SENESCENT FIBROBLASTS BETA-GALACTOSIDASE INDUCED BY EXTRACELLULAR VESICLES OBTAINED FROM MSC-iPSC UNDER THE ACTION OF VARIOUS MODULATORS

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SENESCENT FIBROBLASTS BETA-GALACTOSIDASE INDUCED BY EXTRACELLULAR VESICLES OBTAINED FROM MSC-iPSC UNDER THE ACTION OF VARIOUS MODULATORS (Abstract): The absolute majority of published experimental data involve mesenchymal stem cells, although the possibilities offered by the use of pluripotent stem cells reprogrammed from mesenchymal stem cells are practically infinite. **Aim:** The goal of our study was represented by tracking the effects of stimulation and inhibition of multiple functional, intracellular pathways of senescent dermal fibroblasts in culture, in the presence of microvesicles released from pluripotent stem cells reprogrammed from mesenchymal stem cells. **Material and methods:** We used flow cytometry to measure the production of the β-galactosidase level by dermal fibroblasts in culture after 30 passages and ultraviolet treatment. **Results:** Reduction of β-galactosidase concentrations in senescent dermal fibroblasts was evident upon administration of exosomes (approximately 20% on average) and exosomes co-administered with: LY-294002 (a highly selective PI3K kinase inhibitor, 41% on average); nutlin-3 (which stabilizes the non-genomic actions of p53, respectively the MDM2/p53 pathway, 63% on average); rapamycin (which stimulates autophagy and the mTOR pathway and related ones, 62% on average); and metformin (a pharmacologically active substance, considered a reprogrammer of energy metabolism, 73% on average). **Conclusions:** Exosomes derived from pluripotent stem cells induced from mesenchymal stem cells, administered in the culture medium of senescent dermal fibroblasts, can modify the degree and evolution of the senescence of these last cells by potentiating, activating, or stimulating some intracellular pathways of biological signal transduction, but especially through epigenetic reprogramming of energy metabolism. **Keywords:** MSC-iPSC, ENERGY METABOLISM REPROGRAMMING, EXOSOMES, FIBROBLASTS, MSC, EPIGENETICS.

The skin is the largest human organ and has a multitude of functions. It is well known that the healing of a skin wound/injury activates an impressive number of unique functionalities, which include cascaded cellular mechanisms and which, at the same time, represent a unique process in nature. While regeneration defines the specific replacement of tissue, with reference to the superficial epidermis, mucosa, or fetal skin, skin repair processes actually represent a non-specific form of healing, in which the
injury is healed by extensive fibrosis and scar formation. The clear physiological objective of wound repair in mammals ultimately equates to the formation of scars, a phenomenon that is directly proportional to the extent of the inflammatory process throughout healing (1).

The healing of injuries is thus a complex and dynamic process, at the same time supported by a multitude of cellular processes, which require strict coordination to be able to efficiently repair the damaged tissue. Alteration of cellular behaviors, which occurs for example in diabetes and the aging process, can lead to significant impairment of healing and the appearance of chronic, non-healing lesions. This chronic evolution represents a particularly important socio-economic burden due to its high prevalence and recurrence. Thus, there is a great need for a better understanding of all the complex mechanisms underlying healing (2).

Chronic lesions become superinfected with bacteria, which aggravates the inflammatory process. Keratinocytes in chronic lesions exhibit aberrant activation, resulting in hyperproliferation and impaired local migration. A very large number of cells present at the level of chronic lesions (for example, macrophages and fibroblasts) become evolutionarily senescent and transform into a senescence-associated secretory phenotype (or called SASP), which further perpetuates senescence, activates the massive release of reactive oxygen species (ROS) and further intensifies the inflammatory process. Moreover, the large amounts of final products of advanced glycation that appear contribute, at the same time, to the development of the inflammatory process and cellular senescence at the level of the lesion. Together, all these characteristics produce excessive tissue destruction and alter cellular functions, preventing normal healing (3).

Mesenchymal stem/stromal cells (MSCs), due to their competences to establish skin cells such as fibroblasts and keratinocytes, as well as their special attribute to suppress inflammation in the inflammatory focus of wounds have attracted increasing attention among researchers. Thus, exosomes transfer practical cargoes (e.g., growth factors, cytokines, miRNAs, etc.) from mesenchymal stem cells to target cells, thus affecting extraordinarily important biological functions of recipient skin cells, such as migration, the expansion and also the secretion of particularly important elements of the extracellular matrix (for example, collagen of various types) (4).

