European Journal of Pharmaceutics and Biopharmaceutics 76 (2010) 253–259

Contents lists available at ScienceDirect

European Journal of Pharmaceutics and Biopharmaceutics

journal homepage: www.elsevier.com/locate/ejpb

Research paper

Interaction and self-organization of human mesenchymal stem cells and neuro-blastoma SH-SY5Y cells under co-culture conditions: A novel system for modeling cancer cell micro-environment

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ARTICLE INFO

Article history:
Received 25 February 2010
Accepted in revised form 27 May 2010
Available online 2 June 2010

Keywords:
Cancer stem cell
Cell fusion
Co-culture
Mesenchymal stem cell
Micro-environment
Neuro-blastoma

ABSTRACT

The common drawback of many *in vitro* cell culture systems is the absence of appropriate micro-environment, which is formed by the combination of factors such as cell–cell contacts, extracellular matrix and paracrine regulation. Micro-environmental factors in a tumor tissue can influence physiological status of the cancer cells and their susceptibility to anticancer therapies. Interaction of cancer cells with their micro-environment and regional stem cells, therefore, is of particular interest. Development of *in vitro* systems which allow more accurate modeling of complex relations occurring in real tumor environments can increase efficiency of preclinical assays for screening anticancer drugs. The aim of this work was to study interactions between human mesenchymal stem cells (MSCs) and neuro-blastoma cancer SH-SY5Y cells under co-culture conditions on different coated surfaces to determine the effect of co-existence of cancer and stem cells on each cellular population under various stress conditions. We developed an efficient *in vitro* system for studying individual cancer and stem cell populations during co-culture using differential live fluorescent membrane labeling, and demonstrated self-organization of cancer and stem cells during co-culture on various coated surfaces. Our findings support the evidence that cancer and stem cell interactions play important roles in cellular behavior of cancer cells. These properties can be used in different fields of cancer research, tissue engineering and biotechnology.

1. Introduction

Conventional cell culture assays designed to test new therapeutics are far from representing real tissue dynamics in our body because cells in the organism are influenced by various micro-environmental factors including direct cell–cell communication, autocrine and paracrine signaling, and interactions with 3-dimensional extracellular matrix. Different *in vitro* and *in vivo* models have been employed in an effort to model such complex relations. Boyden chambers, a type of chemotaxis assay, provide cell culture with spatially but not chemically isolated environment [1]. Chambers separated by filters are proper tools for accurate determination of chemotactic behavior, as cells placed into one compartment are allowed to grow and migrate in response to concentration gradient of biologically active compounds in the other partition. Despite technical simplicity, the major drawback of such systems is lack of direct cell–cell interaction between different types of cell populations. Another popular approach for modeling natural micro-environment is the use of organo-typic cultures. In such system, a portion of live tissue is removed from the organ and cultured *in vitro*. Combination of Boyden chambers with organo-typic brain slice model was proposed to study tumor cell invasion into mammalian brain *in vitro* [2,3]. Disadvantage of such systems can be the standardization in preparation of organo-typic cultures and inability to monitor cellular behavior in real time due to 3-dimensional organization of the system. Same problems arise in spheroid culture models [4]. These obstacles were partially solved in organo-typic co-culture assays, in which myelinated axons from embryonic chicken were formed with subsequent co-culture with cancer cells [5]. Due to the 2-dimensional nature of the

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doi:10.1016/j.ejpb.2010.05.012

system, time-lapse videoscopy of live cellular behavior can be observed *in vitro*. However, standardization and interpretation of such organo-typic cultures remains a challenge. Any organo-typic culture system contains many different cell types, and although such heterogeneity allows more accurate modeling of cellular micro-environment, monitoring behavior and effect of individual cellular sub-population is a daunting task. For accurate interpretation of biological effects, there is a need of compromise between complexity of the system and our ability to dissect complex interactions among different cellular components.

Current standard drug screening protocol, which was introduced in 1985 by the National Cancer Institute (NCI), is based on the human tumor cell-line assay. The screen is currently composed of 60 human tumor cell lines (NCI60), representing leukemia, melanoma, and cancers of the lung, colon, brain, ovary, breast, prostate, and kidney (for review see [6]). However, after this screening protocol, potential drugs do not always exert the same activity when they are further tested against solid tumors. This is in part due to poor modeling of *in vivo* tumor micro-environment and its 3-dimensional structure. A decade ago a multi-layered post-confluent cell culture was designed to model solid tumor architecture *in vitro* for drug screening [7]. However, the use of single cell type in this system does not allow modeling of extracellular relations with other cell types in human tissues.

