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The Effect of Murine Herpesvirus 68 (MHV-68) Related Growth Factor 68 (MHGF-68) on the Tumor Progression in Athymic Nude Mice

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Abstract: In this study the effect of MHV-68 related growth factor 68 (MHGF-68) on initiation and progression of tumor growth using fractions in various stages of purification and treated with various chemical methods was demonstrated. The biological sample in the form of culture medium of BHK-21 cells infected with Murine herpesvirus 68 (MHV-68) was fractionated using conventional chemical procedures (dilution by deionized water and physiological solution pH 7.2, centrifugation and extraction with n-butanol and chloroform) and different chromatographic techniques. All obtained fractions were tested for biological activity. In the experiments were used 4 weeks old athymic nude mice, subcutaneously infected with the suspension of tumor Hepa 1c1c7 cells. Within 8 days post administration tumor proliferation was observed. Subsequently intravenously administered MHGF-68 fractions 1B1 and 1D2 with the antiproliferative effect were tested *in vivo* to see their inhibitory activity on the initiation or progression of tumor growth in mice. Tumors in experimental mice treated with MHGF-68 fractions exhibited decreased growth of tumors in comparison to control mice. These data show that tumor xenografts were suppressed after treatment with 1B1 and 1D2 fractions of MHGF-68. Taken together, the promising potential of MHGF-68 in modulating tumor growth could be used as a foundation for development of novel cancer treatment measures with antiproliferative effects.

Keywords: Murine Herpesvirus, Oncogenesis *in vivo*, Tumor Cell Line, Athymic Nude Mice, Putative Growth Factor MHGF-68

1. Introduction

Gammaherpesviruses belong to a group of fascinating DNA viruses including important human pathogens such as Epstein-Barr virus (EBV) and the Kaposi´s sarcoma-associated herpesvirus (KSHV): the causative agents of lymphoproliferative diseases and tumors. A key attribute of these viruses is their ability to establish a lifelong latent infection of the host relatively early after overcoming the primary infection [1]. Despite the huge research efforts, the understanding of establishment of latency, persistence of virus in the host, mechanisms of the immune evasion, and what triggers the viral reactivation from latency is still a major challenge for today´s researchers. Murine herpesvirus 68, first discovered in Slovakia [2], is generally accepted as an animal model for studying human gammaherpesviruses [3-6].

Some herpesviruses and poxviruses code secretory

proteins with structural similarity to cellular growth factors, cytokines and chemokines [7]. First growth factors related to herpesviruses were obtained from alphaherpesviruses: pseudorabies virus (PRGF) and HSV-2 (HSGF-2) [8-10]. It is estimated that viral infections contribute to 15-20% of all human cancers [11]. Herpesviruses encoded genes are able manipulate the host system through targeting important biological processes in infected cells [12]. Growth factors associated with herpesviruses could be obtained from virus free medium of infected cells [10]. PRGF and HSGF-2 exhibited transformation activity *in vitro* [8, 9, 13-16].

Recent studies on MHV-68 confirm that during a particular phase of infection the virus is associated with the compounds resembling growth factors (MHGFs). The purified growth factor-like compound associated with the MHV-68 infection (MHGF-68) is capable of changing cellular morphology of transformed and cancer cells towards the normal ones, and on the other hand, it changes the phenotype of normal cells to transformed ones [17]. The atypical properties of MHGF-68 in various experimental conditions such as the increase of biological activity with the temperature change, urea concentration and UV radiation were characterized [18]. In addition, basic structural characteristics of MHGF-68 were defined [19].

Studies dedicated to the transformation of cells with MHV-68 have shown that cell transformation is closely related to the loss of actin filaments [20, 21]. Recently, a study has been published to confirm the presence of changes in the MRC-5 human cell cytoskeleton following culturing with some MHGF-68 fractions [22]. Similar changes in cell cytoskeleton were found in Hepa1c1c7 and H2 cancer cells after administration of fractions MHGF-68/1B1 and MHGF-68/1D2, to confirm their antiproliferative effect. The disappearance of actin filaments in Hepa1c1c7 cells grown in the presence of MHGF-68/1B1 fraction was very pronounced (Šupolíková, unpublished). By analysis cell´s phenotype and cytoskeleton and cell proliferation rate was confirmed and characterized the proliferation effect of the MHGF-68/68A fraction and the antiproliferative effects of both fractions MHGF-68/1B1 and MHGF-68/1D2 on normal and tumor cells, respectively [23, 24].

