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GLIOBLASTOMA PRODUCES NITRIC OXIDE (NO) IN RESPONSE TO ACTIVATION STIMULUS

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Abstract - Glioblastoma is the most common and lethal primary brain tumor in adults. In order to better understand the nature of the lesion, including the multiple roles of its most important signal molecule NO, a more exact tumor structure simulation in vitro is required. The aim of the presented research is to study the relationship between the level of endogenous NO and mitochondrial function in vitro at individual three-dimensional multicellular objectresolution. A close positive association has been revealed between endogenous NO production and mitochondrial membrane hyperpolarization that can support the higher intracellular level of energy required for cell activation, proliferation and movement.

Keywords - Glioblastoma, 3D Spheroids In Vitro, Nitric Oxide (NO), Mitochondrial Membrane Potential (MMP), Image Analysis.

I. INTRODUCTION

Glioblastoma, the most common and lethal primary brain tumor in adults,with a life expectancy of less than 15months, responds poorly to treatment [1]. The origins of glioblastoma are contradictory. As classified by the World Health Organization, glioblastoma multiforme is a grade III or IV malignant astrocytoma [2].However, because of its inherent heterogeneity and promiscuous histology, morphological classification can often be difficult [3]. Pathological features of glioblastoma include an active invasiveness, strong angiogenesis, necrosis and high resistance to radio- and chemotherapy.

Successful tumor progression and expansion require the supportive conditions created by tumor cells and theirmicroenvironment both by means of direct contact (cell-cell) and remote contact through active molecules secreted by one cell and absorbed by the other. One of the most common and important molecules, nitric oxide (NO), an apparent neuro-transmitter, mediates normal synaptic transmission or neurotoxicitydepending on its concentration level. A high level of NO causesenergy depletion-induced necrosis, while with maintainedenergy levels NO can induce apoptosis via oxidative or endoplasmic reticulum stress. On the other hand, low levels of NO can block cell death via activation pathways or byblocking mitochondrial permeability transition [4]. In the brain, NO hyperpolarizes astrocytic mitochondria, but depolarizes neuronal mitochondria, as has beenshown in primary cultures of cortical rat cells [5]. The balance between intra- and extracellular NO levels plays a role in controlling major cell functions and may have coexisting beneficial and detrimental effects [6]. Endogenous NO is produced in live cells,in two different ways – enzymatic and non-enzymatic –initiatedby both an external signal which reaches the mitochondria from the cytosol [7-8] and an internal (intramitochondrial) signal generated bypositive-negative feedback loops [9].

In the healthy brain, NO is produced mainly by nNOS, while in the diseased brain iNOS isinduced mainly in microglia and astrocytes, and once expressed,chronically produce moderate levels of NO [10-11].In glioma cells and the tumor micro-environment a multitude of roles for NO and NOSs have beendetermined, including regulation of tumor initiation, angiogenesis, invasion, immune system function, therapeutic resistance and brain tumor initiating cell maintenance [12]. Extracellular NO introduction activates glycolysis in astrocytes, and so, prevents further ATP depletion. Early stages of this process arecharacterized by a persistent mitochondrial hyperpolarization and the absence of apoptotic cell death in primary astrocytes [13].

Nowadays, multicellular 3D spheroids are recognized as better models for tumor growth in vitro,in comparison to traditionally used adherent cultures. The most important advantages of 3D multicellular object culturing is its closer likeness tothe native arrangement, which allows overcoming morphologic and functional alterations in the cell,due tonon-physiologicalcultural conditions in vitro [14]. Spheroids are formed bycell aggregation when the cells self-assemble into a spheroid and self-sort with the formation ofthe central core and outer shell [15]. Thereafter, cells proliferate in a spherical structure, wheretheycome in close contact with oneanother [16]. Such cell culturing maintains functional and molecular characteristics of tumors, as well as their morphological and structural features [17], including their physiological response to microenvironmental signals. Thus, glioblastoma spheroids serve as good research toolsfor studying tumor pathogenesis in

vitro, andfor discovery and development ofpotential drugs.

The aim of the presented researchisto evaluate associations between levels of endogenous NO and mitochondrial function during stimulation of glioblastoma spheroids to NO production in vitro at individual 3D multicellular objectresolution. A better understanding of the relationbetween NO production and mitochondrial function in glioblastoma spheroids can provide additional information about lesion pathogenesis and evaluate the potential of NO as a druggable molecule.

II. DETAILS EXPERIMENTAL

2.1. Materials

4,5-diaminofluorescein diacetate (DAF-2DA) was purchased from Calbiochem (La Jolla, CA). Phorbol 12-myristate 13-acetate (PMA), Tetramethyl-rhodamine methyl ester perchlorate (TMRM) and Propidium iodide (PI) were obtained from Sigma-Aldrich (St.Louis, MO, USA). Hoechst 33342 tri-hydrochloride, trihydrate, (MW 615.99) dye was purchased from Invitrogen-Molecular probes (Carlsbad, CA, USA). DMEM, L-glutamine, sodium pyruvate, penicillin, streptomycin, FBS and trypsin were purchased from Biological Industries (Kibbutz Beit Haemek, Israel).