Interaction of cancer and stem cells in tumor initiation and development is becoming a very popular area of research nowadays. Specific molecules secreted by stem cells into the micro-environment might alter growth of cancer cells and their responses to chemotherapeutic agents [8,9]. On the other hand, some pathological stem cells are thought to be the cause of cancers, which is the basis for cancer stem cell hypothesis (for reviews see [10,11]). It is thought that there is a defined population of cancer cells with stem cells properties. This population possesses increased resistance to traditional therapy and serves as a new source of cancer cells, which re-occur after anticancer treatments. Developing screening techniques to target this rare cell population is a challenging task. Recently, Gupta et al. proposed a model based on induction of an epithelial-mesenchymal transition in breast cancer cells to generate cancer stem-like cells [12]. The approach was successful for identifying a drug that was >100-fold more effective than a commonly used breast cancer chemotherapeutic agent in targeting cancer stem cells. However, single cell type mono-layer culture retains a limitation due to inability to model complex spatial and micro-environment parameters.

An origin of cancer disease could also be related to oncogenic transformation of a single stem cell. It is also known that stem cells are able to interact with their micro-environment, influence differentiation status of neighboring cells, and cause their reprogramming. For these very reasons, it might be useful to design new models to study interactions between cancer and stem cells allowing analysis of both cell populations independently. Broadly in line with it, this work aimed to understand the effect of mesenchymal stem cells (MSC) derived from human 3rd molar tooth (wisdom tooth) germs on SH-SY5Y neuro-blastoma cells when co-cultured under various conditions. As the physical and chemical properties of the micro-environment affect cellular behavior, we also investigated whether the cellular organizations of co-cultured MSCs and SH-SY5Y cells may vary on different surfaces.

2. Materials and methods

2.1. Cell cultures

MSCs from 3rd molar tooth germs were isolated as described previously [13,14]. In short, human impacted 3rd molars were surgically removed from healthy patients (11–17 years of age) as part of a prophylactic treatment for orthodontic reasons. Written informed consents were obtained from the patients and their parents following approval by the Institutional Ethics Committee of Istanbul University, Turkey. The entire tooth germ tissue including the dental mesenchyme residing inside the developing crown and its surrounding follicle was excised, minced into small pieces with a sterile scalpel and cultured in Dulbecco's modified essential medium (DMEM, Sigma Chemical Co., St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine and 1% of penicillin–streptomycin-amphotericin solution (PSA) (Biological Industries, Beit Haemek, Israel). After 10–15 days of incubation at 37 °C in a humidified atmosphere of 5% CO_2 in the incubator, the cells reached confluency. The culture medium was changed every other day. Cells were passaged using trypsin–EDTA solution (1×, Sigma Chemical Co., St. Louis, MO, USA).

The human neuro-blastoma cell line SH-SY5Y was obtained commercially (ATCC – American Type Culture Collection, Manassas, VA, USA) (ATCC number: CRL-2266).

Co-cultures were established by plating various ratios of MSCs and SH-SY5Y cells at a final concentration of 6×10^4 cells/well in 12-well plates. Co-cultures were maintained in DMEM with 10% FBS at 37 °C in a humidified atmosphere of 5% CO_2.

All tissue culture flasks and plates were purchased from TPP (TechnoPlasticProducts, Trasadingen, Switzerland), unless otherwise specified.

2.2. Cell labeling

MSCs and SH-SY5Y cells were labeled using PKH26 (red) and PKH67 (green) fluorescent cell linker kit for general cell membrane labeling, according to the manufacturer's instructions (Sigma Chemical Co., St. Louis, MO, USA). In short, trypsinized cells were resuspended in diluent-C buffer and mixed with 2× dye working solution, consisting of diluent-C and fluorescent dye (PKH26 or PKH67). Cell suspension was mixed immediately by gentle pipetting and incubated for 5 min at room temperature. Labeling was terminated by addition of FBS. Labeled cells were washed twice using complete growth medium.

2.3. Cell surface modification

In the experiments, 12-well plates were used. Coating of the wells was done according to the following protocols.

2.3.1. Poly-L-lysin coating

Poly-L-lysine (Sigma Chemical Co., St. Louis, MO, USA) was dissolved in sterile distilled water to yield a working solution (15 μg/mL), 1 mL from which was applied into each well. After incubating for 2 h, plates were rinsed with sterile water (1×) and dried in tissue culture hood before seeding the cells.

