LONG-TERM MULTICELLULAR SPHEROIDS CULTURE AND ANTI-CANCER DRUG ACTIVITY EVALUATION IN A MICROFLUIDIC SYSTEM

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ABSTRACT
In this paper, a microfluidic system for long-term multicellular tumor spheroids culture and analysis is presented. Human carcinoma cells were cultured in an array of microwells for four weeks and a slowdown of the proliferation with maintained viability was observed. The construction of the device enabled observation of decreasing spheroid diameter as an indicator of cell death. Long-term observation of cell viability was possible. An activity of repeated doses of 5-fluorouracil was observed.

KEYWORDS: Multicellular Tumor Spheroid, long-term culture, 5-fluorouracil, drug screening

INTRODUCTION
According to World Health Organization (WHO), cancer is a leading cause of death worldwide [1]. Therefore there is a need for cancer biology research and new anticancer therapies development. Current in vitro cellular models (such as monolayer), which are used in cancer research and preclinical trials, do not correspond with the in vivo situation due to significant differences between in vitro and in vivo environments [2]. The microfluidic device for long-term multicellular tumor spheroid culture and analysis is an alternative drug screening method, which more closely mimic the in vivo environment. The developed microfluidic system was used for 5-fluorouracil cytotoxicity evaluation. One of its major advantages over standard drug screening assays is a possibility of long-term observation of the cytotoxic effect and evaluation of the activity of repeated doses of the drug [3].

THEORY
Lab-on-a-chip devices can mimic the in vivo environment at various levels of its organization [2]. Microfabrication provides engineering of a single cell surroundings, by application of micrometer scale micronishes, subcellular scale microtextures and selective surface modifications. The mechanical properties of the in vivo environment can be imitated due to a possibility of strict control over the fluid perfusion. It is expected, that research on lab-on-a-chip in vivo-like systems will lead to evaluation of the methods that can replace animals in different fields of biomedical research.

The best cellular model for anticancer therapy testing developed so far is a multicellular tumor spheroid (MCTS) [4]. The MCTS presents morphology and physiology similar to a tumor in vivo with the network of cell-cell interactions, three-dimensional structure, presence of natural extracellular matrix and nutrients, metabolites and oxygen gradients. There is an evidence that three-dimensional tumor cell models are more representative for cancer tumor in vivo than two-dimensional ones [2]. The tissue structure determines growth rate of a tumor as well as response to anticancer drugs. Number of methods of MCTS formation were described in the literature and several found their final applications [4]. However, current methods face a number of limitations: cause variation in size or are cost, labor and energy consuming. Moreover, quantitative determination of cellular response in a three-dimensional arrangement is still very problematic and challenging [3]. Coupling of multicellular tumor spheroid with the microfluidic chip technology is a prospective solution for the needs.

EXPERIMENTAL
The geometry of the microsystem (Figure 1) consisted of 50 μm deep and 1 mm wide channels with arrays of 150 μm deep microwells (a diameter of 200 μm). The master was fabricated by microdrilling in poly(methyl methacrylate). A microfluidic system was obtained by double casting of poly(dimethylsiloxane) (PDMS) [5] and bonding using oxygen plasma treatment. PDMS was chosen for the fabrication, due to its gas permeability and hydrophobicity preventing cell adhesion.

HT-29 human carcinoma cell suspension (10⁶ cells/mL) was introduced into the microsystem and due to properties of the PDMS surface, cell aggregation was observed within 24 hours. Unaggregated cells were flushed away with

Figure 1. Cell culture microsystem: (A) a photograph of the fabricated device, (B) confocal laser scanning microscope profiles of the fabricated 3D microchannel consisting culture microwells and (C) MCTSs cultured in the microsystem.
a fresh medium flow. Next, medium was exchanged every 48 hours. Cell viability assay was performed using fluorescent dyes: Calcein-AM and Propidium Iodide. Culture was observed using an inverted fluorescence microscope (Olympus).