The goal of this study is to characterize antiproliferative effects of MHGF-68 *in vivo* by using an athymic mouse model. Selected MHGF-68 fractions in various phases of purification were tested and their potential antiproliferative activity to inhibit the tumor growth was studied. Preliminary *in vivo* data indicate that several MHGF-68 fractions can suppress subcutaneous xenograft growth in mice.

2. Materials and Methods

2.1. Chemicals, Cells and Virus

All the chemicals used in this study were purchased from Merck (Darmstadt, Germany) and Sigma Aldrich (Steinheim, Germany*). In vitro* experiments were done on BHK-21 (hamster kidney fibroblasts), Hepa lc1c7 (mouse epithelial cells derived from hepatocellular carcinoma) and HeLa (human cervical carcinoma), stable adherent cell lines. All cell lines were used from collections of cell lines from Institute of Virology, Biomedical Research Center, Slovak Academy of Sciences in Bratislava. In the experiments was used Murine herpesvirus (MHV-68) – prototype strain isolated from *Myodes glareolus,* taxonomically classified as Murine herpesvirus 4 (MuHV-4) in genus *Rhadinovirus,* subfamily *Gammaherpesvirinae*, family *Herpesviridae* and order *Herpesvirales* [25].

2.2. Sample Purification (Figure 1)

Culture medium, collected from cultured BHK-21 cells infected with MHV-68 containing MHGF was chemically treated as follows: i) dilution with deionized water and physiological solution pH 7.2; ii) evaporation at 60°C followed by addition of deionized water or methanol; iii) centrifugation at 2000 rpm for 5 or 30 min; iv) n-butanol and chloroform extraction. Using another chemical approach, culture media of MHV-68-infected BHK-21 cells were fractionated by chromatographic techniques, such as solid phase extraction at various types of stationary phases, FPLC-Sephadex G15 and RP-HPLC C18 [17, 19].

2.3. MHGF Treatment

The fractionated samples were further tested in BHK-21 and HeLa cell lines in 96-well plates. Cells were treated with 5-10 µl of the culture medium containing MHGF for 30 min in DMEM supplemented with 10% FBS, 1% penicilin/streptomycin, 1% glutamine and 0.1% gentamicin. Cells were observed under a light microscope.

2.4. Animal Experiments

Four weeks old athymic nude mice CD1 were purchased from Charles River Laboratories. The animals had access to standard food and received water *ad libitum*. Ten animals per group were injected subcutaneously into the flank on both sides with the suspension of Hepa 1c1c7 cells (500 000 cells/100µl/mouse) in sterile PBS. After 14 days post administration, when the tumors reached the volume of about 40-80 mm^3, animals were divided into three groups. Each tested group used ten mice. First group was treated with fraction 1B1 of MHGF-68, second group was treated with fraction 1D2 of MHGF-68 and the third group was treated with sterile PBS as a control. Fractions were inoculated intravenously into the tail vein in a 1:10 dilution in PBS with 3 doses of MHGF-68. The tumors were measured before MHGF-68 administration and last measurement was done at day 8. Experiment was terminated due to fast tumor growth. The tumor size was determined by caliper measurements and was calculated according to the formula $W^2*L/2$, where W is the width and L the length of the tumor. Statistical significance was done by students t-test. The animal experiment was approved by The State Veterinary and Food Institute of the Slovak Republic (Facility permit no. SK P 40011; project permit no. 1612/16-221; personal accreditation no. 1570/2012/80/2).

