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Possible Existence of the Relict Mechanism of Cell Protection During Division

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ABSTRACT

Antibacterial properties of the stem cells and human- and plant- derived microvesicles are attracting increasing attention of the research community. Awareness of publications pointing out to the pronounced anti-bacterial properties of microvesicles isolated from the supernatants of different human origin prompted us to test corresponding properties of supernatant isolated using simplified protocol. Extensive research with more than 100 laboratory strains and hospital isolates of Gram-positive and Gram- negative bacteria point out to a pronounced anti-bacterial activity of the supernatant isolated from the cultures of actively dividing human cells. Simultaneous support of cell proliferation homeostasis and anti-bacterial activity led us to the hypotheses suggesting that there exists some archaic (possibly relict) mechanism with bivalent activity, which is provided by certain entities present in the supernatant. Taking into account that dried supernatant does not lose its potency for relatively long time, we would like to attract activity of wide research community to the opening application possibilities, and to the critical discussions over the formulated hypothesis.

Keywords: Cell Culture; Proliferation; Apoptosis; Homeostasis; Supernatant; Microvesicles; Exosomes; Antibacterial Properties

Abbreviations: BAI: Bactericidal Activity Index; ROS: Reactive Oxygen Species; PSB: Percentage of Sensitive Bacteria

Introduction

Significant part of our work related to searching for the compounds with anti-aging and rejuvenation potential involves experiments with cell cultures. It allowed us suggesting express search criteria basing upon age definition for the cell populations, extended concept of the proliferation niche and homeostatic balance of the proliferation and apoptosis in it. Corresponding experiments were carried out with the cultures of human dermal fibroblasts and human blood mononuclear cells. It was also suggested that populations of actively divided cells could be a promising source for substances with anti-aging and rejuvenating activity [1]. It is also commonly accepted that immunity status is one of the factors influencing aging process and potential human longevity (e.g. [2-14]). Thus, envisaging a connection between immunity and longevity, corresponding studies were included into the scope of our work on skin aging and rejuvenation. It was confirmed that corresponding changes in the blood immune profile are correlating with the other measurable factors reflecting skin aging, and express criterion using the difference between chronologic and biologic age was suggested in support of skin state assessment [13,14]. Search of the substances with specific activity linked to the cultures of actively divided cells led to the suggestion for testing a supernatant isolated from such cultures and started a chain of experiments yielding quite interesting results.

Experiments

Initial experiments carried out with the supernatant collected from the cultures of actively dividing cells using filtration and centrifugation as it is common for example with early stages of exosome isolation [15,17] are pointing out to the striking similarities with the known properties of some human origin microvesicles. Namely, addition of the supernatant is promoting the intensification of cell proliferation (e.g. [18-20] for exosomes) and enhance apoptosis (e.g. [21,22] for exosomes). Extensive tests with the cell cultures revealed that addition of corresponding supernatant is intensifying cell division and apoptosis and increasing relative share of the cells in cycle stages just before or immediately after division (rejuvenation of the culture in terms of suggested cell population age definition) simultaneously increasing cell vitality and prolonging cell culture lifespan. These cell experiments were carried out using the protocol described in [1] with the cultures of human dermal fibroblasts (line Hs27, purchased from Merck, Rahway, NJ, USA) and human blood mononuclear cells extracted from the material donated by healthy volunteers using generally accepted protocol [23]. Corresponding cells were cultivated in Dulbecco's Modified Eagle cell cultivation Medium DMEM/F-12 by Gibco (Waltham, NJ, USA) following common protocols (e.g. [24,25]). Fibroblast viability was determined by colorimetric analysis using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide using MTT assay [26]. Mononuclear cell viability was assessed using Trypan Blue Exclusion Test according to common protocol [27]. Flow cytometry analysis of the cell cycle stage distribution profile was carried out with a Cyto FLEX system (Beckman Coulter Inc., Brea, CA, USA), monitoring specific markers using cell cycle stage and apoptosis analysis assays. Cell cultivation, cell vitality assessment and flow cytometry analysis were carried out as described in detail in [1]. Some of cell culture experiments were carried out using the cultivation media without addition of antibiotics without significant difference in the achieved cell culture lifespan, hinting towards the possibility of supernatant's antimicrobial activity. Aware of significant interest towards the antibacterial properties of the stem cells (e.g. [28-34]) and microvesicles reflected in a significant number of publications (e.g. [35-40]) we have conducted a series of tests with Gram-positive and Gram-negative bacteria.