2.2. Procedures

Glioblastoma spheroid formation and experimental procedure. Human glioblastoma A-172 cell line was grown initially as monolayer in a standard Petri dish until cells reached about 70-80% confluence. Following cell dissociation usingtrypsin, 150 µl cell-suspension (1-1.2×106 cells/mL) was loaded onto a 250µm picowell array as described previously [18] and leftfor A-172 spheroid formation over72h. Three-day A-172 spheroids were triple stained on-array withDAF-2DA, TMRM and Hoechst dyes (final concentrations 10 µM, 100 nM and 5µg/mL, respectively) for simultaneousmeasurement of intracellular NO level, mitochondrial membrane potential (MMP) and total number of cells per single 3D object. After initialimage acquisition, PMA (1 µg/mL) was introduced into the device in order to stimulate spheroids to produceNO. DMSO was added to thecontrol such that its concentration in both the control and experiment remained identical. Early kinetic measurements were performed during the firsthour after stimulant addition, while transmitted light and fluorescent images were acquired every 10 minutes. The kinetics of NO level and MMP changes within each individual spheroid were evaluated by fluorescent intensity (FI) deviation during exposure to PMA by imaging system.

Image Analysis and Statistics.The change in fluorescent signals within each individual 3D spheroid was measured using a motorized inverted IX81 microscope equipped with an incubator system for maintaining optimal conditions (37ºC, 5% CO2). Spheroids were illuminated by a mercury light source.Emitted fluorescence was imaged using a CoolSNAP HQ monochrome CCD camera. Digital analysis of cellular fluorescence was performed by Olympus Cell^P software. In order to evaluate the FI of a single spheroid, the dark current value was first subtracted from the acquired images. Then, regions of interest were defined and outlined for the bright field image separately for each spheroid and its surroundings.

Each test was performed in duplicate. Mean and standard deviation (SD) for each measured parameter werecalculated for the different spheroid populations under investigation. Comparisons between groups were performed using the t-test for groups with Gaussian distributions, and ANOVA for small groups. A paired two sample of means t-test was used to compare two groups of cell-clusters before and after treatment. Statistical significance of differences was determined at $P < 0.05$.

III. RESULTS AND DISCUSSION

Thedevice comprising microchamber arrays (Fig. 1a) was used to generate and grow 3D multicellular objects, keeping their positions throughout the growth, staining, activation, inducer introduction and repeated kinetic measurement process. More than 700 homogenous A-172 spheroids were grown in each device (Fig.1b). Although spheroid size and volume was easily controlled by the initial number of cells in each picowell (Fig.1c), no strong correlation was found between number of cells initiating its growth and final spheroid volume (0.68). Spheroidviability was evaluated by the morphology change and vital dye staining, and demonstrated good survival for 7 days (data not presented).

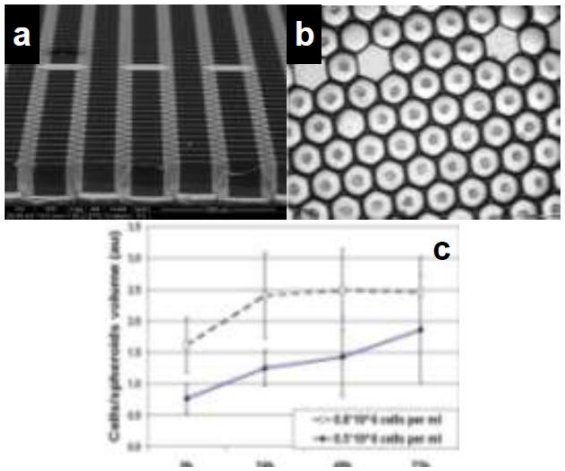

Fig.1. (a) SEM micrograph of the 250 µm microchamber structure. (b) Bright field image of A-172 GBM spheroids within the microchamber array at 72h after individual cell seeding. Scale bar 500 µm. (c) Relationship between cumulativevolume of cells loaded into each microchamber (0 h) and change in volume of A-172 spheroid formed at 24, 48 and 72h after seeding.

Three-day A-172 spheroids were triple stained on-array (Fig.2) as described in Methods. Classic cell activator PMAis the specific inducer of the PKC familywhose various isoform expressions have been demonstrated in malignant glioblastoma patient samples [19]. Long-term incubation with PMA induced migration of A-172 cells mediated byPKC activation [20] that promotes tumor invasion and so formation of metastases. PMA was introduced to the device for stimulation of NO production within spheroids. Spheroid DAF mean FI value immediately afterPMA addition was 164.4±19.8 au, whereas values forcontrol samplesin whichDMSO was introduced, were 168.5±11 au respectively,indicatinginitial homogenous DAF staining both in experimental and control devices.

