Biochemical Effects of Some Endoplasmic Reticulum Stress Inducers on Mesenchymal Stem Cells *in vitro*

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Our studies aimed the effects of some endoplasmic reticulum stress inducers (thapsigargin, a Ca^{2+}-ATP-ase inhibitor; tunicamycin, a protein N-glycosylation inhibitor; brefeldin A, a protein transport inhibitor; paraquat, an enhancer of reactive oxygen species production; A23187, a Ca^{2+} ionophore), as well as some antioxidants (N-acetylcysteine; dithiothreitol, a disulfide bond formation inhibitor) on apoptosis of cultured rat mesenchymal stem cells. The analyze of obtained results evidenced that paraquat, a common and effective herbicide, induced the apoptosis of the isolated rat mesenchymal stem cells in a larger proportion as compared to other chemicals as follows: paraquat > thapsigargin > tunicamycin ≅A23187 > brefeldin A. Dithiothreitol was effective as a reducer of mesenchymal stem cells apoptosis when was administered as co-treatment for paraquat for 24 h. In contrast, N-acetylcysteine, another potent antioxidant, had no protective effects against paraquat apoptotic effects.

Keywords: mesenchymal stem cell, rat, paraquat, thapsigargin, dithiothreitol

The efficiency of mesenchymal stem cells treatments is decreased by their poor survival capacities. The most incriminated condition is represented by the deprivation of growth factors supported by donor mesenchymal stem cells. When mesenchymal stem cells were grown in serum-deprived conditions and stained with Hoechst 33342/propidium iodide, it was demonstrated through Western blotting an important apoptosis in the first 72 hours of treatment. Some altered protein expressions were found for caspase 3, caspase 8, glucose regulated protein 78/ kDa and C/EBP homologous protein. The analyzed data from these experiments demonstrated that the important apoptosis of serum-deprived mesenchymal stem cells within 72 hours extensively included the major apoptotic pathways, represented by mitochondrial, extrinsic and endoplasmic reticulum stress ones. The parallel increased expression of C/EBP homologous protein and decreased expression of Bcl-2 proteins suggested a synergistic involvement of both mitochondria and endoplasmic reticulum in serum-deprived mesenchymal stem cells apoptosis [1].

The diseases affecting the distal lung such as the fibrosis or acute lung injury are basically including as pathophysiological mechanism the alveolar hypoxia, which might be harmful when the apoptosis of alveolar epithelial cells is highly stimulated. When human mesenchymal stem cells, of allogenic origin, were administered as treatments in mouse models of pulmonary injury, these types of cells were able to provide a protective effect of paracrine kind, reducing the lung inflammatory processes and concomitant fibrosis. The observed protective effects of mesenchymal stem cells treatments in such experimental models were associated with their conditioning culture medium properties. Alveolar epithelial cells of rat origin, when exposed to hypoxic conditions *in vitro* (only 1.5% O_2 for 24 h), showed an increased accumulation of HIF-1 factor (hypoxia-inducible factor) and an enhanced apoptosis. Both anterior mentioned conditions were reduced by a HIF-1 inhibitor or an antioxidant (oxidative stress reduction) treatment. The hypoxic apoptosis of alveolar epithelial cells was significantly decreased by concomitant culture with human allogenic mesenchymal stem cells. Human allogenic mesenchymal stem cells were able to reduce HIF-1 factor expression, as well as reactive oxygen species accumulation, the underlying mechanisms being represented by an enhancement of antioxidant enzymes and their biologic activities. In the same time, human allogenic mesenchymal stem cells treatments highly reduced the expressed Bnip3 and C/EBP homologous protein and augmented the expression of anti-apoptotic Bcl-2 proteins. The clear conclusions of these studies are that human allogenic mesenchymal stem cells treatments would have a protective effect against hypoxia-induced apoptosis of alveolar epithelial cells in co-culture, the paracrine effect being able to prevent the enhanced expression and accumulation of hypoxia-inducible factor alpha and reactive oxygen species, and being partially dependent on keratinocyte and hepatocyte growth factors [2].

