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研究課題名（和文）Mesenchymal stem cell function to prevent regulated cell death

研究課題名（英文）Mesenchymal stem cell function to prevent regulated cell death

研究代表者
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研究成果の概要（和文）：ヒト間葉系幹細胞（ＭＳＣ）は、成人骨髄および脂肪組織などの複数の生物学的組織、臍帯および胎盤などの新生児組織から単離され、そして現在多くの臨床試験で使用されている。ＭＳＣは、対宿主性疾患を含む重度の変性疾患および炎症性疾患の治療的介入に使用される。変性疾患または炎症性疾患におけるＭＳＣ療法の成功は、ＭＳＣの生物学的機能の頑健性にかかっている。我々は、ＭＳＣがストレスを受けた細胞と相互作用し、それによって全体的な細胞生存および機能を改善することを解明した。この生物学的機能はほぼ決定的であり、そしてＭＳＣの治療効果のかなりの部分に関与している可能性がある。

研究成果の学術的意義や社会的意義
MSCs is a remarkable adult stem cell for cell therapy and advantageous for responding to various medical demands. The significance of our work resides in the use of MSC death modulation function to be exploited in the clinic for degenerative and inflammatory diseases in cell therapy based on MSCs.

研究成果の概要（英文）：Human Mesenchymal Stem Cells (MSCs) are isolated from biological tissues including adult bone-marrow and adipose tissues and neonatal tissues such as umbilical cord and placenta. A number of clinical trials are using MSCs for therapeutic interventions in severe degenerative and/or inflammatory diseases, including Alzheimer’s disease, Crohn’s disease and graft-versus-host disease. The success of MSC therapy in degenerative and/or inflammatory diseases depends on the robustness of the biological functions of MSCs. We and others have shown that MSCs interact with stressed cells, thereby improving the overall cells’ survival and function. From this aspect, an unexpected function of MSCs have been unraveled, and apprehended as MSC death modulation function. This biological function appears decisive and could carry a significant part of the therapeutic effects of MSCs.

研究分野：44010

キーワード：Mesenchymal stem cells　cell function　cell death　cell therapy

１．研究開始当初の背景

Human Mesenchymal Stem Cells (MSCs) are isolated from multiple biological tissues – adult bone-marrow and adipose tissues and neonatal tissues such as umbilical cord and placenta. A number of clinical trials are using MSCs for therapeutic interventions in severe degenerative and/or inflammatory diseases, including Alzheimer's disease, Crohn's disease and graft-versus-host disease. The success of MSC therapy in degenerative and/or inflammatory diseases depends on the robustness of the biological functions of MSCs. We and others have shown that MSCs interact with stressed cells, thereby improving the overall cells' survival and function. From this aspect, an unexpected function of MSCs have been unraveled, and apprehended as MSC death modulation function. This biological function appears decisive and could carry a significant part of the therapeutic effects of MSCs.

２．研究の目的

Our research aims are fundamentally centered 1) to decipher the mechanisms of action of MSCs in preventing RCD and, 2) to bring biological basis to implement selection protocols for MSCs to improve and ensure MSC therapy efficacy in clinical setting.

３．研究の方法

Our methods to achieve our research aims, were first to implement a comprehensive cell banking of human MSCs from different donors obtained from different biological sources, i.e. bone marrow (BM-MSCs) and adipose tissue (ASCs). These MSCs were characterized biologically ex vivo and further used in vitro in different system of co-cultures with either human macrophages or epithelial cells that were stimulated with specific inducers of either apoptosis, necroptosis or pyroptosis. Our data suggest that gap-junction, extracellular vesicles (EVs) and mitochondria transfer from MSCs to macrophages and epithelial cells undergoing RCD signalling, improve cell survival and cell functions.BM-MSCs and ASCs are both established as CD14-CD34-CD45- non-hematopoietic cells, and phenotypically characterized as CD73+CD90+CD105+ cells by flow cytometry analysis. Cell bank is established for each donor with a unique identification at passages P1 to P10. Human primary macrophages and alveolar epithelial cells, as well as monocyte-derived macrophages (THP-1) and lung epithelial cells (A549) cell lines are challenged with specific cell death inducers, such as bortezomib (necroptosis), etoposide (apoptosis), CH-11 (apoptosis), ATP (pyroptosis), nigericin (pyroptosis) or nanoparticles (pyroptosis). Stimulated cells are then co-cultured with MSCs in vitro at varying cell ratios to estimate MSC ability to shield cells from RCD signaling and execution. Cell death is measured by trypan blue dye exclusion, MTS, propidium iodide, annexin V, LDH release and cytokine release of IL-1β, IL-6, IL-10 and TNF-α. To compare MSC ability to shield RCD, various MSC products (various donors and biological sources), are tested in limiting dilution analysis (LDA). Transfer of mitochondria from MSCs to stressed cells are studied by fluorescence microscopy, confocal microscopy and transmission electron microscopy imaging. We observed that function of MSCs to modulate RCD, is not equally distributed among all MSCs. Therefore, we are developing potency tests in vitro to identify those MSCs with optimal function. Of note, selection criteria for subsets of MSCs that are intended for cell therapy in human diseases is of vital and immediate concern. Within my laboratory, we are optimizing in vitro functional assays for determining biological potency of BM-MSCs and ASCs from different donors.

