Characterization of bioactive substances MHGF-68 on tumour cell lines with LiveFlow In Vitro Technology

Original Paper

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Abstract As part of experimental research, growth factor-like substances associated with MHV-68, named MHGF-68, were discovered in our laboratory. MHGF-68 activity was manifested by the ability to alter cell morphology, that is, normal phenotype to transformed, resp. suppresses the transformed phenotype of tumour cells. The aim of the experiments was to monitor the effect of MHGF-68 on the change of the cell actin cytoskeleton in the tumour cell line Hepa1c1c7, as well as the normal cell line NIH3T3, and compare conventional stationary cultivation and dynamic cultivation conditions using a LiveFlow system (In Vitro Technologies). LiveFlow is an advanced system to test the impact of different compounds on the cell cultures, which allows simulation of *in vivo* conditions thanks to continuous flow of cultivation medium. MHGF-68 was prepared with the infection of BHK-21 cells with MHV-68 virus under non-permissive conditions (41°C). After dynamic cultivation with MHGF-68, we observed changes in morphology on Hepa1c1c7 cells. In cells cultured in a dynamic environment, we observed more pronounced changes in cell morphology in comparison with cells cultured statically. We observed no changes in the cytoskeletal structures in the NIH 3T3 cell line affected by MHGF-68 in both types of cultivation. The advantage of LiveFlow in comparison to *in vivo* testing is that the experiments performed in this system are less time and money consuming. Dynamic cultivation in the LiveFlow system is suitable for optimizing experiments before testing substances *in vivo*.

Keywords MHV-68 – bioactive substances MHGF-68 – cytoskeletal structure – LiveFlow system – tumour cell line

INTRODUCTION

In vitro cultures are a tool for preliminary investigation cell behaviour. They allow to control most of the experimental variables and permit quantitative analysis. In comparison to *in vivo*, *in vitro* models are highly controllable, with a reduction in time and cost (Table 1). Common model for *in vitro* studies is the cell monolayer cultured in static conditions. Ideally, an *in vitro* model should come as close as possible to the *in vivo* situation (Giusti et al., 2014). LiveFlow is an advanced system to test the impact of different compounds on the cell cultures, which allows simulation of *in vivo* conditions thanks to continuous flow of cultivation medium; with the possibility of exact regulation flow speed, IVTech technology is based on compact, user-friendly and transparent cell culture chambers (LiveBox) with shape and dimensions

similar to the 24-well plate wells. The main advantage of LiveFlow is the possibility to simulate different tissues and view them in real time, maintaining the same protocols used in traditional cell culture experiments. In this study, we treated cells with MHGF-68 and observed the effect on the cell morphology after cultivation with stationary conditions and after dynamic cultivation resembling *in vivo* conditions with a LiveFlow system (In Vitro Technologies). MHGF-68 compounds are bioactive compounds that resemble cellular growth factors and were isolated in our laboratory in 2015 (Šupolíková et al., 2015). There are already known herpesviruses and poxviruses which have genes encoding secretory proteins with structural similarity to cellular growth factors (Konvalina et al., 2002). The first growth factors related

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Table 1. Comparison of IVTech, *in vitro* and *in vivo* testing.

In vitro	In vivo	IVTech (advanced cell culture systems)
Lack of human complexity	Ethically controversial	Human organ environment simulation
Lack of side effects tests	Time ineffective	Multi-organ models
Lack of geometrical complexity	Expensive (2–30 times more than <i>in vitro</i>)	3D and dynamic cell cultures
Cells cultivated in static conditions	No high-throughput monitoring	Real time monitoring