After 4 days of culture, a cytostatic drug (5-fluorouracil) was introduced into the microsystem with different dosing regimen (every day or every second day) and different concentrations (2 mM and 5 mM). Concentrations of drug that exhibit anticancer activity to three-dimensional cellular models are known to be at least 10 fold higher than concentrations effective to monolayer cultured cells [4]. Therefore 5-fluorouracil concentrations used in the experiments were relatively high. The geometry and construction of the microsystem enables flushing away of unaggregated (including dead) cells while viable MCTSs remain inside microwells (Figure 2). Therefore decreasing diameter of MCTS can be observed and measured as an indicator of decreasing cell viability. Cell culture medium was introduced to the microsystem using a syringe pump with the flow rate of 4.5 µL/min for 30 minutes. The average velocity of the medium in the channel was 0.5 mm/s. The conditions of flow rate were the same for each experiment.

RESULTS AND DISCUSSION

The multicellular tumor spheroids were cultured in the microsystem for four weeks and maintained high viability (Figure 3). The MCTS growth rate obtained was significantly lower in the microsystem than in a non-adhesive Petri dish. After two weeks of the culture growth slowdown was observed. Spheroids reached diameters between 150-200 µm and remained stable for the following two weeks. High cell viability was determined after the whole period of culture (Figure 3:C). The characteristic, layered structure of the spheroids was observed: viable proliferating and quiescent cells exhibiting green fluorescence (Calcein-AM, indicator of the viable cells) surrounding the necrotic core (red fluorescence of Propidium Iodide, indicator of the necrotic cells). High cell viability and the stability of the number of cells (directly proportional to the volume of the culture) proved that human cells can be cultured for a long time in the limited space inside the microfluidic environment. Quiescent proliferation, which is a characteristic of an in vivo tissue, was observed. It is the evidence that microfluidic environment can mimic the in vivo conditions, as a homeostasis-like state was achieved.

The 5-fluorouracil was introduced to cell culture microsystems after four days of cultures – time of the most intense cell growth. Conditions of the medium flow rate were set precisely and kept the same for each experiment. The study of the shear stress caused by the medium flow of the average velocity of 0.5 mm/s was performed earlier [6] and it was found harmless for cultured cells. The results of the 5-fluorouracil activity are presented in Figure 4. It is clear, that it is possible to distinguish spheroids exposed to different concentrations of the drug using the measurements of the spheroids’ decreasing diameters. The main advantage of the developed assay is a possibility of long-term data collecting. Current cell viability assays require termination of the culture and provide single measurement point of a single culture [4]. On the contrary, the assay performed in the microdevice enabled observation of cellular response during several days after exposition to the drug. This advantage was used for evaluation of cytotoxicity of repeated doses (Figure 4:B). 5-Fluorouracil was introduced into two cell culture microsystems at the same concentration (5 mM) and within different regimes (i.e. every day or every second day). It was observed, that higher frequency of drug dosing resulted in faster MCTS diameter decrease. In the experiment with every second day dosing a MCTS growth was ob-
served during first days of exposition to the drug, while dose repeated 24 hours after the first dose, and next, every day, resulted in rapid cell death.

CONCLUSION

The presented microfluidic system was successfully used for long-term multicellular tumor spheroid cultivation. A homeostasis-like state of the cultured cell model was observed: after 10-14 days of culture spheroids reached required diameters and no cell growth was observed for the following two weeks. High viability was maintained within this time. It is a solution for cell-based studies in an in vivo-like microfluidic environment.

The microsystem was used for evaluation of 5-fluorouracil effect on HT-29 cells. It provided an unique opportunity of evaluation of cytotoxicity of repeated doses of the drug. Moreover, observation of decreasing MCTS dimensions is a low-cost and easy-to-conduct mean of a quantitative determination of a three-dimensional cellular model response to a applied drug. The assay is suitable for long-term observation of multicellular tumor spheroid response, in a contrary to other viability assays requiring termination of a culture.

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