The same parameter for spheroid surroundings was evaluated at85.3±12.8 au in experiment and 89.8±7.6 au in control measurements without significant difference. For every subsequent image, the FI ratio between that measurementand the first measurement for each individual spheroid and its immediate environment was calculated. Fig.3 presents adiagram of the time dependent alternations inNO levelsforthe PMA-treated and control spheroids. In the untreated spheroids (control), the average NO ratio values indicate that no change had occurred in NO formation rate. However, PMA-treated spheroids showed higher relative rates and results are similar forthe NO alternations inthe spheroids' immediate environment. A minimal increase of FI signal in subsequent measurements following PMA treatment is a result of fading. In order to illuminate the fading phenomenon, FI measurements were performed attwo time points only: immediately after PMA additionand after30 min of exposure to the reagent. The DAF FI change within 3D spheroids doubled after PMA introduction, in comparison to non-stimulated control (22% in contrast to 9%, P< 0.001) and indicates an intracellular NO level increase. The average of NO ratio values was 1.22±0.07 and 1.09±0.05 for the PMA-activated and control spheroids, respectively.

Electrochemical measurements of NO production by glioblastoma cells in vitropreviously revealed that A-172 cells, when cultured as traditional 2Dmonolayer, do not produce any detectable free radical species. Following activation, the cells displayed significant rates of NO production, reached a steady state level within 1 min whichwas sustained for at least 7 min [21].Upon performance ofcombined optical-electrochemical measurement, immediate initial NO generation was detected during the first 30-60 sec [6].

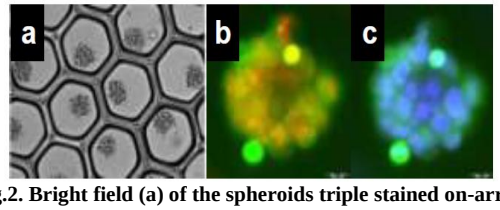

Fig.2. Bright field (a) of the spheroids triple stained on-array with DAF-2DA (green), TMRM (red) and Hoechst dye (violet). Scale bar 200 µm. Overlays of DAF2-DA and TMRM (b)and Hoechst and DAF2-DA (c) are presented. Scale bar 20 µm.

Alinear increase of MMPfollowing PMA introduction was observed in the spheroids over50 min by achange of the TMRM FI value (Fig. 3b). TMRM FI in the control spheroids also increased during this time, but atasignificantly lower rate. The increase of TMRM FI,with no change in the surrounding environment is attributed tohyperpolarization of mitochondrial membranes in the cells which compose the A-172 spheroids, during exposure to appropriate inducer.

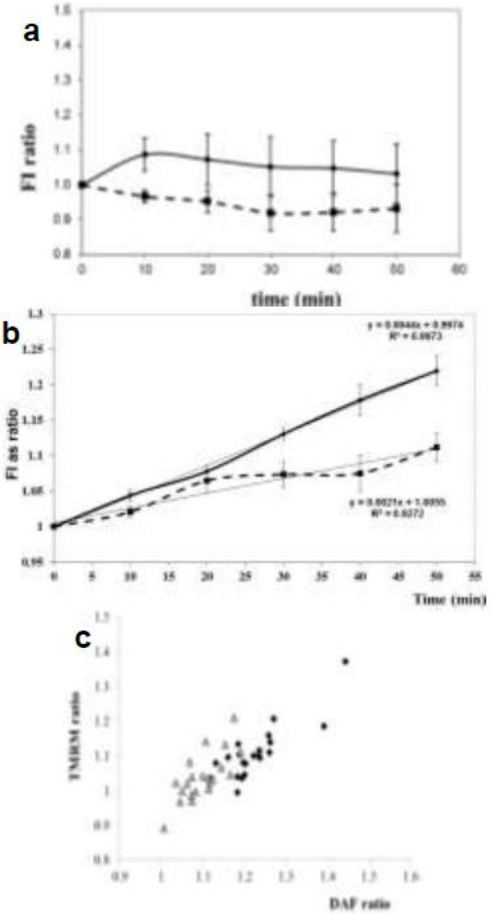

Fig. 3. Changes of NO level (a) and MMP (b) in individual spheroids are presented as FI ratios with (solid line) and without treatment (dashed line) with PMA. (c) The scatter histogram presents NO level vs MMP change for one hour after PMA addition (black rhombus) andforcontrol (unfilled triangles).

In two time point experiments, a high correlation was found between NO and MMP ratio at single spheroid level (Fig.3c).Theclose positive association between NO production and MMP change was evaluated as 84% in the stimulated spheroids and 79% in the control samples.

IV. CONCLUSION

Switching the growth mode to 3D conditions enables glioblastoma cells to produce endogenous NO in response to external stimulus, followed by

mitochondrial hyperpolarization that can maintain the higher intracellular level of energy necessaryfor cell activation, proliferation and movement.

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