2.3.2. Fibronectin coating

Fibronectin (Sigma Chemical Co., St. Louis, MO, USA) was diluted in sterile distilled water and a working solution of 10 μg/mL was prepared. Each well was incubated for 4 h at room temperature with 1 mL of this solution. Fibronectin suspension was then aspirated, and plates were dehydrated in tissue culture hood before plating the cells.

2.3.3. Collagen coating

A vial of lyophilized collagen (Roche, Mannheim, Germany) was dissolved in 5 mL of sterile 0.2% (v/v) acetic-acid (Fluka, Buchs, Switzerland). A volume of 10 μL of this 2 mg/mL final concentration solution was poured into each well, followed by spreading of the collagen with sterile plastic microbiological loop on the

surface. Plates were incubated for 1 h at room temperature in tissue culture hood. Collagen-coated wells were washed with medium once before plating of the cells.

2.3.4. Matrigel coating

Matrigel is an *in vitro* tube formation assay that demonstrates angio-genic potential of cells. After thawing on ice, 200 μL of Matrigel (BD Biosciences, San Diego, CA, USA) was placed into each compartment of a 12-well plate on ice, using cooled pipette tips. For Matrigel polymerization, plates were incubated at 37 °C for 30 min before seeding of the cells.

2.3.5. Gelatin coating

A volume of 1 mL of 0.1% gelatin (Millipore, Billerica, MA, USA) in sterile water was put into each well and incubated for 1 h at room temperature in a tissue culture hood. The gelatin solution was then removed from the wells just before plating the cells.

2.4. Mitomycin-C treatment

For inactivation of mitotic division of SH-SY5Y cells, a standard mitomycin-C treatment was applied. In brief, cells were grown to reach 70–80% confluency. The medium was changed to mitomycin-C solution (10 μL/mL) prepared in PBS and incubated for 2 h, followed by washing 4× with PBS.

2.5. Cell viability assay

Conditions for oxidative stress were achieved by incubating cultures with 300 μM hydrogen peroxide (H_2O_2) for 20 h in a humidified atmosphere of 5% CO_2. Cells were trypsinized into single cell suspension, washed in PBS, and dead cells were stained using propidium-iodide (PI). Cell suspension was analyzed using Becton Dickinson FACSCalibur flow-cytometry system (Becton Dickinson, San Jose, CA, USA).

2.6. Statistical calculations

Standard error and Student's *t*-test values were calculated using Microsoft Excel.

3. Results

MSCs from human 3rd molar tooth germs displayed the following surface antigen profile: CD29+, CD73+, CD90+, CD105+, CD14−, CD34−, CD45−, CD133−, CD166−, as demonstrated previously [14]. Incubation of MSCs and SH-SY5Y cells with PKH26 or PKH67 fluorescent dyes resulted in labeling of virtually every cell (data not shown). When grown as co-culture, labeled MSCs and SH-SY5Y cells were readily distinguished by their unique fluorescence emission (Fig. 1). Despite the fact that both cell populations were introduced in co-culture simultaneously as single cell suspensions, we observed rapid self-organization of MSCs and SH-SY5Y cells resembling channel-like structures and island-like formations, respectively.

In order to determine the effect of extracellular matrix components on self-organization of co-cultures, MSCs and SH-SY5Y cells were plated at different ratios on tissue culture plastic surfaces coated with poly-L-lysine, Matrigel, fibronectin, gelatin or collagen (Fig. 2). No significant differences were observed in self-organization of co-cultures on non-coated and surfaces coated with poly-L-lysine, fibronectin, gelatin, or collagen. Surprisingly it was detected that some cells emitted both green and red fluorescent light, the mark of a rare fusion event between cancer and stem cells (Fig. 3).