４．研究成果

First, we developed in vitro tests as to assess different RCD including apoptosis, necroptosis and pyroptosis in human macrophages and epithelial cells and the potency of MSCs in modulating those RCD signaling.

a) Experimental model of macrophage pyroptosis mediated by Caspase-1.

Pyroptosis is featured with production and secretion of inflammatory cytokines that is rapidly followed by plasma membrane rupture and release of intracellular content (**Fig.**

1, Fig. 2 and Fig. 3). Pyroptosis is intrinsically inflammatory. Pyroptosis can be induced in macrophages (MΦ) by extracellular ATP, nigericin (Nig) and nanoparticles (NPs) that is measured with lactate dehydrogenase release (LDH) and IL-1β secretion.

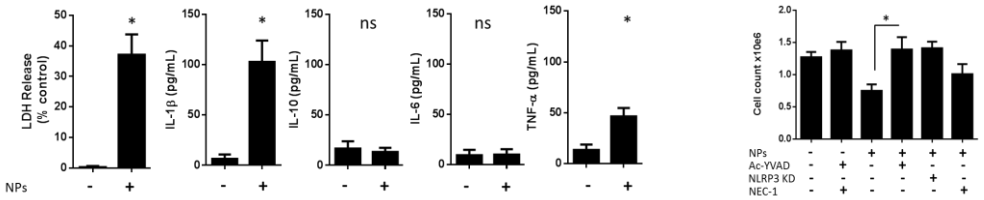
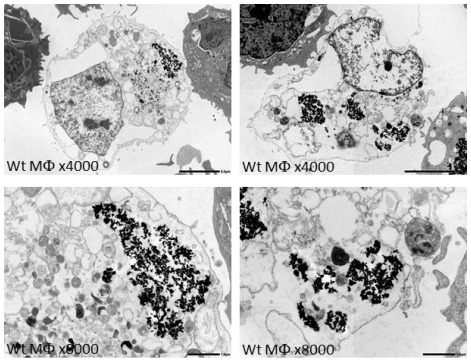


Figure 1. Herein, monocyte-derived macrophage cell line, THP-1 stimulated with phorbol myristate acetate (PMA), and in presence of NPs induce pyroptosis with LDH release and IL-1 β secretion at 18h post-stimulation but not of IL-6 measured in supernatants (n=6, *p<0.05 is considered significant, student's t-test). Cell death by pyroptosis is significantly attenuated in monocyte-derived macrophages in presence of NPs for 18h with an inhibitor of Caspase-1 (Ac-YVAD) but not with an inhibitor of RIPK1 (NEC-1).

Transmission electron microscopy (TEM) show morphological features of pyroptosis occurring in THP-1 monocyte-derived macrophages following 18h exposure to NPs (**Fig. 2 and Fig. 3**). Cell membranes are disrupted. That are the most evident morphological feature shown on TEM after NPs endocytosis by macrophages and pyroptosis execution. Cell death by pyroptosis is significantly attenuated in monocyte-derived macrophages in presence of NPs for 18h with an inhibitor of Caspase-1 (Ac-YVAD) but not with an inhibitor of RIPK1 (NEC-1). This attests for a cell death by pyroptosis but not necroptosis (n=6, *p<0.05 is considered significant, student's t-test).

Figure 2. Transmission electron microscope (TEM) analysis of monocyte-derived macrophage cell line, THP-1 stimulated with phorbol myristate acetate (PMA), in presence of NPs. TEM show morphological features of pyroptosis with cell membrane rupture in macrophages following endocytosis of NPs 18h post-stimulation (n=4). Magnification x4000 (top) and x8000 (down).



b) NLRP-3 genetic alteration turns pyroptosis into apoptosis.