3. Results and Discussion

Morphological changes observed in the cell lines incubated with MHGF may be attributed to their biological activity, responsible for changing the normal phenotype to transformed, and/or their biological activity suppressing the transformed phenotype of cancer cells. Data show that in some samples, fractions did not affect biological activities, while in others it caused a loss of activity responsible for the change of phenotype of normal cells to a transformed or a loss of activity suppressing the transformed phenotype of cancer cells (Figure 1). The fractions affected biological activity of the samples in three ways: i) loss of activity responsible for the change of transformed phenotype with preserved activity suppressing transformed phenotype of cancer cells ii) both biological activities were preserved iii) loss of activity suppressing transformed phenotype of cancer cells with preserved activity responsible for the change of transformed phenotype.

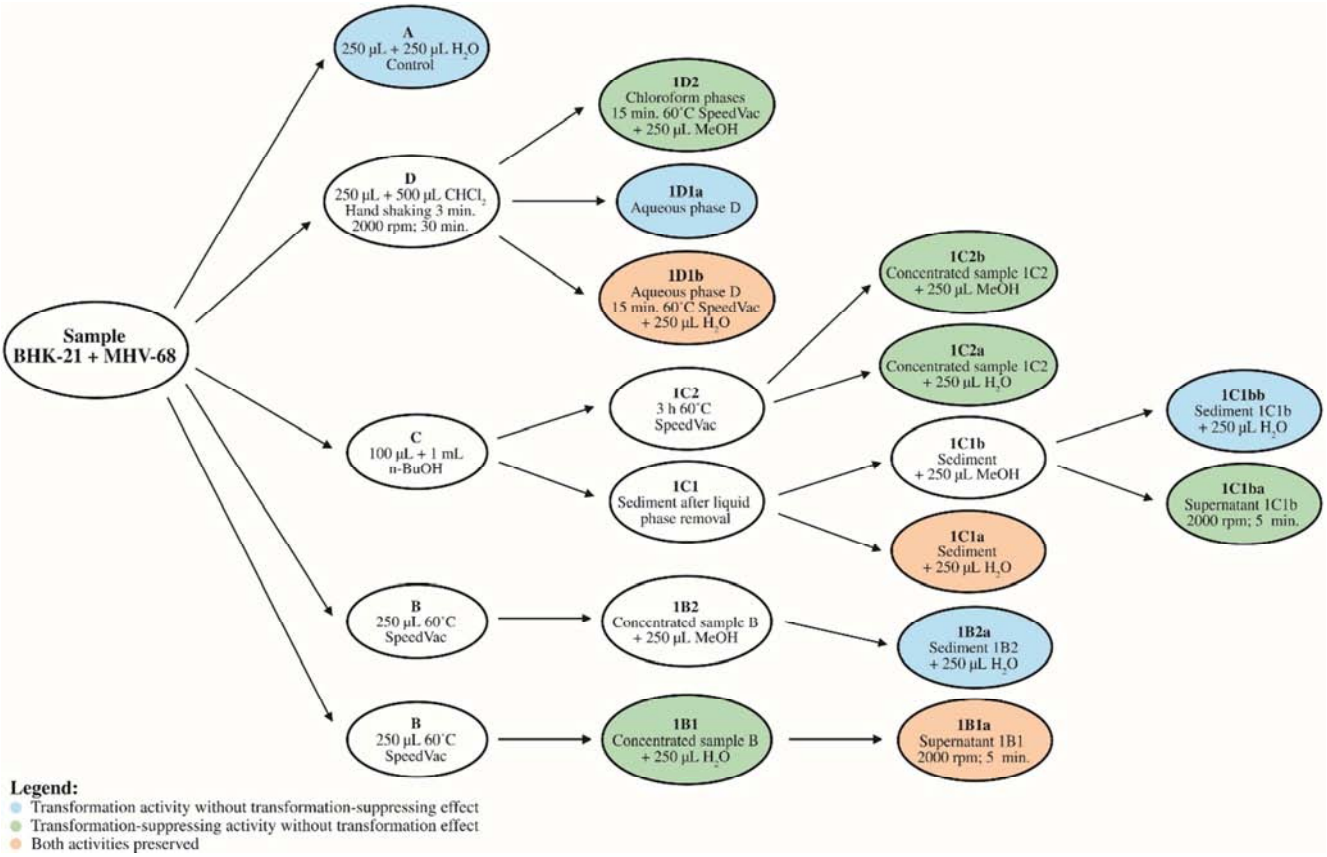

Figure 1. *Schematic representation of the treatment of MHGF samples using basic chemical procedures.*