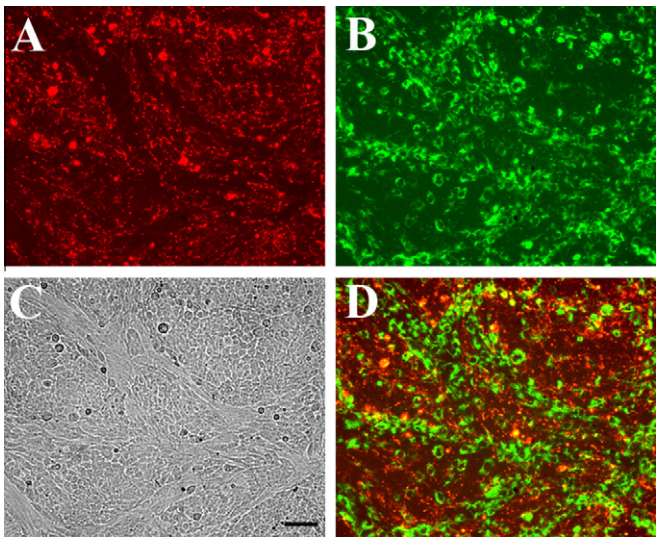

Fig. 1. MSC and SH-SY5Y cells after 3 days co-culture, self-organization pattern can be observed with channel-like structures formed by MSCs (green fluorescence) and island-like formations made of SH-SY5Y (red fluorescence). Scale bar: 100 μm. (A) SH-SY5Y cells labeled with PKH26 (red fluorescence). (B) MSCs labeled with PKH67 (green fluorescence). (C) Bright field image. (D) Merged image of panels A&B. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Short-term incubation of co-cultures for 28 h on thin layer Matrigel resulted in tube-like structure formation by MSCs (Fig. 5C). However, this pattern was transient with co-cultures transforming into a unique pattern within 3–4 days: flying saucer-like organization with centrally located MSCs and an aureole of SH-SY5Y cells was observed (Figs. 2C, I, O, and 4).

In order to determine the nature of interactions between MSCs and SH-SY5Y cells, MSCs were incubated with condition medium (CM) of normal SH-SY5Y cells and mitomycin-C treated SH-SY5Y cells on Matrigel. CM had no significant effect on tube-like structure formation of MSCs. On the other hand, when MSCs were co-cultured with normal SH-SY5Y cells and mitomycin-C treated SH-SY5Y cells, significant tube-like structure formation was observed (Figs. 5 and 6).

To determine the effect of MSCs (labeled with PKH67 green fluorescent dye) on the viability of SH-SY5Y cells (non-labeled), co-cultures were subjected to oxidative stress induced by H_2O_2 treatment. Cell viability of individual cell populations was assayed using PI-staining and flow-cytometry (Fig. 7A). This allowed monitoring of these two cell populations by their green fluorescence intensity. PI-staining was used to determine percentage of dead cells. Our results indicate that the viability of neuro-blastoma SH-SY5Y cells increased 2.1–3.5-times in the presence of MSCs under oxidative stress conditions (Fig. 7B).

4. Discussion

Current *in vitro* systems to model tumor micro-environment posses various draw-backs which limit their use for advanced anti-cancer drug screening. Developing new designs which are mimicking cancer cell interactions with normal somatic and stem cells more accurately might help understanding the mechanisms underlying tumor initiation, development, and resistance to therapeutic strategies that have been developed so far.

In this report, we describe an *in vitro* system for studying cancer and stem cell interactions using live cell fluorescent imaging. Co-culture of human neuro-blastoma SH-SY5Y cells and MSCs resulted in rapid self-organization of cells into distinct pat-

