Biochemical Effects of Collagen-Cultured Mesenchymal Stem Cells on Endothelial Progenitor Cells *In Vitro*

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We tempted to explore the biochemical effects, represented by apoptosis, triggered by conditioned medium, obtained as a result of culturing mesenchymal stem cells for 24 h, on endothelial progenitor cells. The mesenchymal stem cells were developed on collagen fibers in the presence of thapsigargin, concanavalin A, leptin and ghrelin. Meanwhile, were used the following inhibitors: AG490, a JAK2 inhibitor; PD98059, a MEK/ERK inhibitor; and LY294002, a PI3K/Akt signaling inhibitor. When we analyzed the results, we observed that the conditioned medium, obtained from mesenchymal stem cells cultivation, applied in a 10% concentration for 24 H , induced apoptosis of endothelial progenitor cells in different degrees: 10 μM leptin> ghrelin>>concanavalin A ≅ apelin. Furthermore, LY294002 and AG490-conditioned medium of mesenchymal stem cells reduced the apoptotic effects induced by leptin on developed endothelial progenitor cells. The very first conclusion is that the inhibition of PI3K/Akt signaling pathway is blocking the apoptotic effects of leptin stimulation of mesenchymal stem cells population. The inhibition of JAK/STAT pathways seems to be less effective.

Keywords: mesenchymal stem cell, endothelial progenitor stem cells, leptin, ghrelin, PI3K/Akt signaling

Vascular repairing and the forming of *de novo* blood vessels are implying more and more circulating cellular types into these processes. Starting from this point, there is large debate if these circulating cells, having clear characteristics of the hematopoietic lines and being involved in angiogenesis or angioneogenesis, could be defined as endothelial progenitor cells or not. Generally, the term endothelial progenitor cells should include some very specific cells having exclusive features. Thus, an endothelial progenitor cell is a circulating one associating progeny capacities through clonal proliferation and differentiation potentiated toward endothelial lineage. Moreover, such cells must be equipped with the capacities to develop in vitro tubes very similar to vessels with lumen (defined by the vacuolation of the cytoplasm ability). Endothelial progenitor cells would have the capacity to induce the appearance of basement membranes through secretion and thus the formation of stable human blood vessels when they are implanted, using or not any type of scaffolds. Finally, such progenitor cells would include the capacities to accept the total integration inside the general circulatory system of the host and to have the shining potential to endure remodeling and to be the starting point for intimal tissue of arteries, veins and capillaries. So, there is evidence that the circulating endothelial-colony forming cells having the above mentioned characteristics are rare, while most other called endothelial progenitor cells, derived from bone marrow, in larger numbers, are not [1].

Physiological and/or pathological vascular repairing and, ultimately, its fine mechanisms, will obviously be advantaged by the knowledge of endothelial progenitor cells functioning and source. Besides, the possibility to isolate and *in vitro* culturing of endothelial progenitor cells will open unforeseen and unintended possibilities for clinical testing in regenerative and cancer medicine [2-6].

Other published data consider the endothelial progenitor cells as being circulatory ones able to widely express several markers identical to those found on the surface of vascular endothelial cells [7-10]. Likewise, the endothelial progenitor cells would have the ability to join the hypoxia and/or ischemia sites, initiating the angiogenesis and/or neoangiogenesis. This moment, there is no specific marker described for the putative endothelial progenitor cells, Thus, as a result, only some surrogate markers are generally used to characterize the abilities of potential endothelial progenitor cells. The increasingly obvious conclusion of recent studies is represented by the fact that the same bunches of markers are expressed by endothelial progenitor cells as well as by circulating hematopoietic-derived cells subsets. Thus, their discrimination involves a broad genetic analysis from the point of view of expression and functional assays. The above discriminatory expensive and elaborated approaches are not applied as routine [11].