The goal of our study was represented by the tracking of the effects of stimulation and inhibition of multiple intracellular functional pathways of senescent dermal fibroblasts in culture, in the presence of microvesicles released from pluripotent stem cells reprogrammed from mesenchymal stem cells.

MATERIAL AND METHODS

The laboratory research that we carried out aimed at quantifying the evolution of the degree of senescence of dermal fibroblasts (senescent cells) by evaluating β-galactosidase through flow cytometry (5, 6), in a bi-culture, in the presence of pluripotent stem cells reprogrammed from mesenchymal stem cells (in culture) or under the action of exosomes/microvesicles released from MSC-iPSCs, in the form of concentrated supernatant, administered in the culture medium.

Storage rat dermal fibroblasts (cell line no. 1) were grown under standard laborato-
ry conditions and tested for β-galactosidase through flow cytometry after passage 1, passage 10, passage 20, and passage 30. To obtain senescent dermal fibroblasts, they were irradiated with ultraviolet, λ=315 nm, 80 mJ/cm², and then multiplied by means of 30 consecutive passages.

To obtain MSC-iPSCs, we used the episomal method with iPSC reprogramming vectors and the specific Stemline culture medium (Invitrogen), in full agreement with the manufacturer’s specifications. We preferred this technique because the use and manipulation of potentially genetic material included in the “dangerous” chapter are totally excluded. The growth and multiplication of pluripotent stem cells reprogrammed from mesenchymal stem cells were done using an Alvetex 3D bio support-a gift from Reinnervate-, since a series of other experiments demonstrated the maximum efficiency for this type of support.

For the large-scale evaluation of possible interactions between the cell lines we used, first of all, a series of preconditioning factors for MSC-iPSCs: 1% O₂, 1 µM angiotensin II, vitamin E 0.5 µM, and low doses of lipopolysaccharide (0.1 µM) (7).

For exosome separation, the culture medium of pluripotent stem cells induced from rat aortic smooth muscle cells was centrifuged up to 100,000xg (8).

The active substances we used for the various experiments and protocols including senescent dermal fibroblasts were: 1 µM LY294002, 1 µM nutlin-3, 1 µM rapamycin, and 2 µM metformin.

All the experimental results obtained during the present research were processed entirely by statistical methodologies, using a number of well-known and optimized tests over time and for medical research, among which the One-Way ANOVA test should be mentioned, which was also completed, only occasionally, with the Student-Newman-Keuls test.

RESULTS

During the development and multiplication processes, strictly monitored, MSC-iPSCs presented a series of characteristics of embryonic morphology, constantly observed in cell culture conditions, being otherwise undifferentiated, even organizing themselves in the form of aggregates/organoids, demonstrating it therefore clearly belongs to this cell type.

To verify the viability of experimentally reprogrammed pluripotent stem cells from mesenchymal stem cells, grown on 3D Alvetex bio support, preconditioned with 1% O₂, angiotensin II, vitamin E, and low doses of lipopolysaccharide, we used 0.1 μM 5-carboxyfluorescein (succinimidyl ester), with excitation/emission maxima 498/517 nm (green fluorescence) and flow cytometry as stated. The best qualitative and quantitative development in the case of pluripotent stem cells experimentally reprogrammed from mesenchymal stem cells, starting from the 2nd passage in culture, we obtained when we multiplied them in a preconditioning medium that contained a concentration of only 1% O₂. We considered this increase to always be 100% (n=5 experiments for each set, data not shown, personal observation). Comparatively, development in the presence of angiotensin II in the preconditioning medium was 79.23±2.74% (n=5), in the presence of vitamin E it was 72.39±2.87% (n=5) and in the presence of lipopolysaccharide was 76.72±1.79% (n=5).

In addition, the most important β-galactosidase reduction effects in senescent dermal fibroblasts in monoculture, in the presence of exosomes/microvesicles at a
Senescent fibroblasts beta-galactosidase induced by extracellular vesicles obtained from MSC-iPSC under the action of various modulators

concentration of 0.01%, obtained in concentrated form by ultracentrifugation of the MSC-iPSC supernatant, were observed when we developed these latter cell types in a medium preconditioned with 1% O₂. Thus, under these conditions, we found amplification of β-galactosidase reduction in senescent dermal fibroblasts by approximately 20% (on average).

