. Manufacturer recommendations and concentrations were followed.

Results and disucssions

The mixed spheroids progress and viability was explored by measuring the area (at 2, 3 and 5 weeks) and the viability at 5 weeks using the Ethidium homodimer assay. The mixed spheroid conditions were compared between them and with hASC single cell line spheroids. The conditions in the setup included hASC – 2000 cells/spheroid; hASC+HUVEC – ratio 1:3 (6000 cells/spheroid); hASC+NDHF – ratio 1:1 (4000 cells/spheroid) and hASC + HUVEC + NDHF – ratio 1:3:1 (10,000 cells/spheroid).

3D self-assembly in mixed two or three cell line cultures

hASC, HUVECs and NDHF were cultured individually or in mixtures in agarose coated 96-well plates. hASC monoculture was used as control. Due to 1% matrigel presence in the culture media and plate spinning, the spheroids formed overnight. Images were analysed and spheroids measured in Fiji_ImageJ. While we have used 2-10x10^3 cells per spheroid for the 4 conditions we start imaging at day 7 and continued imaging the plate at 2, 3 and 5 weeks respectively. To maintain normal proliferation conditions, media in the wells with spheroids was refreshed weekly.

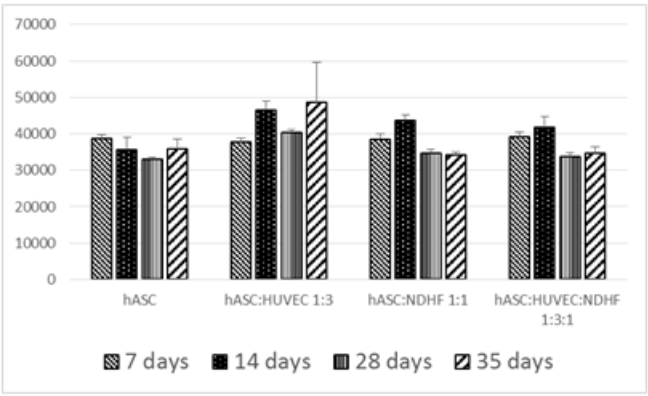

Fig. 1. Spheroid size for the four described conditions, hASC – single cell line spheroids, and combinations with HUVECs and NHF respectively. Measurements were performed at 1, 2, 3 and 5 weeks respectively

Size measurements (fig. 1) showed a low proliferation rate expressed by low spheroid size variations. Lack of spheroid size increase beyond week 1 is probably due either to the hypoxic core that keeps cells quiescent or to differentiation due to co-culturing hASC with HUVECs or fibroblasts. In all setups, area varied in the range 33000-48000 µm^2 and the spheroid circularity was constant (0.8 to 0.9). Values for hASC:HUVECs 1:3 in week 5 show an elevated standard deviation caused by some unusual measurements failure in ImageJ, probably due to uneven gel generated by debris in some replicate wells. The small size variations are not a good indicator of cell status, as the starting cell number was different.

Media influence on mixed spheroid proliferation

To evaluate spheroid proliferation at 5 weeks we have compared not only different spheroid composition but also the media used for their maintenance. DMEM with 10% FCS and EGM were used for spheroid maintenance, media being changed in all wells on a weekly basis. At 5 weeks, the ATP levels were evaluated for 4 replicates of each condition, using the CellTiter Glo endpoint assay. Data showed that DMEM did not support cell proliferation in any of the spheroids, the ATP levels (expressed by Relative Luminescence Units) being low in all spheroid types (fig. 2A). Conversely, EGM sustain cell proliferation, ATP levels at 5 weeks being 10 fold higher than the levels recorded for DMEM incubation (fig. 2A).

While calculating the cell proliferation status in the mixed spheroids compared to the hASC control spheroids, it is important to remember that the starting cell-seeding concentrations were different for the 4 conditions. Thus, the proliferation rate in the corresponding spheroids, reproduced by the ATP levels (expressed in RFU units) is supposed to be different. However, there were noticed no major differences among the investigated conditions, except the triple combination. In order to normalize the proliferation ration to the initial cell seeding values, a ratio between areas measured in ImageJ was reported to the initial cell number seed. Then the resulted values were represented in a different graph, giving a closer picture of the spheroid fate for each condition (fig. 2B) As predicted, cell proliferation is more important in controls (single cell line hASC spheroids) than in the mixed spheroids. Cell proliferation was 61.2% of the control in the combination hASC+HUVEC and 32.9% of the control for the combination hASC+NDHF. It is to notice that addition of NDHF to the hASC+HUVEC spheroids increased the ATP levels in those spheroids at 37.7% of the control.