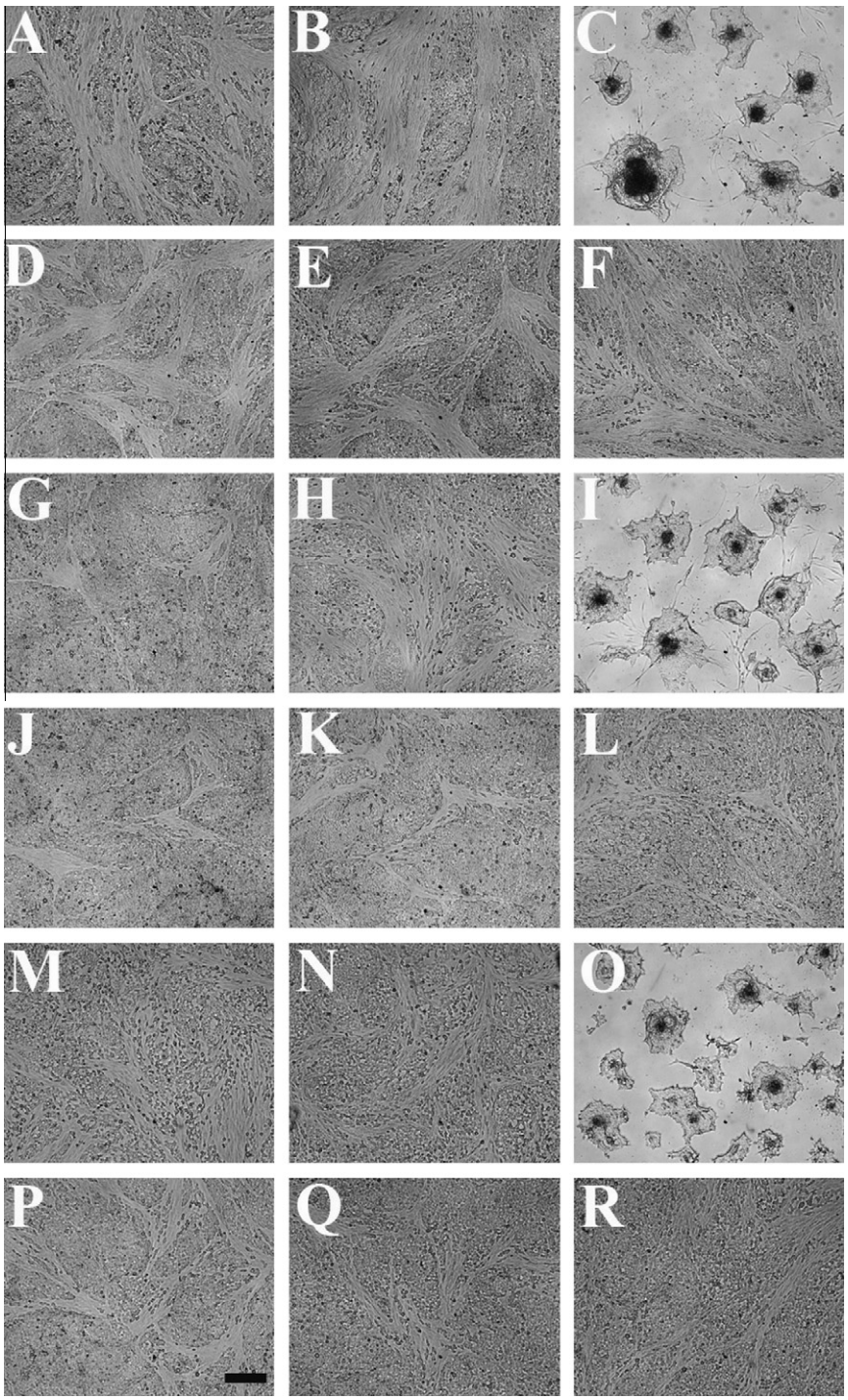

Fig. 2. Co-culture of MSCs and SH-SY5Y cells on different coated surfaces. Island-like distribution of SH-SY5Y was observed on all tested surfaces, except Matrigel. Flying saucer-like self-organization was observed on a thin layer of Matrigel with MSCs forming dense core and an aureole of SH-SY5Y neuro-blastoma cells around them. Scale bar: 200 μm. (A–F) MSC/SH-SY5Y plating ratio 3:1. (G–L) MSC/SH-SY5Y plating ratio 1:1. (M–R) MSC/SH-SY5Y plating ratio 1:3. Tissue culture plastic treatment: untreated (A, G, M), poly-L-lysine (B, H, N), Matrigel (C, I, O), fibronectin (D, J, P), gelatin (E, K, Q), collagen (F, L, R).

terns. The majority of well-known surface modifications, such as poly-L-lysine, fibronectin, gelatin, and collagen, caused cells to form similar self-organization pattern: channel-like organization of MSCs and island-like structure of SH-SY5Y cells. This pattern resembles tumor cross section and thus can be viewed as simplified 2-dimensional *in vitro* model of cancers. Similar organo-typic system could be obtained by thin sectioning of tumor-containing tissues; however, co-cultures allow more reproducible results due to efficient self-organization properties of defined cell populations. Since the complexity of our 2-dimensional co-culture system surpasses that of a single-type tumor cell-line assay, it is possible that it might be used for a more accurate, yet simplified tumor micro-environment modeling, thus providing a more realistic drug screening target.

Exposure to oxidative stress demonstrated significant increase in the viability of neuro-blastoma cells in co-culture conditions.