Using the cultures of progenitor cells of endothelial type should provide the basis for the research of their involvement in the genesis of vascular tissues. Therefore, the methods used to prepare endothelial progenitor cells are very different, as well as the results obtained using these cells. The most studied type of endothelial progenitor cells is the mononuclear one, that is the bunch of circulating mononuclear cells. Variate conditions were tested focusing on the development of circulating mononuclear cells, respectively the volume of necessary blood, the effective anticoagulant, the matrices used for coatings, and the concentrations of fetal bovine serum in media of culture. There were tested the time needed till the appearance of the very first endothelial cells colonies, the correlation of their number with the number of circulating endothelial progenitor cells, as well as their development and functioning as compared to those of HUVECs. The success of endothelial progenitor cells culturing was ensured by heparin presence, 30 mL of blood as minimum quantity, fibronectin as one of the best matrices for coatings and the use of endothelial growing media-2 including a concentration of 20% fetal bovine serum. The mean time necessary for the appearance of the very first endothelial cells colonies is about 13 days. The cells isolated and cultured in the above mentioned conditions were endothelial-like ones, considering both from the morphological and phenotypical point of view. On the other side, the endothelial progenitor cells isolated as described were less committed to grow and form vascular tissues as

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compared to HUVECs. The already described conditions are basically allowing the using of a small volume of blood and a reduced time appearance of derived endothelial-like cells colonies as other described methods [12].

The goal of our studies was represented by the apoptotic effects of the culture medium obtained from rat mesenchymal stem cells developed on collagen matrix (fibers) on isolated circulating endothelial progenitor cells. The mesenchymal stem cells were stimulated with thapsigargin, concanavalin A, leptin, ghrelin, and were used the following inhibitors: AG490, a JAK2 inhibitor; PD98059, a MEK/ERK inhibitor; and LY294002, a PI3K/Akt signaling inhibitor.

Experimental part

The mesenchymal stem cells were obtained as previously described [13], a method we adapted started from previous work [14]. The mesenchymal stem cells were obtained from the bone marrow of the femural and tibial rat Wistar males, weighting 180-200 g from Bãneasa source. The obtained cells were multiplied and established using α-MEM medium (Sigma-Aldrich), supplemented with 10% FBS (Sigma-Aldrich). The considered appropriated cells, meaning the nucleated ones, were developed using Petri culture dishes for at least 6 days. Rat mesenchymal stem cells as obtained through our protocols were equalized with the final monolayers as previously described [15]. All the expetimental analysis was performed on a FACS caliber flow cytometer (Becton Dickinson Immuno-cytometry Systems).

When mesenchymal stem cells were used for the experiments, they were further developed on collagen matrix, obtained rat tail, carefully prepared in accordance with the provided instructions (Sigma-Aldrich).

The optimal observed density for the obtained rat mesenchymal stem cells was 10.000/mL. Separate lots of study were carefully stimulated with 10 μM concanavalin A, thapsigargin, ghrelin and leptin. In some experiments, the following inhibitors were used as 10 μM co-treatment for 24 h: AG490, a JAK2 inhibitor; PD98059, a MEK/ERK inhibitor; and LY294002, a PI3K/Akt signaling inhibitor.

The medium in which rat mesenchymal stem cells were developed, named conditioned medium, was further applied in a ratio of 10% to the culture medium of endothelial progenitor cells, to stimulate the last ones for 24 h.

The development of endothelial progenitor cells in our experiments had as starting point the rat circulating mononuclear cells. The functional transformed cells we obtained in our study were isolated, characterized and cultured in agreement with a previous described and adapted technique [12].

To characterize the apoptosis index and the proportion of viable endothelial progenitor cells we further applied the techniques described [16-19].

All the protocols including Wistar rats were specifically approved by the Ethics Committee of the Grigore T. Popa University of Medicine and Pharmacy from Iasi [20-22].

Results and discussions

When we analyzed the results, we observed that the conditioned medium, obtained from mesenchymal stem cells cultivation, applied in a 10% concentration for 24 h, induced apoptosis of endothelial progenitor cells in different degrees: 10μM leptin (fig. 1) > 10 μM ghrelin (fig. 2) >> 10 μM concanavalin A (fig. 3) ≅ 10 μM apelin (fig. 4). Furthermore, 10 μM LY294002 (fig. 5) and 10 μM AG490 (fig. 6) - conditioned medium of mesenchymal stem cells reduced the apoptotic effects induced by leptin on developed endothelial progenitor cells.