In a series of experiments, a number of senescent dermal fibroblasts were treated with 1 µM LY294002, considered to be a highly selective PI3K kinase inhibitor, as well as 0.01% exosomes obtained from the supernatant of MSC-iPSC. We found amplification of β-galactosidase reduction in senescent dermal fibroblasts by approximately 41% (on average) when MSC-iPSC were multiplied in a medium preconditioned with 1% O₂ (fig. 1).

In the second series of experiments, a different batch of senescent dermal fibroblasts was treated with 1 µM nutlin-3, which stabilizes with great efficiency the non-genomic actions of p53 (MDM2/p53 pathway), as well as 0.01% exosomes obtained from the supernatant of MSC-iPSC. We found amplification of β-galactosidase reduction in senescent dermal fibroblasts by approximately 63% (on average) when MSC-iPSC were multiplied in a medium preconditioned with 1% O₂ (fig. 2).

In the third series of experiments, an important number of senescent dermal fibroblasts were treated with 1 µM rapamycin, which stimulates autophagy and the mTOR pathway and related ones, as well as 0.01% exosomes obtained from the supernatant of MSC-iPSC. We found amplification of β-galactosidase reduction in senescent dermal fibroblasts by approximately 62% (on average) when MSC-iPSC were multiplied in a medium preconditioned with 1% O₂ (fig. 3).

Finally, in the fourth series of experiments, a totally different batch of senescent dermal fibroblasts was treated with 2 µM metformin, a pharmacologically active substance, considered a reprogrammer of energy metabolism, as well as 0.01% exosomes obtained from the supernatant of MSC-iPSC. We found amplification of β-galactosidase reduction in senescent dermal fibroblasts by approximately 73% (on average) when MSC-iPSC were multiplied in a medium preconditioned with 1% O₂ (fig. 4).

Fig. 1. Quantification by flow cytometry of β-galactosidase in senescent dermal fibroblasts in monoculture, when multiplied in the presence of LY294002 (1 µM) and MSC-iPSC-released exosomes, preconditioned in 1% O₂ medium. p<0.05 as compared to the normal culture medium.
Fig. 2. Measurement of β-galactosidase in senescent dermal fibroblasts, when developed in the presence of nutlin-3 (1 µM) and MSC-iPSC-released vesicles, preconditioned in 1% O₂ medium. p<0.01 as compared to the normal culture medium.

Fig. 3. Determination of β-galactosidase in senescent dermal fibroblasts, when cultured in the presence of rapamycin (1 µM) and MSC-iPSC-released extracellular vesicles, preconditioned in 1% O₂ medium. p<0.01 as compared to the normal culture medium.

Fig. 4. Evaluation of β-galactosidase in senescent dermal fibroblasts in monoculture, when grown in the presence of metformin (2 µM) and MSC-iPSC-released extracellular microvesicles, preconditioned in 1% O₂ medium. p<0.01 as compared to the normal culture medium.
DISCUSSION

Mesenchymal stem cells (MSCs) have the potential to be used in cell-based regenerative therapies. Currently, hundreds of clinical trials are using MSCs for the treatment of various diseases. However, MSCs are low in number in adult tissues; they show heterogeneity depending on the cell source and show limited proliferative capacity and early senescence in vitro cultures. These elements negatively affect the regenerative capacity of MSCs and therefore limit their use for clinical applications. As a result, unique methods to generate induced MSCs (iMSCs) from induced pluripotent stem cells have actually been verified. Advancement and optimization of procedures for generating iMSCs from induced pluripotent stem cells is necessary to assess their regenerative capacity in vivo and in vitro. In addition, it is essential to compare iMSCs with primary MSCs (separated from adult tissues) regarding their safety and efficacy. Careful examination of the promising properties of iMSCs in vitro and their long-term interaction habits in animals is essential for their transfer from bench to patient (9, 10).

Human derived pluripotent stem cells (hiPSCs) are indispensable resources for the production of premium, separated cells in unrestricted quantities for both basic research studies and scientific use. They are particularly useful for studying human disease systems in vitro, making it possible to avoid the ethical problems of human embryonic stem cell research. However, there are substantial constraints when using traditional flat culture approaches, which specifically impair cell growth, differentiation performance, maintenance of stability, and facilitation of multicellular 3D structure. Embryoid bodies, which are multicellular aggregates spontaneously created from iPSCs in the suspension system, can help address these issues. Due to the unique microenvironment and cell communication within the structure of embryoid bodies, which a 2D culture system cannot achieve, they have actually been widely applied in hiPSC-derived differentiation and show considerable advantages especially in culture scale-up, performance improvement differentiation and ex vivo simulation. Embryoid bodies can also be used in early prediction of the differentiation capacity of iPSCs (11, 12).