Subsequently, MHGF-68 fractions 1B1 and 1D2 were tested *in vivo* and their potential antiproliferative activity to inhibit the tumor growth in mice was studied. In comparison to control mice both fractions reduced the tumor growth. The final volume of tumor at the day 8 from the fraction 1B1 and 1D2 treated mice was lower by 45% (1B1) and 42% (1D2) than in control mice, respectively. Within the comparison of fractions, in fraction 1D2 treated mice, the decrease of the tumor volume was drastic in the last three days, while in fraction 1B1 the tumor volume decrease was continuous during the whole treatment. However, the changes in the tumor growth were not statistically significant (Table 1).

Table 1. *Tumor volumes in control and experimental mice.*

Group of athymic nude mice	Tumor volume [mm^3]			
	day 0	day 2	day 5	day 8
Experimental mice with tumor + 1B1 fraction	67,68 ± 30,84	156,82 ± 101,22	190,32 ± 110,86	221,02 ± 134,06
Experimental mice with tumor + 1D2 fraction	61,75 ± 40,01	246,32 ± 142,32	302,44 ± 139,07	232,70 ± 121,36
Experimental mice with tumor + PBS	68,38 ± 21,60	204,49 ± 58,08	290,81 ± 138,86	396,25 ± 157,75

Tumor volume after administration of MHGF-68 fractions with antiproliferative effects in experimental mice was calculated. Tumors, in comparison to control, treated with fraction 1B1 were lower by 45% and with fraction 1D2 by 42%. However, the changes in the tumor growth were not statistically significant. Tumor volumes are average volumes taken from 10 tumors. Day 0 represents the starting point of MHGF-68 administration. Experiment was done on 10 mice in each group.

Histological findings did not confirm substantial differences in the tumor tissue between control and experimental mice.

Cytologically, there were tumor formations/masses consisting of a little differentiated population of cells with cellular and nuclear pleiomorphia with atypia, mitoses (Figures 2 - 4). Tumor cells from experimental mice treated with MHGF-68 fractions resembled the phenotype of transformed cells 68/NIH3T3 cultured with MHGF-68 [17, 20]. Finally, MHGF-68 fractions 1B1 and 1D2 manifesting antiproliferative effects *in vitro*, were further tested *in vivo*. The data show that subcutaneous xenografts were suppressed after the treatment with both 1B1 and 1D2 fractions, although with a various response. Taken together, the promising potential of MHGF-68 in the modulation of tumor growth could be used as a foundation for development of novel cancer treatment measures with antiproliferative effects.

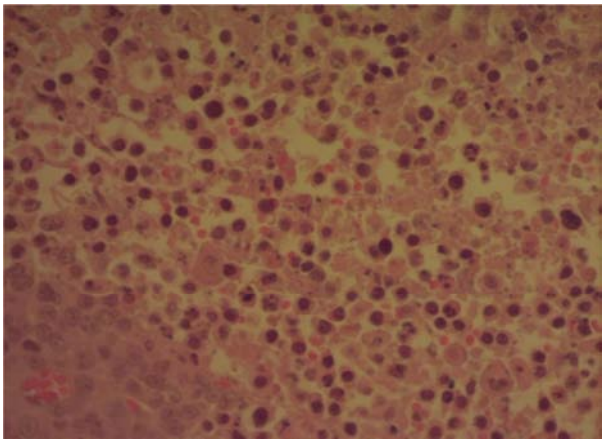

Figure 2. *Histological staining of tumor tissue with hematoxylin-eosin. Figure presents the tumor tissue of mouse only after subcutaneously administration of suspension tumor cells Hepa 1c1c7. This mouse was treated with sterile PBS as a control. Cytologically, there were tumor formations of a little differentiated population of cells with cellular and nuclear pleiomorphia with atypia, mitoses. Observed by light microscope at 400x magnification (Carl Zeiss Axio).*