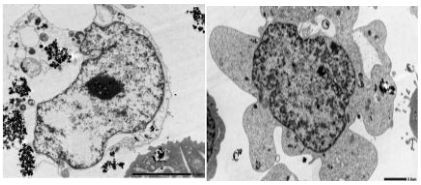


Figure 3. Transmission electron microscope (TEM) analysis of monocyte-derived macrophage cell line, THP-1 stimulated with phorbol myristate acetate (PMA), and in presence of NPs. THP-1 wild-type (top) versus THP-1 knock down for NLRP3 (down) TEM images show morphological features of pyroptosis and plasma membrane rupture in wild-type macrophages following endocytosis of NPs 18h post-stimulation (top), whilst in NLRP3 knock down macrophages apoptosis features are revealed with membrane blebs, (n=4). Magnification x4000.

Hence, inhibition of pyroptosis pathways may lead to apoptosis. The interest of using MSCs appear then obvious since MSCs show abilities to prevent any RCD including of pyroptosis and apoptosis (**Fig. 4, Fig. 5, Fig. 6, Fig. 7 and Fig. 8**).

c) Human mesenchymal stem cell characterization

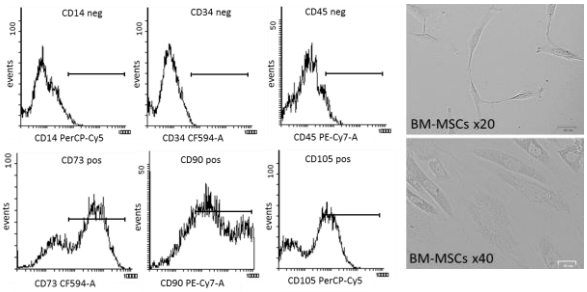


Figure 4. Human mesenchymal stem cells derived from bone marrow (BM-MSCs) or adipose tissue (ASCs). BM-MSCs and ASCs are negative for CD14, CD34, CD45 and positive for CD73 (ecto-5′-nucleotidase), CD90 (Thy1) and CD105 (endoglin). MSCs show a typical spindle-shape morphology. Data show representative flow cytometer analysis and optical microscope magnification x20 and x 40 photographs (n=12).

d) Supravital staining of human mesenchymal stem cells

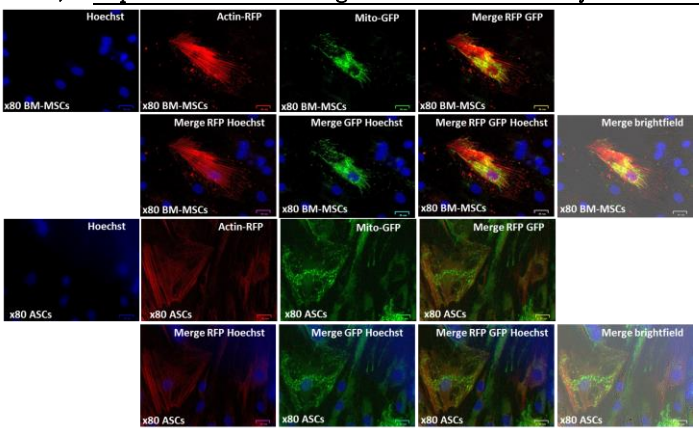


Figure 5. Human mesenchymal stem cell (BM-MSCs and ASCs) representative supravital staining of actin and mitochondria with transfection of plasmid constructs expressing genes for fluorescent fusion proteins that target specific intracellular structures. Images were acquired with a fluorescence microscope using alive (supravital) BM-MSCs and ASCs (n=6).

e) MSCs shield macrophages from cell death

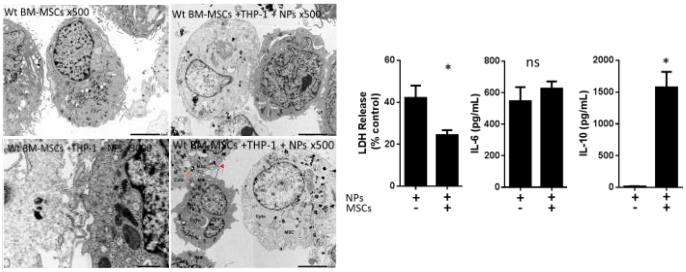


Figure 6. TEM show BM-MSCs in adjacent interactions with macrophages undergoing pyroptosis. The close interactions likely involve together gap-junction (GJ), tunneling nanotubes (TNTs) and extracellular vesicles (EVs) as shown (red and orange arrows) on TEM. Those interactions would allow transfer of mitochondria from MSCs to macrophages and thus prevent cell death by pyroptosis. LDH release is reduced by nearly half while IL-6 and IL-10 secretion are dramatically increased in presence of MSCs (n=6, *p<0.05 is considered significant, student's t-test).