For such experiments supernatant separation from the cell cultures was carried out using filtration and centrifugation as it is common for example during the exosome isolation [15-17,41,42]. Supernatant was subjected to triplicate procedure of freeze-thaw-centrifugation- filtering (freezing was carried out at -20°C followed thawing at 20°C). After that supernatant was dried overnight in glass Petri dishes at 37°C at atmospheric pressure. Dried supernatant was gently removed from the glass surfaces by a nylon scraper and stored in dark glass vials at room temperature. Antibacterial activity was tested using a number of test strains and clinical isolates of Gram-negative and Gram-positive bacteria: *Escherichia coli* (including *E. coli* K12 and *E. coli* ATCC 25922); *Pseudomonas aeruginosa* (including *P. aeruginosa* ATCC 27853); Staphylococcus (including *S. aureus* ATCC 25923, S. aureus ATCC 6538P, *S. xylosus* 55/5, *S. epidermidis* ICSI 711); *Micrococcus luteus var. lysodeikticus* ATCC 15307; *Klebsiella pneumoniae* and over one hundred clinical strains of Gram-negative microorganisms highly resistant to antibiotics (including strains of *E. coli, Acinetobacter baumannii, Klebsiella pneumoniae and Pseudomonas aeruginosa*).

Antibacterial activity of supernatant was tested using common methodology (e.g. [43]). Bacteria were preliminary cultivated for 20 minutes in agaric media. Suspension of the bacterial cells in isotonic NaCl solution with and without added supernatant was introduced into a plastic 96-well sterile plate, followed by addition of cultivation broth and incubation at 37 oC for four hours. Optical density of bacterial cultures in the wells was measured with a Multiscan Accent microplate photometer (Thermo Electron, Finland) at λ = 492 nm. Each of the wells contained bacterial cells with the concentration of $5*10^8$ 1/mL. Test concentration of supernatant in the wells was 250, 500 and 750 µg/mL (referring to the weight of dried supernatant). For the assessment of supernatant action, Bactericidal Activity Index (BAI) was calculated as a relative drop of the measured density values for the wells with added supernatant Vs control (no supernatant addition). Experiments were repeated three times. Although amount of data on the supernatant action on the test strains of bacteria is not fully adequate for thorough statistical analysis, it was possible to formulate certain conclusions. Following Table presents preliminary results of the experiments with some bacterial strains (Table 1).

Table 1: Average values of Bactericidal Activity Index*.

Average values of Bactericidal Activity Index*			
Supernatant Concentration	**250 µg/mL**	**500 µg/mL**	**750 µg/mL**
Strains of bacteria			
E. coli K12	+	++	+++
E. coli 25922	+++	+++	+++
P. aeruginosa 27853	+	++	+++
S. aureus 25923	+++	+++	+++
S. aureus 6538P	+++	+++	+++
S. xylosus 55/5,	+++	+++	+++
S. epidermidis 711	+	+	++

Note: '+' indicates BAI over 10%; '++' - BAI over 25%; '+++' - BAI over 50%.