Spheroid viability at 3 and 5 weeks

Spheroid viability was evaluated at 3 and 5 weeks by the endpoint assay, using Ethidium homodimer (EthD) for

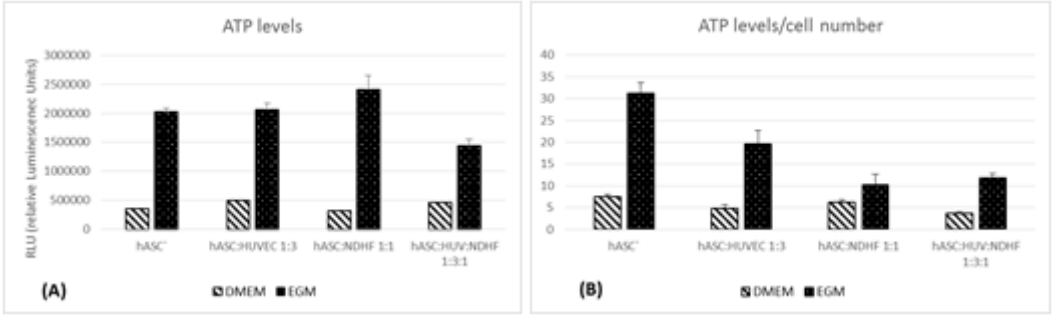

Fig. 2. (A) Cell proliferation in the 3D constructs of the four described conditions and maintained either in DMEM with 10%FCS or EGM for 5 weeks. Cell proliferation is expressed by ATP levels detected by CellTiterGlo assay. (B) Corrected cell proliferation in the 4 described conditions in DMEM or EGM for 5 weeks. Correction was performed reporting the ATP levels to the initial cell number in spheroids, for each condition

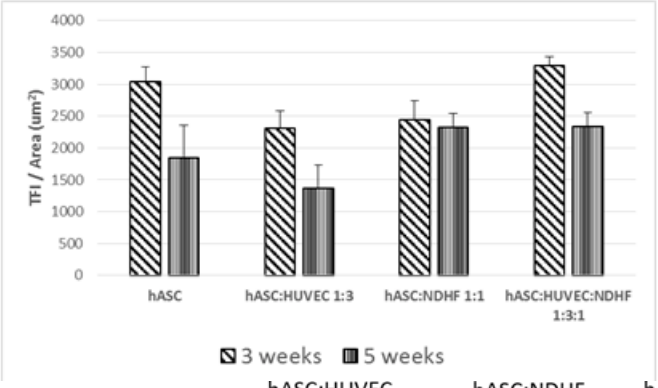

Fig. 3. Spheroid viability was evaluated at 3 and 5 weeks by the endpoint assay, using Ethidium homodimer (EthD). Normalization was performed by reporting the TFI for each condition to the corresponding spheroid size.

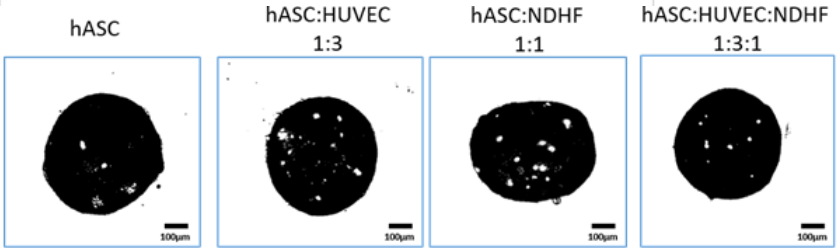

Fig. 4. Spheroid viability at 5 weeks of incubation in EGM (changed weekly). Lower number of dead cells (light spots) in the complex spheroids hASC+HUVEC mixed with NDHF compared to those without NDHF.

dead cells and Hoechst 33342 to detect total number of cells in the spheroid. Cell viability in the spheroids was evaluated by subtracting total fluorescence intensity (TFI) for dead cells (EthD) from total fluorescence induced by Hoechst stain. Normalization was performed by reporting the TFI for each condition to the corresponding spheroid size. Spheroid size was expressed by its projection area (μm^2) and was measured in FIJI_ImageJ.

It is to mention that viability at 5 weeks was lower than at 3 weeks, despite media change. The most important viability drop was recorded for the single cell spheroids hASC; this demonstrates once again the importance of the microenvironment in cell survival in 3D cultures. Viability for hASC+HUVEC mixed spheroids was lower than the combination with NDHF on both measuring points (3 and 5 weeks). NDHF addition to 3d construct improved viability in long term culture (5 weeks); At 5 weeks, viability dropped for single cell line spheroids while addition of NDHF to hASC maintained the viability at the same level at 5 weeks. Thus, the viability improvement was noticed for the most complex mixture, while using NDHF.

Samples from every condition processed in ImageJ showed a lower number of dead cells (light spots) in the complex spheroids hASC+HUVEC mixed with NDHF compared to those without NDHF (fig. 4).