The MSC death modulation function appears to contribute a part of the therapeutic effects of MSCs for various diseases. This function is implemented predominantly by MSCs through modes of actions implying direct cell-to-cell interactions requiring either cell contact with gap junctions and TNTs, or with EVs (**Fig. 6**). Together, these modes of actions might be interdependent in ultimately controlling the transfer of mitochondria from MSCs to unfit cells for the full potency of the MSC death modulation function.

f) Transfer of mitochondria in macrophages in presence of ATP or Nigericin

ATP stimulation of macrophages in co-culture with ASCs

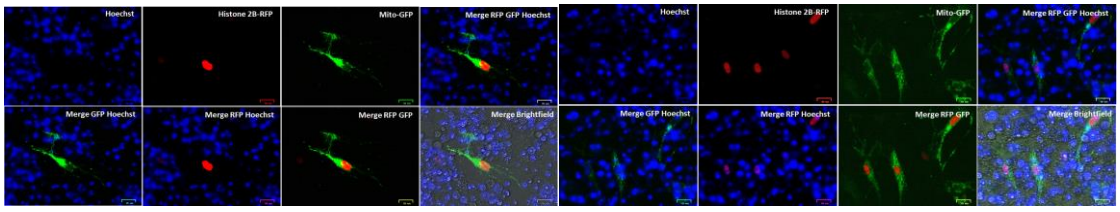


Figure 7. Human mesenchymal stem cell supravital staining of histone 2B and mitochondria with constructs expressing fluorescent fusion proteins targeted to specific intracellular structures. The fluorescent protein is introduced by transfection of plasmid containing DNA coding the assigned fluorescent protein. Images were acquired with a fluorescence microscope using alive ASCs in co-culture with monocytes derived macrophages in presence of ATP or nigericin. A representative image shows a transfer of mitochondria from BM-MSCs to macrophages via TNTs whether stimulated with ATP (top) or nigericin (down) (n=6).

g) MSCs shield from cell death both macrophages and epithelial cells

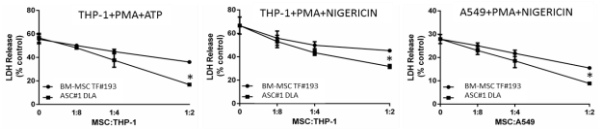


Figure 8. BM-MSCs (TF193) and ASCs (#1 DLA) reduce cell death induced by ATP and nigericin in human macrophages and epithelial cells. The data show a decrease in LDH release in presence of MSCs. ASCs compared to BM-MSCs possess a higher potential to shield from cell death in those conditions (n=6, *p<0.05 is considered significant, student's t-test).

Our in vitro potency test discriminates between MSCs with enhanced capabilities (ASC#1

DLA) to modulate cell death in human macrophages and epithelial cells stimulated with either ATP and/or nigericin and those of lesser capabilities (BM-MSCs TF#193).

５．主な発表論文等

〔雑誌論文〕（計３件）

1. Naji A (corresponding author), Eitoku M, Favier B, Deschaseaux F, Rouas-Freiss N, Suganuma N. Biological functions of mesenchymal stem cells and clinical implications. (peer reviewed). Cellular and Molecular Life Sciences, 1-26 2019. https://doi.org/10.1007/s00018-019-03125-1
2. Naji A (corresponding author), Favier B, Deschaseaux F, Eitoku M, Rouas-Freiss N, Suganuma N. Mesenchymal stem/stromal cell function in modulating cell death. (peer reviewed). Stem Cell Research & Therapy, 10(1), 56, 2019. https://doi.org/10.1186/s13287-019-1158-4
3. Naji A (corresponding author), Suganuma N, Espagnolle N, Yagyu KI, Baba N, Sensebé L, Deschaseaux F. Rationale for determining the functional potency of mesenchymal stem cells in preventing regulated cell death for therapeutic use. (peer reviewed). Stem Cells Translational Medicine, 2017; 6(3): 713-719. https://doi.org/10.5966/sctm.2016-0289

〔学会発表〕（計１件）

Naji A Mesenchymal stem cell functions and clinical application. 9th Symposium on Cell Therapy, FujiSoft Tissue Engineering, Tokyo, Japan, 27th January. 2018.

〔図書〕（計０件）

〔産業財産権〕

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名称：
発明者：
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取得年：
国内外の別：

〔その他〕

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