Test results indicate that although supernatant was showing clear antibacterial activity in almost all cases, it was generally lower with the strains of the coagulase-negative *S. xylosus*, *S. epidermidis* and *Micrococcus luteus*. Corresponding tests were also performed with the large number of bacterial isolates in order to determine if antibacterial properties of corresponding supernatant are not limited to a certain particular case. Corresponding percentage of sensitive bacteria (PSB, calculated basing on the tests with BAI>10%) was PSB~60% for E. coli (20 isolates) and *A. baumannii* (40 isolates), PSB~40% for P. aeruginosa (40 isolates) and PSB~90% for K. pneumonia (70 iso-

lates). Cited tests of antibacterial activity were carried out with the supernatant extracted from the cultures of commercially acquired human dermal fibroblasts. Preliminary tests carried out with the supernatant extracted from the cultures of *ex-vivo* human dermal fibroblasts and human mononuclear cell cultures indicate a presence of antibacterial properties. Activity of freshly isolated supernatant in solution at room temperature was decaying with half-lifetime of about 36 hours, similar to what was reported for the exosomes in [44]. The same time, it was possible to store the supernatant dried at room or slightly elevated temperature without vacuum assistance at room temperature for few month without significant degradation of its activity, while the exosome storage needs lyophilisation or freeze-drying and temperatures of about -80^{0}C (e.g. [44,45]).

Hypotheses

It is quite interesting that auxins and gibberellins, sometimes referred to as the 'plant growth hormones', responsible for the cell elongation and expansion in plants (e.g. [46,47]), are found in the callus and other areas with active cell proliferation and tissue growth. Moreover, it is suggested that gibberellins regulate major aspects of plant growth and development (e.g. [48]). Gibberellins are also known to have antibacterial properties (e.g. [49,50]). This carries certain similarities with the properties of the mammalian microvesicles and the supernatant isolated from the actively divided mammalian (human) cell cultures described earlier. In both cases, seemingly same substance(s) are involved in the regulation of the cell proliferation, and simultaneously provide antibacterial protection for the cells during the division, i.e. supposedly most vulnerable development stage.

It is known that different reactive oxygen species (ROS) play an important role in the immune response (e.g. [51-53]. The same time, ROS presence especially in the elevated concentrations can disrupt and even arrest the cell division cycle [54,55]. It is possible that complex mechanisms with ROS, which involvement providing antimicrobial properties [56] are balancing between the need of decreasing ROS concentration in the vicinity of dividing cells to prevent damage and increasing its concentration elsewhere for more effective defence. Moreover, it is feasible that there exists alternative antimicrobial defence system not related to the ROS which is quite universal at least in the action against different types of bacteria. Is surprising that such mechanism seems to be provided by the same compounds or entities that are involved in the cell proliferation control in both human and plants. This leads to the formulation of a rather interesting hypothesis, suggesting that there exists some very archaic (possibly relict) mechanism with bivalent activity, which is provided by certain entities transported in the liquid phase. One hand, such entities are participating in the cell proliferation homeostasis. On the other hand, they are incorporated into nonspecific immunity and protection of proliferating cells from bacterial (and possibly viral) aggression. Used level of abstraction and available data do not allow speculations on the type of such entities (microvesicles, exosomes, chemical compounds or something completely different). It seems reasonable that since such entities are acting at the cellular and intercellular level they should easily diffuse in a liquid medium or carried by a solution. It means that search and isolation of such entities should be carried out from the supernatant isolated from cultures of actively dividing cells.

Concluding Remarks

In conclusion, we believe that hypothesis about existence of archaic innate immunity mechanism involving entities that simultaneously support cell proliferation homeostasis deserves wider attention from the research community. Although this hypothesis does not seem intrinsically controversial, focused multidisciplinary studies are needed to prove or disprove it. Main purpose of present communication is attracting wider attention of research community to the discussed problems. Antibacterial potential of the supernatant isolated from the cultures of actively dividing cells for a variety of Gram-positive and Gram-negative bacteria including some strains resistant to traditional antibiotics is also very promising, and studies of the possible mechanisms involved are under the way. It should be also noted that such supernatant shows certain potential to overcome two of the critical challenges hampering wide therapeutic applications of the isolated exosomes, namely the long-term storage problems and relatively low yield [35]. Only in-depth studies could allow turning promising potential of such supernatants into viable products and technologies. Further progress in the discussed research areas strongly depends on the critical evaluation and discussions from the wider community of specialists.

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