Regarding the size of the mixed spheroids, it is impressive the effect the fibroblasts are playing in adjusting the size and compactness. Due to complex interactions and cross-talk between fibroblasts, endothelial and stem cells, viability in complex mixed spheroids is better than in combinations that are lacking spheroids. The interaction between hASCs and HUVECs was previously documented [17]. Alkaline phosphatase activity, osteocalcin and BMP-2 expression are enhanced at least in bi-dimensional co-cultures of hASCs and HUVECs in a ratio of 1:1. It was proven that hASC co-cultured with endothelial cells are more differentiated on the osteogenic lineage. At the same time it was shown that HUVECs cultured alone are generating a rapid increase in BMP-2 levels, which will promote hASC osteogenic differentiation [5]. Thus, combining the HUVECs and hASCc in three-dimensional setup will increase not only cell-cell interactions but also will favour paracrine signalling between the two cell lines involved. hASC differentiation on the osteogenic line will reduce proliferation rate, that was noticed on our experiment by the reduction of ATP levels at 5 weeks, in the mixed 3D cultures of hASC and HUVECs. Moreover, addition of NDHF amplified this effect (fig. 2) so we may conclude that the differentiation process was enhanced by fibroblast presence in the combined three dimensional culture.

Cell viability in 3d constructs depends not only on direct contact between cells of different origins and cell differentiation but also in chemical composition of the culture media. Differences in 3D mixtures viability while using DMEM or EGM2 are potentially due to important differences in chemical formulation. Thus, EGM2 formulation contains hEGF, 0.5 mL; VEGF, 0.5 mL; R3-IGF-1, 0.5 mL; Ascorbic Acid, 0.5 mL; Hydrocortisone, 0.2 mL; hFGF-β, 2.0 mL. DMEM does not include any of these growth factors that ae supporting mainly endothelial cell proliferation. However, fibroblast presence in the 3D construct is supposed to provide the FGF and VEGF required for endothelial cell survival and proliferation. Even though the chemical composition of DMEM Is theoretically able to support hASC survival, endothelial cells maintenance and proliferation cannot be supported just by the fibroblasts presence in the 3D construct. Thus, culture media chemical formulation remains a permanent challenge while using complex 3d cell constructs. Not only are growth factors important but also the presence of ascorbic acid and hydrocortisone. Addition of cholera toxin supports cell proliferation but it is not a physiological option for these co-cultures. Addition of Na-pyruvate is also improving cell proliferation and survival. L-Glutamine is another chemical compound of the culture media, compulsory for cell proliferation. L-Glutamine is, nevertheless, subject to chemical degradation in about 7 days, then usage of Glutamax is recommended.

Data regarding co-cultured spheroids of hMSC and endothelial cells were previously published [6 - 18]. However, to our knowledge, none of the published studies dealt with combined culture of hASC, HUV and fibroblasts in the same spheroid. Our results are showing not only that these three cell lines are dealing well with the three-dimensional environment but also that the fibroblasts are improving this construct survival, making it more suitable for usage in bone tissue healing or tissue engineering. Thus, in our results, hASC and endothelial cells show self-assembly ability in a three-dimensional culture, either in the presence or absence of the fibroblasts. At the same

time, the cell ratio we used seemed to be well tolerated in the 3D context, while the compactness on circularity of the construct were visually improved by fibroblast addition.

The influence of HUVECs on enhancing hMSC osteogenic differentiation was published before. The role of HUVECs as enhancers of ALP activity either in 2D or 3D cultures was shown extensively [19-21]. The cell number in spheroids is important for the hypoxic central core [22, 23].

In our study, the onset cell number started with 2000 cells in single cell cultures and raised up to 10,000 cells in co-cultured samples, keeping an intermediate value between the cited studies. At 5 weeks we noticed that the sample size was quite even, despite the huge difference in initial cell number. Quiescence in these constructs is not an unexpected event; however, even if the cell number in the samples that included fibroblasts was 5-fold higher than in single cell cultures, the spheroid size was almost the same. The same notice can be made according to viability. Fibroblasts presence improved the viability, considering the cell number in the co-cultured spheroids. At the same time, fibroblast addition to hASC+HUVEC spheroids lowered proliferation rate compared to the samples with no fibroblasts. This effect can be due either to increased cell number in the spheroid, inducing cell quiescence in a larger hypoxic core or to hASC differentiation.

In a previous paper were studied the biochemical effects of collagen-cultured mesenchymal stem cells on isolated vascular smooth muscle cells [24].

Conclusions

hASC, HUVEC and NDHF three dimensional construct show good viability at 5 weeks of culture in EGM media while the fibroblasts are improving this construct survival, making it more suitable for usage in bone tissue healing or tissue engineering. The cell ratio 1:3:1 hASC:HUVEC:NDHF is well tolerated in the 3D context, and fibroblast addition improves spheroid circularity and compactness.

Continuously improved microenvironment for stem cell based therapies will help understanding their behaviour while being embedded in polymer or inorganic matrices or porous biomaterials.

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Manuscript received: 21.12.2016