to herpesviruses were obtained from the alphaherpesviruses, specifically pseudorabies virus (PRGF) and herpes simplex virus (HSV) type 1 and type 2 (HSGF-1 and HSGF-2) (Golais et al., 1990; Golais et al., 1992). MHGF-68 compounds were obtained by the cultivation of BHK-21 cells infected with murine herpesvirus-68 (MHV-68) under non-permissive conditions for viral replication (cultivation at 41°C). The viral origin of MHGF-68 was confirmed with a panel of monoclonal antibodies directed against viral glycoprotein B (Šupolíková et al., 2015). These substances are able to change the cell morphology from normal to transformed phenotype and vice versa (Šupolíková et al., 2018). Basic chemical separation of MHGF-68 substances on FPLC column with Sephadex G15 in the absence of salts resulted in loss of transformation activity, while the ability to suppress transformation remained conserved. On the other hand, both effects were conserved when separated on the same column but washed with phosphate buffered saline (PBS). MHGF-68 substances were separated on two biologically active components MHGF_A and MHGF_B after separation on the different column (RP-HPLC C18) using methanol–water phase. Function of these components is to change the normal cell phenotype to transformed phenotype (Šupolíková et al., 2015).

In our experiments, we have confirmed that MHGF-68 has a two-component character and its Mr is < 1000. We used MALDI technique, which is also suitable for checking the samples during the purification procedure for checking the mass of the protein/peptide content after fractionation after RP-HPLC, FPLC respectively (Olejníková *et al.*, unpublished data). HPCL-MS showed that every fraction offers different types and different amount of ions during the ionization, which means that the fractions consist of different types of (bio)chemical substances. Chemometrical techniques showed that there are (bio)chemical substances responsible for biological activity. Detailed structure of those compounds needs further study and subsequent experimental work (Vojs Staňová, personal communication). Based on preliminary, the results of the solid-phase extraction (SPE) experiments show that MHGF-68 biologically active substances contain in its molecule a phenolic structure, as evidenced by this that the substances are ionized only at high pH = 9 and pH = 11. This fact is also confirmed by the fact that these substances are not captured on the C8 column, but they are captured on a functional group column propylamine, which behaves

as a weak annex. At the same time, these substances, despite ionization, are partially captured on phenyl and cyanide column (Vojs Staňová et al., 2015). The aim of the present study was to compare the effect of MHGF-68 on selected cell lines in conditions of stationary *in vitro* cultivation and dynamic *in vitro* cultivation in LiveFlow apparatus.

MATERIALS AND METHODS

MHGF-68

MHGF-68 compounds were obtained by the cultivation of BHK-21 cells (baby hamster kidney) infected with murine herpesvirus-68 (MHV-68). Monolayers of BHK-21 cells were cultivated in Dulbecco's modified Eagle's medium (DMEM) enriched with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin, 1% L-glutamine and 0.1% gentamicin. Subsequently, cells were infected with MHV-68 with multiplicity of infection (MOI) 0.01 and incubated for 24 hours in 41°C. Afterwards, cells were transferred to 37°C and cultivated for another 24 hours. After cultivations, media from these cells were pooled, lyophilized and stored in +4°C until other experiments were performed (Šupolíková et al., 2015). The fraction of MHGF-68 used in experiments was acquired by FPLC separation on Sephadex G15 column, eluted by redistilled water, so the transformation suppressing activity remained. Experimental conditions: 5 ml of medium obtained from infected cells MHV-68 was loaded onto an HPLC analyser (Shimadzu, Kyoto, Japan) with column of dimensions 950 x 20 mm containing sorbent Sephadex G15 Fine (Pharmacia, Lund, Sweden). Deionized was used as the mobile phase water or saline (pH 7.2). Flow mobile phase was 0.8 ml/min. Fractions were collected based on the signal from the UV detector at the wave detectors lengths 220 and 254 nm (Vojs Staňová et al., 2015).

Cells

Experiments were performed on adherent fibroblast cell lines Hepa1c1c7 and NIH3T3. Cell line Hepa1c1c7 (ATCC® CRL-2026™) is a stabilized tumour cell line isolated from murine hepatocellular carcinoma, whereas cell line NIH3T3 is a normal cell line derived from murine embryonal cells (ATCC® CRL1658™). Cells were cultivated in DMEM enriched with 7%

Figure 1. LiveFlow system scheme. Blue and green – LiveBox with samples, parallel circuits of medium, Red – controls, series circuit of medium.

Figure 2. Scheme of dynamic cultivation using LiveFlow (IVTech). LiveBox with adherent cell line on the glass slide inside, reservoir with cultivation medium and LiveFlow ensuring continual flow of the medium.