Some experiments were aiming the roles of microRNA 138 (miR-138) in osteoporosis. The involved cells were osteoblasts (MC3T3-E1 cell type) and the injury was induced using hydrogen peroxide. The cells were transfected in the view of miR-138 as well as TIMP-1 expressed levels modulation in such osteoblasts. Hydrogen peroxide was able to inhibit the development of MC3T3-E1 cells and induced proven stress of endoplasmic reticulum, concomitantly doubled by miR-138 enhanced expression. The studies showed that miR-138 overexpression was able to promote the apoptosis of osteoblasts *in vitro* as well as *in vivo* experiments. It was able to negatively regulate the expression of TIMP-1 in MC3T3-E1 cells. The downregulation of TIMP-1 basically mediated the apoptotic effects induced by endoplasmic reticulum stress of MC3T3-E1 cells (osteoblasts type). The results showed that

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osteoporosis associates apoptosis induced by endoplasmic reticulum stress and miR-138 consecutive induction, further activating at least partially the TIMP-1 factor [3].

Our studies involved the effects of some endoplasmic reticulum stress inducers (thapsigargin, a Ca^{2+}-ATP-ase inhibitor; tunicamycin, a protein N-glycosylation inhibitor; brefeldin A, a protein transport inhibitor; paraquat, an enhancer of reactive oxygen species production; A23187, a Ca^{2+} ionophore), as well as some antioxidants (N-acetylcysteine; dithiothreitol, a disulfide bond formation inhibitor) on apoptosis of cultured rat mesenchymal stem cells.

Experimental part

The mesenchymal stem cells used in our experiments were obtained and cultured as previously described [4]. Chemically-induced apoptosis of rat isolated mesenchymal stem cells was measured using well-established protocols within our laboratory for multiple lines of cells and used the flow cytometry techniques [5-6].

Chemically-induced apoptosis of rat isolated mesenchymal stem cells was assessed after the treatment for 24 h in culture with thapsigargin (Ca^{2+}-ATP-ase inhibitor), tunicamycin (protein N-glycosylation inhibitor), brefeldin A (protein transport inhibitor), paraquat (enhancer of reactive oxygen species production), A23187 (Ca^{2+} ionophore), all in concentrations of 1µM. For some experiments we also concomitantly administered some antioxidants: N-acetylcysteine and dithiothreitol (disulfide bond formation inhibitor), in concentrations of 1µM.

All the actual protocols involving Wistar rats (from Baneasa source) were previously approved by the Ethics Committee of the Grigore T. Popa University of Medicine and Pharmacy from Iasi.

Results and discussions

When we were starting the analysis of obtained results we were able to evidence that paraquat, a common and effective herbicide, induced the apoptosis of the isolated rat mesenchymal stem cells in a larger proportion as compared to other chemicals: paraquat (fig. 1) > thapsigargin (fig. 2) > tunicamycin (fig. 3) ≅ A23187 (fig. 4) > brefeldin A (fig. 5). Dithiothreitol was effective as a reducer of mesenchymal stem cells apoptosis when was administered as co-treatment for paraquat for 24 h (fig. 6). On the other hand, N-acetylcysteine, another potent antioxidant, had no protective effects against paraquat apoptotic effects.

The inhibitory effects of dithiothreitol, a powerful antioxidant, on paraquat-inducing apoptotic effects were very important from the point of view of analysis of the obtained results.

N-acetylcysteine, another powerful antioxidant used in experiments, wasn't effective as a preventive agent againt paraquat apoptotic effects on isolated rat mesenchymal stem cells *in vitro*.

The intimate molecular mechanisms involved by the above interesting effects remain to be developed by our future studies on apoptosis of mesenchymal stem cells.