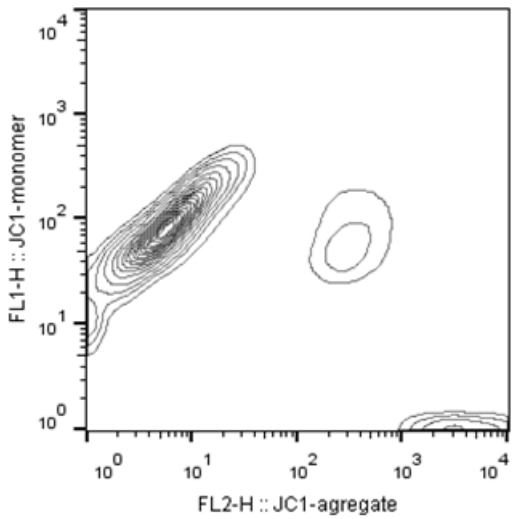

Fig. 1. When stimulated by conditioned culture medium obtained from mesenchymal stem cells treated with 10 μM leptin for 24 h , the developed endothelial progenitor cells were apoptotic in a proportion of 79% on average (representative experiment)

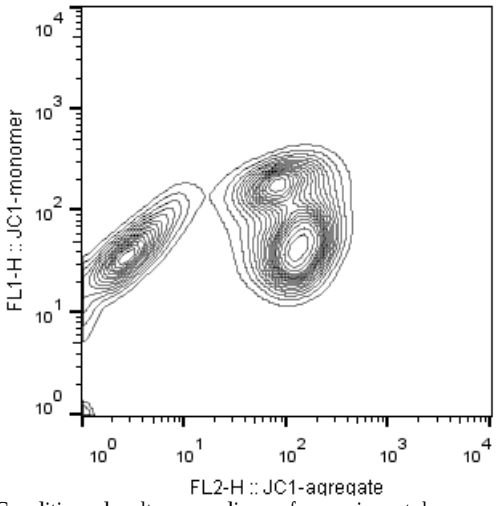

Fig. 2. Conditioned culture medium of experimental mesenchymal stem cells (10%), previously treated for 24 h with 10 μM ghrelin, had as a consequence an apoptosis proportion of 53% on average of isolated endothelial progenitor cells (representative flow cytometry)

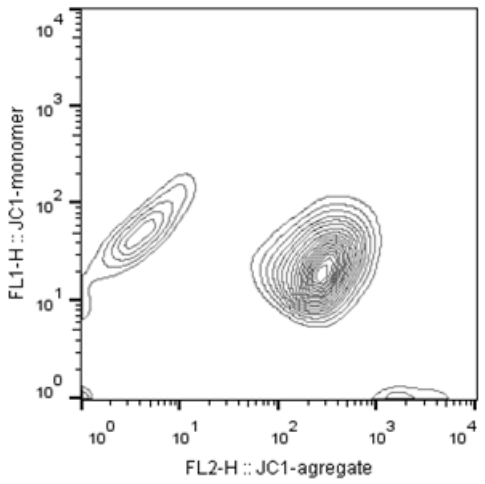

Fig. 3. When stimulated by conditioned culture medium obtained from mesenchymal stem cells treated with 10 μM concanavalin A for 24 h, the developed endothelial progenitor cells were apoptotic in a proportion of 22% on average (representative experiment)

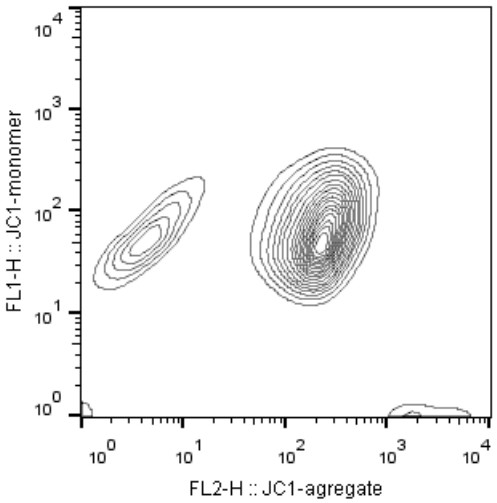

Fig. 4. Conditioned culture medium of experimental mesenchymal stem cells (10%), previously treated for 24 h with 10 μM apelin, had as a consequence an apoptosis proportion of 29% on average of isolated endothelial progenitor cells (representative flow cytometry)

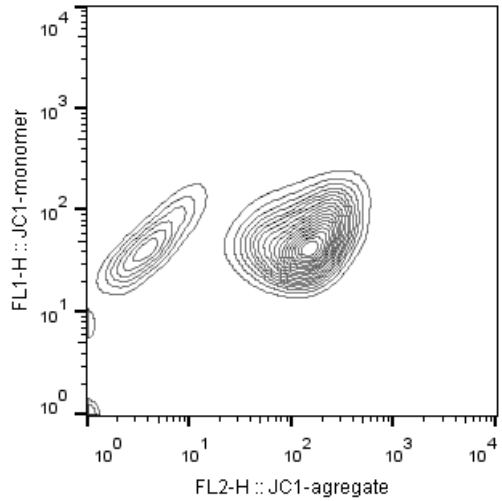

Fig. 5. When stimulated by conditioned culture medium obtained from mesenchymal stem cells treated with 10 μM leptin A and 10 μM LY294002 for 24 h, the developed endothelial progenitor cells were apoptotic in a proportion of 35% on average (representative experiment)