FBS, 1% PSA (antibiotics: 100 U/ml penicillin/streptomycin, 100 µg/ml amphotericin) and 1% L-glutamine, and they were incubated in 37°C with 5% CO₂.

Cell cultivation in dynamic environment with the use of LiveFlow system

Sterile circular glass cover slides (20 mm in diameter) were placed in 6-well plates (1 slide per well). 1×10^5 cells were

seeded onto glass slides in volume of 0.25 ml per slide. After a 20-minute-long incubation, 2 ml of DMEM was added to each well. After a 24-hour-long cultivation, glass slides were placed into LiveFlow system, as shown in Figure 1, and the cultivation medium with MHGF-68 (diluted 1:100) was added. In every experiment, control untreated cells (without MHGF-68) were used. Volume of circulating medium was 7.5 ml per LiveBox (1 slide). Cells were cultivated 24, 48 and 72 hours with continuous flow of medium at a speed of 180 µl per minute (Figure 2). Incubation was performed at 37°C, 5% CO₂. For information: LiveFlow system is commercially manufactured by In Vitro Technologies (in short IVTech) and distributed by Scintilla spol. s r.o.

Cell cultivation in static conditions

Sterile circular glass cover slides (20 mm in diameter) were placed in 6-well plates (1 slide per well). 1×10^5 cells were seeded onto glass slides in volume of 0.25 ml per slide. After a 20-minute-long incubation, 2 ml of DMEM was added to each well. After a 24-hour-long cultivation, the medium was exchanged with a medium with MHGF-68 (diluted 1:100). In every experiment, control untreated cells (without MHGF-68) were used. Cells were cultivated for 24, 48 and 72 hours at 37°C, 5% CO₂.

Immunofluorescence assay

After incubation, the cells were fixed with 4% paraformaldehyde solution and permeabilized with 0.01% Triton X-100 (PBS solution). Alexa Fluor 555 conjugated with Phalloidin was used to visualize actin filaments. To visualize cell nuclei, DAPI stain was used. Prepared slides were observed using Leica TCS SP8 AOBS (Leica Microsystems, Germany) confocal microscope with HC PL APO CS2 63x/1.40 OIL lens.

RESULTS AND DISCUSSION

The cytoskeleton plays an important role in the regulation of various cellular processes associated with transformation, such as proliferation, contact inhibition, and cell growth in thin agar or apoptosis. In general, loss of actin filaments is considered a marker of oncogenicity (Pawlak and Helfman, 2001; Etienne-Manneville, 2004; Shutova and Alexandrova, 2010). In this study, morphological changes in cellular actin cytoskeleton after treatment with bioactive substances MHGF-68 were observed. These changes were significantly more pronounced on Hepa1c1c7 cells, especially after dynamic cultivation in LiveFlow system. After dynamic cultivation in LiveFlow, the shape of untreated (control) cells was rhomboidal (Figure 3A, B, C). Actin cytoskeleton of untreated cells was diffusive, probably depolymerized, with small aggregations of actin molecules with filamentous actin near cell margin (Figure 3C). Cells treated with MHGF-68 after 24 hours of cultivation had enlarged nuclei and changed

Figure 3. Distribution of actin filaments in Hepa1c1c7 cells incubated with MHGF-68 after dynamic cultivation. Hepa1c1c7 cells were incubated with MHGF-68. After 0, 24 and 72 h, the cells were fixed and labeled with Alexa Fluor 555 phalloidin. Nuclei were stained with DAPI. The intracellular distribution of actin filaments (green) and nuclei (blue) were imaged by confocal laser scanning fluorescence microscopy (Leica TCS SP8 AOBS) with HC PL APO CS2 63x/1.40 OIL lens.