Both mesenchymal stromal cells and fibroblasts can be isolated from almost all organs. Mesenchymal stromal cells represent the most favorable cellular healing agents with regenerative potential, and fibroblasts are among the most abundant cell types with the ability to preserve homeostasis. Due to the promising and attractive properties of MSCs, they have been well studied and thus their differentiation capacities, immunomodulatory potentials, as well as gene expression profiles are determined. Fibroblasts and mesenchymal stromal cells have been observed to have comparable morphology, gene expression patterns, surface markers, expansion, differentiation, and immunomodulatory capabilities. Therefore, it is difficult to differentiate these two types of cells. Epigenetic signatures, i.e., the methylation patterns of cells, are the only appealing functional distinction between them. Such substantial similarities show that these two cells may be associated with each other (13, 14, 15).

Cellular senescence is currently recognized as one of the nine characteristics of the aging phenomenon. Recent experimental data show, on the other hand, the major involvement of senescent cells in
tissue aging and even more so in some age-related diseases. Skin is considered to be an ideal model for the study of aging phenomena. If features of senescence are evident with skin aging, the senescent phenotype is different in different skin cell types. Moreover, the origin of cellular senescence in the skin is still far from being known and multiple origins are possible. These aspects reflect the mosaic nature of skin aging (16).

Skin aging is the most visible and obvious manifestation of the body's aging and can directly serve as a predictor of life expectancy and health status. However, it is the human desire for very long-lasting beauty that further exacerbates the interest in this subject, and therefore considerable means and efforts are devoted to studying the mechanisms of skin aging through both basic and applied research. Any type of interest, medical and non-medical, is generally welcome for the development of research in the field of the skin system, it being clear that all the results of these studies can be of great help in our attempt to deepen the understanding of complex molecular and biological processes, the type of cellular and developmental signaling, as well as the immunological type, manifested at the level of this vast organ. In fact, the skin is an ideal and relatively handy organ for observing and analyzing the impact of possible extrinsic and intrinsic driving factors of aging (17, 18).

At the skin level, aging is manifested by the appearance of photodamage and skin atrophy, with the consequent reduction of underlying tissues and impaired barrier function. To determine whether rapamycin, a drug already approved by the US authorities, which targets the mechanistic component of the mTOR complex, can reduce senescence and, at the same time, markers of aging in human skin, an exploratory, placebo-controlled interventional study was conducted, in a strictly controlled clinical dermatology setting. Topical rapamycin was observed to significantly reduce p16INK4A protein expression, consistent with a significant reduction in cellular senescence. This tissue change was accompanied by an important and relative improvement in the clinical appearance of the skin and even histological markers of aging, as well as an important increase in collagen type VII, which is an essential component of basement membrane integrity (19).

Increasing evidence suggests that chronic inflammation and senescence underlie the development of many severe age-related diseases, given that both biological processes appear to be directly proportional to the degree to which the body ages. However, until now, the existence of any specific gene related to inflammation or senescence that is common to different species or different tissues has remained unknown. Thus, these potential markers of aging could greatly help to identify possible targets for therapeutic interventions in conditions associated with aging phenomena and could also help us to deepen our understanding of the main mechanisms of aging. In addition, it was very interesting to note the experimental fact that genes involved in aging-related processes tend to be much more tightly controlled in long-lived people compared to middle-lived people. These observations could suggest the existence of a general genetic level, which would affect the lifespan of any individual. Taken as a whole, such a descriptive study has made a major contribution to a better understanding of the genetic signatures common to aging phenomena, as well as the patterns of
aging that may be tissue-specific in particular (20).