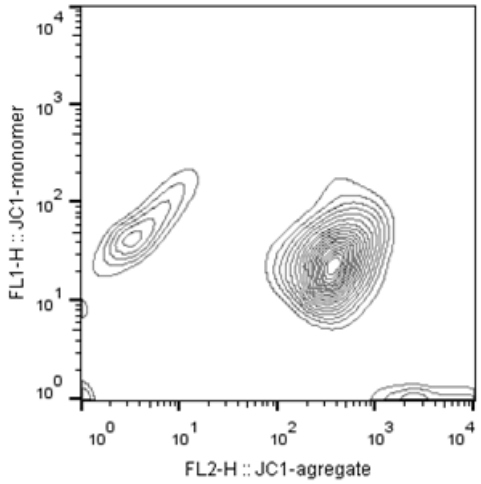

Fig. 6. Conditioned culture medium of experimental mesenchymal stem cells (10%), previously treated for 24 h with 10 μM leptin and 10 μM AG490, had as a consequence an apoptosis proportion of 37% on average of isolated endothelial progenitor cells (representative flow cytometry)

The fine mechanisms underlying the above described effects were not explored in deep, remaining as goals for future studies.

The oxygen increased needs of the tissues might be covered through angiogenesis or neoangiogenesis, such a developing process being entirely dependent on endothelial stem and/or progenitor cells. The actual scientific data state that the involvement of adult stem cells in the above mentioned processes is far away of being clear. The very first experiments done in mice found that there exist resident stem cells of endothelial type in large arteries and veins, being CD157(+). That means such stem cells are positive for bone marrow stromal antigen-1 (bst1) detection. When implanted heterologously in liver's mice, they'll generate endothelial cells as of portal vein, sinusoids and central vein types. *In vitro*, they'll develop into colonies, starting from single cells. As an injury consequence, these resident stem cells will generate entirely the structures of vasculature. These data strongly support the idea of a hierarchy of endothelium inside the blood vessels. Moreover, the experimental data proved that the implanted vascular stem cells were able to bear the sinusoids as well as large vessels in the recipient normal liver for time exceeding one year. Meanwhile, such resident vascular stem cells redeemed the mice bleeding phenotypes when a hemophilia model was tested. These studies showed that the resident vascular stem cells (adult) are associating self-renewal abilities and, thus, the peripheral blood vessels are equipped with a putative potential for vascular regeneration in various condition [23].

The research involving the features and functioning of stem cells or progenitor cells are enlarged and comprises all types of precursor cells and methods [24-33].

Moreover, we are able to recycle a lot of wastes around us to develop the matrices for transferring and development of stem cells and progenitor cells in vitro and in vivo [34-44].

Calcification of bones and several vascular tissues was observed when mesenchymal stem cells were used in conjunction with hydroxyapatite/tricalcium phosphate assemblies [45].

Further studies are necessary to evidence the effects of some benzopyran derivatives, including without being exclusive, KL-1492 and KL-1507. Such derivatives were demonstrated to altering the functions of ATP-dependent K^+ channels (KATP). They should be studied applied to the interactions of different stem cells with vascular and perivascular tissues [46].

Since angiotensin peptides and derivatives are deeply involved in physiological and pathological conditions, the angiotensin-aldosterone system and modulated oxidative stress should also be extensively studied in a close relationship with known and established mesenchymal stem cells [46, 47].

Conclusions

The goal of our studies was represented by the apoptotic effects of the culture medium obtained from rat mesenchymal stem cells developed on collagen matrix (fibers) on isolated circulating endothelial progenitor cells. The mesenchymal stem cells were stimulated with thapsigargin, concanavalin A, leptin, ghrelin, and were used the following inhibitors: AG490, a JAK2 inhibitor; PD98059, a MEK/ERK inhibitor; and LY294002, a PI3K/Akt signaling inhibitor.

The fine mechanisms underlying the above described effects were not explored in deep, remaining as goals for future studies.

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