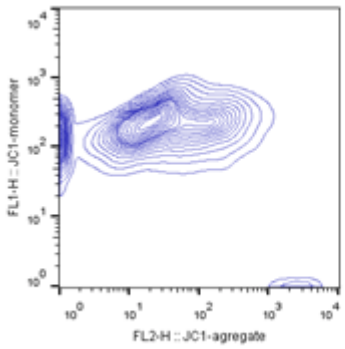

Fig. 1. When 1 µM paraquat was used as treatment for 24 h, the isolated cultured rat mesenchymal stem cells were subjected to apoptosis in a proportion of 63.78±2.35% (7 experiments, representative experiment presented)

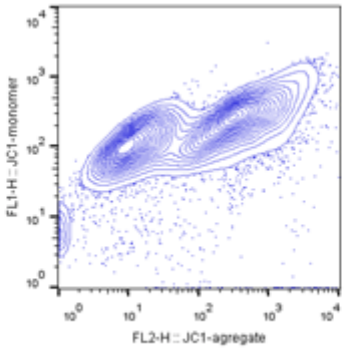

Fig. 2. The treatment of mesenchymal stem cells with 1 µM thapsigargin induced apoptosis in a proportion of 36.6±1.85% (7 experiments, representative flow cytometry)

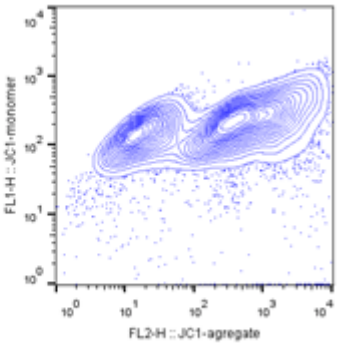

Fig. 3. When were treated with 1 µM tunicamycin for 24 h the isolated rat mesenchymal stem cells in culture were associating apoptosis in a proportion of 28.2±1.11% (7 experiments, representative experiment presented)

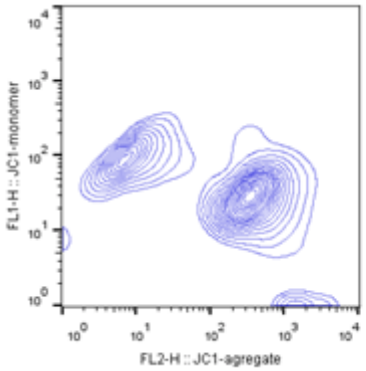

Fig. 4. A23187, 1 µM, a Ca^{2+}-ionophore, induced an apoptotic index of 25.37±1.72% on mesenchymal stem cells from rat origin in culture (7 experiments, representative flow cytometry).

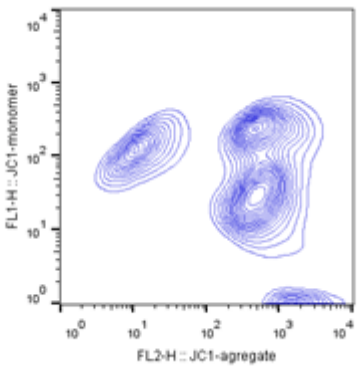

Fig. 5. When isolated mesenchymal stem cells were treated with 1 µM for 24 h, the cultured isolated rat mesenchymal stem cells were associating apoptosis in a proportion of 18.8±2.17% (7 experiments, representative experiment presented).

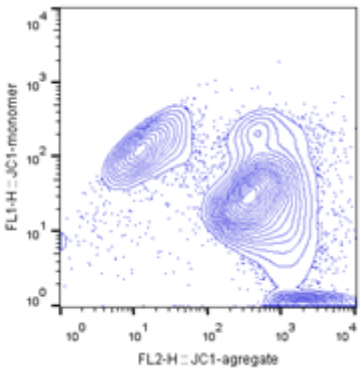

Fig. 6. Dithiothreitol, a disulfide bond formation inhibitor, reduced significantly the apoptotic index of cultured isolated mesenchymal stem cells to 16.27±1.64%, when was administered as 1 µM co-treatment for 24 h for 1 µM paraquat (representative flow cytometry).