A Hepa1c1c7 untreated, 0 h cultivation **B** Hepa1c1c7 untreated, 24 h cultivation **C** Hepa1c1c7 untreated, 72 h cultivation **D** Hepa1c1c7 + MHGF-68, 0 h cultivation **E** Hepa1c1c7 + MHGF-68, 24 h cultivation **F** Hepa1c1c7 + MHGF-68, 72 h cultivation

shape (Figure 3E). After 72 hours, cells gained spindle-like shape (Figure 3F). On the contrary, highly organized actin filaments and high granulation of cytoplasm were found in whole-cell volume of cells incubated with MHGF-68 (Figure 3D). Any significant changes in NIH 3T3 cells were not detected. In comparison with dynamic cultivation, cytoskeleton structure of Hepa1c1c7 cells after static cultivation was different. Highly organized actin filaments were not present in the whole volume of cells, but were largely arranged along the edges of the cells (Figure 4E, F). After 72 hours of static cultivation, signs of spindle-shaped cell formation were observed (Figure 4F). On the normal NIH 3T3 cell line cultured with MHGF-68, we did not observe any changes in the cell cytoskeleton during the dynamic culture (data not shown). Comparison of 72 hours static and dynamic cultivation of Hepa1c1c7 with MHGF-68 showed that during stationary cultivation, changes were less visible (Figure 5). In the case of untreated cells, the formation of highly organized actin filaments and the enlargement of nuclei were observed only after dynamic cultivation in LiveFlow (Figure 5D). Our results from monitoring the effect of MHGF-68 on cell proliferation, metabolic activity and viability of the cells were confirmed by xCELLigence analyser too (Šuplíková et al., unpublished data). We found that the effect of the MHGF-68 fractions was relatively short-lived and decreased with longer

time of cultivation. Since the chemical structure of MHGF-68 fractions is not yet known, the described biological activities require further studies.

CONCLUSION

Changes of cell size and shape and distribution of actin filaments were observed during both types of cultivation in normal and tumour cell line. Comparison of stationary and dynamic cultivation showed that the effect of MHGF-68 on cytoskeletal structures is more significant during dynamic cultivation. Regarding quantitative determination of the activity of bioactive compounds MHGF-68, it is more suitable to use stationary cultivation. We confirmed by MTT assay that the effect of MHGF-68 in a static cultivation showed a significant inhibitory effect on the proliferation and viability of NIH 3T3 and Hepa1c1c7 cells and that the effect in the dynamic cultivation did not have a significant effect on the proliferation and viability of Hepa1c1c7 cells (data not shown). However, dynamic cultivation in LiveFlow system is suitable for the optimization of experiments before *in vivo* tests on laboratory animals, as it simulates conditions in live organisms by continuous flow of culture medium.

Figure 4: Distribution of actin filaments in Hepa1c1c7 cells incubated with MHGF-68 after static cultivation. Hepa1c1c7 cells were incubated with MHGF-68. After 0, 24 and 72 h, the cells were fixed and labeled with Alexa Fluor 555 phalloidin. Nuclei were stained with DAPI. The intracellular distribution of actin filaments (green) and nuclei (blue) were imaged by confocal laser scanning fluorescence microscopy (Leica TCS SP8 AOBS) with HC PL APO CS2 63x/1.40 OIL lens. **A** Hepa1c1c7 untreated, 0 h cultivation **B** Hepa1c1c7 untreated, 24 h cultivation **C** Hepa1c1c7 untreated, 72 h cultivation **D** Hepa1c1c7 + MHGF-68, 0 h cultivation **E** Hepa1c1c7 + MHGF-68, 24 h cultivation **F** Hepa1c1c7 + MHGF-68, 72 h cultivation

Figure 5: Distribution of actin filaments in Hepa1c1c7 cells incubated 72 h with MHGF-68 after static and dynamic cultivation. Hepa1c1c7 cells were incubated with MHGF-68. After 72 h, the cells were fixed and labeled with Alexa Fluor 555 phalloidin. Nuclei were stained with DAPI. The intracellular distribution of actin filaments (green) and nuclei (blue) were imaged by confocal laser scanning fluorescence microscopy (Leica TCS SP8 AOBS) with HC PL APO CS2 63x/1.40 OIL lens. **A** Hepa1c1c7 untreated, static cultivation **B** Hepa1c1c7 + MHGF-68, static cultivation **C** Hepa1c1c7 untreated, dynamic cultivation **D** Hepa1c1c7 + MHGF-68, dynamic cultivation

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