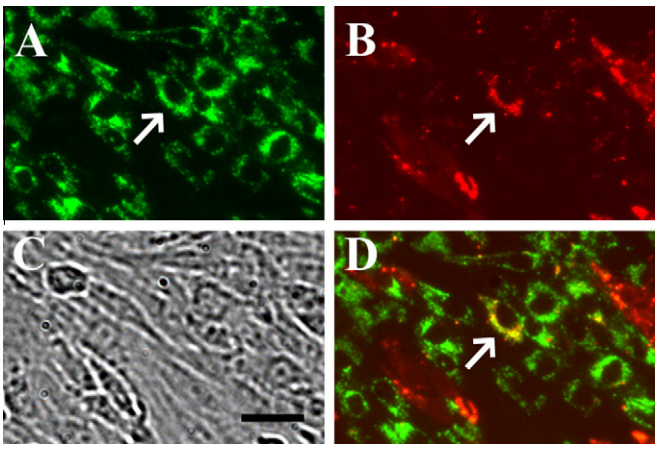

Fig. 3. Fusion between MSCs and SH-SY5Y cells (2 days co-culture). Cell fusion (arrow) was detected by observing cells, labeled with both PKH26 (red) and PKH67 (green) fluorescent dyes. Scale bar: 50 μm. (A) MSCs labeled with PKH26. (B) SH-SY5Y cells labeled with PKH67. (C) Bright field image. (D) Merged image. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

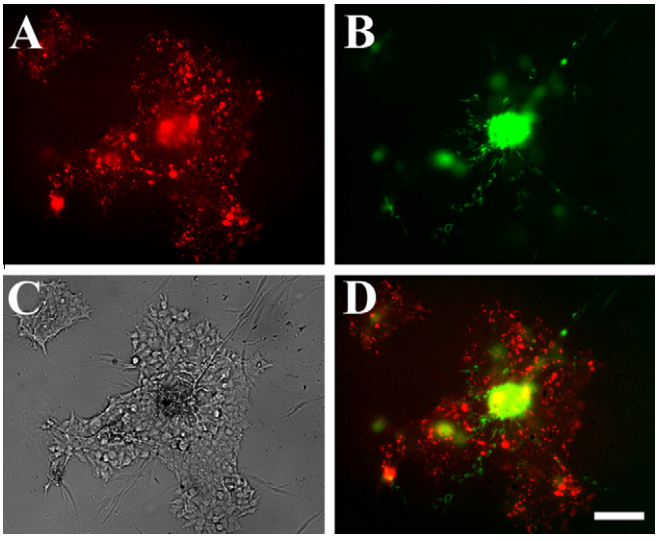

Fig. 4. MSC and SH-SY5Y cells 4 days after co-culture on Matrigel. Flying saucer-like self-organization pattern is composed of dense MSCs core (green fluorescence) surrounded by an aureole of neuro-blastoma SH-SY5Y cells (red fluorescence). Scale bar: 100 μm. (A) SH-SY5Y cells labeled with PKH26. (B) MSCs labeled with PKH67. (C) Bright field image. (D) Merged image of panels A&B. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

This observation is in good correlation with previous reports demonstrating that cancer cells display more resistant phenotype in 3-dimensional *in vitro* models when compared to mono-layer cultures [7,15]. Interestingly, MSCs exhibited remarkable resilience toward oxidative stress, which might be attributable to a low endogenous level of reactive oxygen species (ROS) in stem cells [16,17] and cancer stem cells [18]. It is also not surprising that MSCs become more viable under oxidative stress in a co-culture setting with cancer cells, inasmuch as it is known that cancer cells dynamically secrete different biologically active molecules which possess protective properties (*i.e.* to promote cancer cell survival, proliferation, and metastasis formation). Research into the effects of oxidative stress on viability of cancer cells in their micro-environment is important as oxidative stress is one of the primary

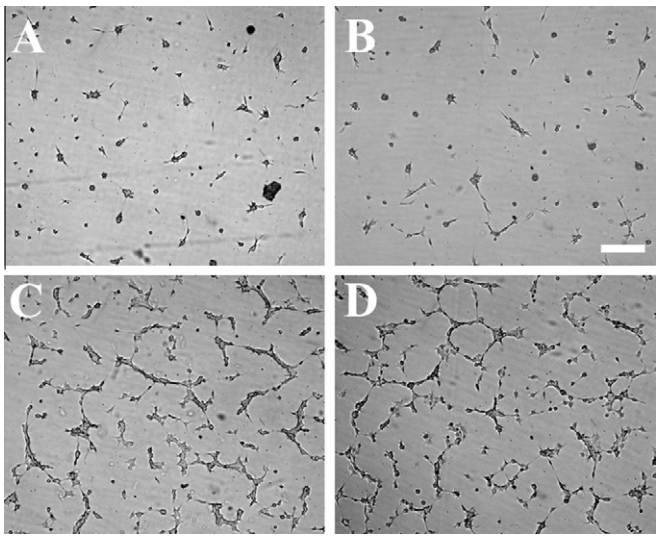

Fig. 5. MSCs and SH-SY5Y cells after co-culture on Matrigel for 28 h. SH-SY5Y conditioned media had no significant effect on tube-like structure formation by MSCs on Matrigel. Co-culture of MSCs with either untreated or mitomycin-C-treated SH-SY5Y cells resulted in significant increase in tube-like structure formation by MSCs. Scale bar: 100 μm. Bright field microscopy: (A) MSCs; (B) MSCs treated with SH-SY5Y conditioned media; (C) MSCs and SH-SY5Y cells co-culture; (D) MSCs and mitomycin-C treated SH-SY5Y cells co-culture.