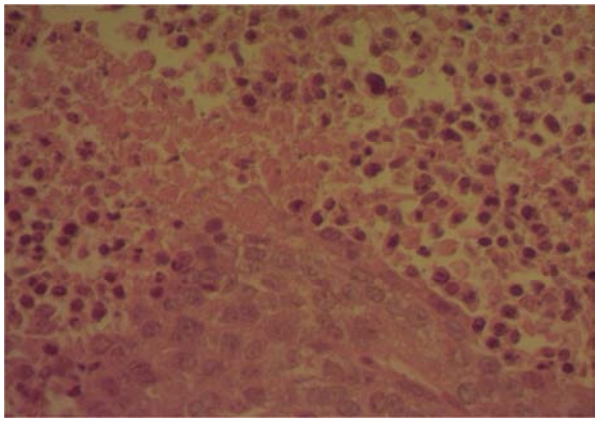

Figure 3. *Histological staining of tumor tissue with hematoxylin-eosin. Figure presents the tumor tissue of mouse after subcutaneously administration of suspension tumor cells Hepa 1c1c7. This mouse was treated with fraction 1B1 of MHGF-68. Fraction was inoculated intravenously into the tail vein of mouse. Cytologically, there were tumor formations of a little differentiated population of cells with cellular and nuclear pleiomorphia with atypia, mitoses. Observed by light microscope at 400x magnification (Carl Zeiss Axio).*

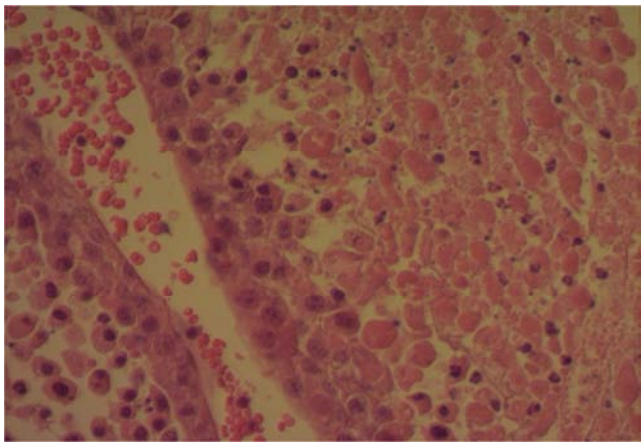

Figure 4. *Histological staining of tumor tissue with hematoxylin-eosin. Figure presents the tumor tissue of mouse after subcutaneously administration of suspension tumor cells Hepa 1c1c7. This mouse was treated with fraction 1D2 of MHGF-68. Fraction was inoculated intravenously into the tail vein of mouse. Cytologically, there were tumor formations of a little differentiated population of cells with cellular and nuclear pleiomorphia with atypia, mitoses. Observed by light microscope at 400x magnification (Carl Zeiss Axio).*

4. Conclusion

Samples from the culture medium of BHK-21 cells infected with MHV-68 were fractionated using conventional chemical, chromatographic and separation techniques. Obtained fractions were tested for biological activity. Subsequently, the MHGF-68 fractions with antiproliferative activity were tested *in vivo* to see their inhibitory activity on tumor growth in mice. In this study were used four weeks old athymic nude mice CD1, subcutaneously infected with the suspension of Hepa 1c1c7 cells. Within 8 days post administration of cells, tumor proliferation was observed. MHGF-68 fractions 1B1 and 1D2 with the antiproliferative effect were intravenously administered to mice and they were tested for inhibition of progression of tumor growth. Tumors in experimental mice treated with MHGF-68 fractions exhibited decreased growth of tumors in comparison to control mice. These data show that tumor xenografts were suppressed after the treatment with 1B1 and 1D2 fractions of MHGF-68. Histological findings did not confirm substantial differences in the tumor tissue between control and experimental mice. Cytologically, there were tumor formations/masses consisting of a little differentiated population of cells with cellular and nuclear pleiomorphia with atypia, mitoses. In further studies tumor tissues collected from mice after administration of MHGF-68 will be studied in more detail by immuno-histochemical methods. Taken together, the promising potential of MHGF-68 in the modulation of tumor growth could be used as a foundation for development of novel cancer treatment measures with antiproliferative effects.

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