Senolytics are a relatively new class of drugs believed to selectively eliminate senescent cells. Thus, the first senolytic drugs, which include dasatinib, quercetin, fisetin, and navitoclax, were discovered using a hypothesis-only approach. The most aggressive senescent cells are highly resistant to apoptosis and show hyper-stimulated anti-apoptotic pathways, which can protect them from their own secretory phenotype associated with senescence, a fact that allows them to survive, even if the neighboring cells are eliminated. On the other hand, senolytics transiently deactivate these pro-survival complexes / pathways, inducing apoptosis of those senescent cells, which have a tissue-destructive SASP. Since it takes weeks to restore senescent cell mass, senolytics can be administered intermittently in a hit-and-run approach. In a number of preclinical models, it has been shown that senolytics can delay, prevent, or significantly improve body fragility, tumorigenesis, but also cardiovascular, neuropsychiatric, hepatic, renal, and musculoskeletal disorders, pulmonary conditions, but also eye, hematological, metabolic, and skin conditions, as well as organ transplant complications, but also those of radiotherapy and, last but not least, those of cancer treatment. Early pilot tests for a number of senolytics strongly suggest that they do indeed decrease senescent cell mass, reduce inflammatory processes, and improve frailty in humans. Clinical trials involving their use in diabetes, idiopathic pulmonary fibrosis, Alzheimer's or COVID-19, osteoarthritis, osteoporosis, eye disease, bone marrow transplantation, and last but not least, in the case of the childhood cancer survivor, are in progress or have just begun (21, 22, 23).

Senescent cells, which are able to release factors in the microenvironment that cause inflammation and dysfunction, assimilating to the so-called secretory phenotype associated with senescence (also known as SASP), increase numerically with aging and, at the same time, at the level of multiple etiological sites chronic diseases. Senolytic-type pharmacological compounds, including the combination of dasatinib and quercetin, can selectively eliminate senescent cells through a mechanism of transient deactivation of pro-survival complexes, which protect them from their own apoptotic environment. Thus, in the first clinical trial in which senolytics were used, dasatinib and quercetin significantly improved physical functions in patients with idiopathic pulmonary fibrosis, a fatal disease inextricably associated with senescence. However, to date, no research has directly demonstrated that senolytics can reduce the number of senescent cells in humans. On the other hand, in the case of the combination with dasatinib and quercetin, the mass of senescent cells in humans would be significantly reduced (24).

CONCLUSIONS

The most important β-galactosidase reduction effects in senescent dermal fibroblasts in monoculture, in the presence of exosomes/microvesicles at a concentration of 0.01%, obtained by ultracentrifugation of the supernatant of pluripotent stem cells reprogrammed from mesenchymal stem cells, were obtained when we grew these latter types of cells in medium preconditioned with 1% O₂.

Reduction of β-galactosidase concentrations in senescent dermal fibroblasts was
evident upon administration of exosomes (approximately 20% on average) and exosomes co-administered with: LY-294002 (a highly selective PI3K kinase inhibitor, 41% on average); nutlin-3 (which stabilizes the non-genomic actions of p53, respectively the MDM2/p53 pathway, 63% on average); rapamycin (which stimulates autophagy and the mTOR pathway and related ones, 62% on average); and metformin (a pharmacologically active substance, considered a reprogrammer of energy metabolism, 73% on average).

All these results suggest that exosomes derived from pluripotent stem cells induced from mesenchymal stem cells, administered in the culture medium of senescent dermal fibroblasts, can modify the degree and evolution of the senescence of these last cells by potentiating, activating, or stimulating some intracellular pathways of biological signal transduction, but especially through epigenetic reprogramming of energy metabolism.

CONFLICT OF INTEREST

AND FUNDING

The authors declare that there is no conflict of interest, and they received no specific funding regarding this scientific research

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Senescent fibroblasts beta-galactosidase induced by extracellular vesicles obtained from MSC-iPSC under the action of various modulators


**NEWS**

**HYPERMAGNESEMIA REDUCES THE RISK OF INTRACRANIAL ANEURYSMS:**
**A MENDELIAN RANDOMIZATION STUDY ON 23,829 INDIVIDUALS OF EUROPEAN ANCESTRY**

Serum magnesium levels have been extensively studied for subarachnoid hemorrhage after intracranial aneurysm (IA) rupture as a treatment for vasospasm however its influence regarding aneurysm formation and progression has not been researched yet. The authors have used a mendelian randomization approach for 5 single nucleotide polymorphisms as variables for serum magnesium in a genome-wide association study in 23,829 individuals of European ancestry. The results proved an association between higher serum magnesium concentration and lower risk of all IAs, as well as unruptured and ruptured IAs examined separately. Also, at least partially, the results suggest that magnesium mediates the role of blood pressure in IA. Overall, this study is the first to identify a potential causal association between serum magnesium concentrations and risk of IA and it provides preliminary evidence for further research regarding the role of magnesium in IA management (Larsson SC, Gill D. Association of Serum Magnesium Levels With Risk of Intracranial Aneurysm: A Mendelian Randomization Study. *Neurology* 2021: 10.1212/WNL.0000000000012244 / doi:10.1212/wnl.0000000000012244).

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