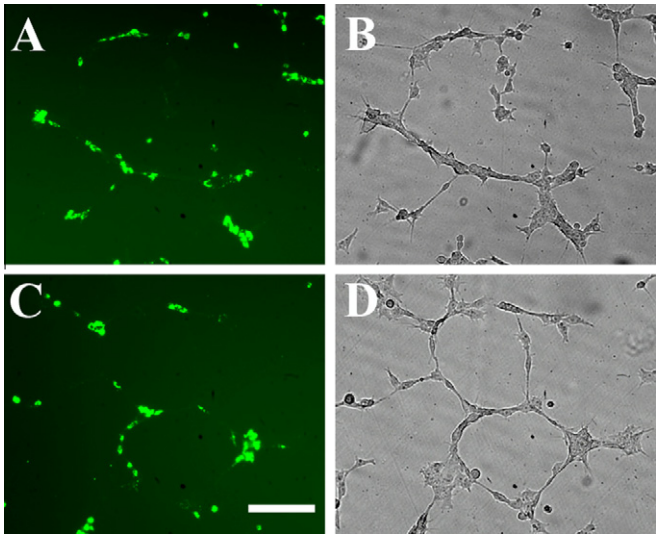

Fig. 6. MSCs and SH-SY5Y cells after co-culture on Matrigel for 28 h. Fluorescently labeled MSCs (green) are forming tube-like structures on Matrigel in co-culture with neuro-blastoma SH-SY5Y cells (unlabeled). Scale bar: 100 μm. Fluorescent and bright field microscopy (MSCs are labeled with PKH67): (A and B) MSCs and SH-SY5Y cells co-culture; (C and D) MSCs and mitomycin-C treated SH-SY5Y cells co-culture. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

modes of action of radiation therapy. Using oxidative stress, this study has demonstrated adequate response of our test system to anticancer treatment; however, authors are planning to expand drug panels for evaluating this system, as well as to use it in novel drug screening applications in the future.

Cancer stem cell hypothesis postulates the existence of certain population of cancer initiating cells, which are resistant to common anticancer treatment (for review see [19]). Cell fusion of cancer cells with adult stem cells was also suggested to play an important role in "oncogenic resistance", a phenomenon seen with