A variety of aspects of cellular physiology and pathology are modulated and including the intracellular calcium pathways. The pathophysiological mechanisms of cardiovascular diseases, cancerogenesis, diabetes mellitus, hepatitis (e.g., steatosis) are all related to abnormal regulation of intracellular calcium pathways. Calcium signal transduction control involves a plethora of proteins, channels, transporters and enzymes, well-coordinated and orchestrated to fine tune all the temporally and spatially precise intracellular calcium signals. The kappelmeister of this orchestration is a Ca^{2+}-ATP-ase pump located in sarco/endoplasmic reticulum (named SERCA). The 14 variants of SERCA described are responsible for the re-capture and re-entering of released calcium into the cytosol, re-establishing the homeostasis of intracellular calcium. When the expression of these calcium pumps is altered, the cells are undergoing tumoral transformation or apoptosis, almost entirely induced by endoplasmic reticulum stress. The cell development and survival, as well as cell death (apotosis, autophagy, etc.) are both controlled by intracellular calcium concentrations and fluxes, SERCA pumps being a nodal turnover of all physiological and many pathological processes. This axis presents an extraordinary therapeutic potential since might allow targeting of multiple diseases [7].

Tumoral treatment has gain success through the appliance of strategies based on immunogenic cell death. That means at least the downregulation of several factors as CD47 or PD-L1. The Wharton jelly stem cells, isolated from human umbilical cord, used themselves or their conditioned medium from cultures, were described to have antitumoral effects. The mechanisms underlying such antitumoral effects are not clear. The known fact associated with Wharton jelly stem cells is that they are having immunomodulatory capacities. When lymphoma cells were co-cultured with Wharton jelly stem cells concentrates, they showed large mitochondrial and chromatin changes, evidencing augmented apoptotic index and decreased cellular viability, as well as endoplasmic reticulum functional alteration. Danger associated molecular patterns, activated and maturated dendritic cell pool, extracellular HMGB1 were all increased in lymphoma treated cells as compared to control ones. On the other hand, CD47 and PD-L1 molecules were reduced when compared to control, untreated cells. The augmented expression of multiple suppressor molecules for tumors as IL-6, miRNA-146a, miRNA-126, as well as MCP-1, IL-8 and IL-12 in Wharton jelly stem cells suggests that one or multiples of the anterior mentioned molecules are involved in immunogenic cell death [8].

The cells undergoing osteogenic differentiation are totally influenced by the architecture of the used biomaterials. From this point of view, the development of biofunctional surfaces and structures is closely related to our understanding of topographical signal transduction. The osteogenic differentiation and unfolded protein response are inter-related processes as was recently evidenced. The unfolded protein response pathway is linked with endoplasmic reticulum stress. The performed experiments included many surface topographies constructed on titanium foils and tested the involvement of pathways underlined by endoplasmic reticulum stress and unfolded protein response. The activation of endoplasmic reticulum stress and PERK-eIF2 alpha-ATF4 pathways were deeply dependent on topography and time, conditioning further the osteogenic capabilities. Furthermore, the tuning of endoplasmic reticulum stress by thapsigargin evidenced the osteogenic differentiation capabilities under mild stress, in contrast with its inhibition under excessive one. The final conclusion is that unfolded protein response is finely involved in osteogenic differentiation triggering and development, opening the door for knowledge on signal transduction related to topography [9].

From the point of view of our results it would be very interesting to apply the protocols for other cellular systems, stem or mature or both in co-culture. Thus we will be able to find out the involvement of endoplasmic reticulum stress and unfolded protein response in the functionality of young cells [10-19].

Endoplasmic reticulum stress could be induced by several plastics components, released in large amounts in the environment and absorbed in the body as it happens with paraquat herbicide [20-21]. Our goal should be the improvement of the quality of our life through the reducing of the environmental aggression [22-29].

Conclusions

Our studies aimed the effects of some endoplasmic reticulum stress inducers (thapsigargin, a Ca^{2+}-ATP-ase inhibitor; tunicamycin, a protein N-glycosylation inhibitor; brefeldin A, a protein transport inhibitor; paraquat, an enhancer of reactive oxygen species production; A23187, a Ca^{2+} ionophore), as well as some antioxidants (N-acetylcysteine; dithiothreitol, a disulfide bond formation inhibitor), on apoptosis of cultured rat mesenchymal stem cells.

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