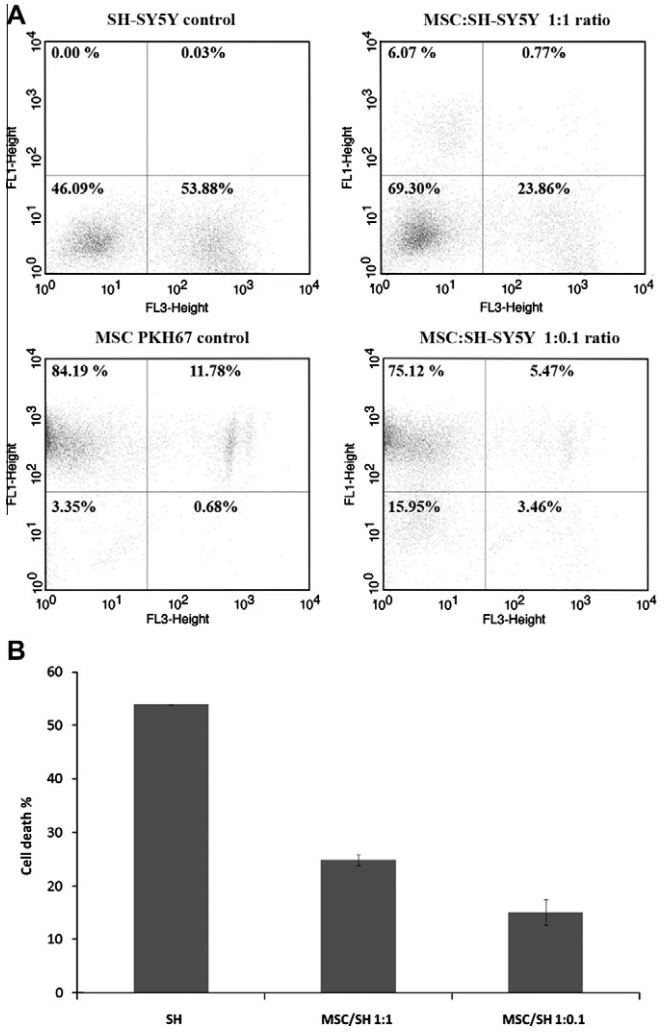

Fig. 7. Flow-cytometry. (A) Flow-cytometry analysis of MSCs' viability in co-culture with SH-SY5Y cells after 20 h treatment with H_2O_2. Unlabeled SH-SY5Y and PKH67-labeled MSCs were plated at different ratios. Necrotic cells were visualized with PI-staining. Green channel (FL1): PKH67; red channel (FL3): PI. (B) Diagram representation of SH-SY5Y viability (flow-cytometry data) in co-culture with MSCs after 20 h treatment with H_2O_2. Unlabeled SH-SY5Y and PKH67-labeled MSCs were plated at different ratios. Co-culture of SH-SY5Y with MSCs significantly increased resistance of neuro-blastoma cells toward oxidative stress conditions. Error bars represent standard error between replicates in the experiment ($n = 2$).

recurring cancers that is characterized by both increased malignancy and resistance toward anticancer therapy [20]. In our experimental model, we observed rare cell fusion events between tumor and MSCs. Such intimate interaction is of particular interest, since residual cancer cell populations surviving after conventional treatment may be enriched for sub-populations of cells with both tumor-initiating and mesenchymal features [21]. We suggest that fluorescent membrane labeling kits in combination with co-culture of cancer and stem cells might be used with fluorescence activated cell sorting (FACS) as a promising approach to obtain pure cell fusion cultures for further analyses.

An interesting self-organization of MSCs and neuro-blastoma co-cultures was observed on thin layer Matrigel. The core of the individual cellular aggregates was composed of MSCs surrounded by flat aureole of SH-SY5Y cells. During the first several hours of co-culture on Matrigel, MSCs formed transient tube-like structures but rapidly re-organized into a more permanent flying saucer-like architecture within a few days. This type of co-culture self-organization might represent interaction of cancer and stem cells during tumor metastasis. Indeed, it has been demonstrated that bone marrow-derived hematopoietic progenitor cells can form pre-metastatic clusters which are permissive niches for incoming tumor cells [22]. Although SH-SY5Y cells demonstrated poor growth *per se* on Matrigel, they readily grew around MSC aggregates in co-culture. Under all tested tissue culture conditions (poly-L-lysine, Matrigel, fibronectin, gelatin, collagen, control plastic) the proliferation rate of neuro-blastoma cells significantly surpassed MSCs, which resulted in compression of MSC cultures. As such, longer term monitoring of co-cultures is rather problematic. Therefore, using additional relevant parameters for malignant behavior other then oxidative stress, such as proliferation, does not appear to be technically feasible due to the specifics of our system and the intrinsic significant level in proliferation activity of cancer and MSCs (with cancer cells overtaking MSCs). However, developing rapid test-systems for screening anticancer compounds requires fast self-organizing co-cultures; and therefore, our short-term study may indeed be used as springboard for future trials.

We also demonstrated that direct cell–cell interaction between MSCs and neuro-blastoma SH-SY5Y cells is required for permanent tube-like structure formation by MSCs on Matrigel. Interestingly, mitotic inactivation of cancer cells did not have significant effect on the stimulation of tube-like structure formation, indicating that cancer cell proliferation was not required for that process. This observation raises the possibility that inactivated cancer cells can be used for therapeutic angiogenesis, or in a more general perspective, as bioreactors to provide cell–cell and paracrine stimulation to increase cell viability and stimulate regeneration in various degenerative conditions.

In summary, we developed an efficient *in vitro* system for studying individual cancer and stem cell populations during co-culture using differential live fluorescent labeling and demonstrated self-organization of cancer and stem cells during co-culture on various coated surfaces. These properties can be potentially used in different fields of cancer research, tissue engineering and biotechnology which require self-assembling of various cell types in mono-layers or thin films.

Acknowledgements

This study was supported by Yeditepe University (Turkey), grants from the Russian Foundation for Basic Research, Russian Federal Agency for Science and Innovations government contracts FCP, and by *Asklepios-Med* (Hungary). AAR was supported in part by NATO reintegration grant NR.RIG.983007.

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