RESEARCH ARTICLE

Inhibitory Effects of Dunning Rat Prostate Tumor Fluid on Proliferation of the Metastatic MAT-LyLu Cell Line

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Abstract

Tumor fluid accumulation occurs in both human cancer and experimental tumor models. Solid tumors show a tendency to tumor fluid accumulation because of their anatomical and physiological features and this may be influenced by molecular factors. Fluid accumulation in the peri-tumor area also occurs in the Dunning model of rat prostate cancer as the tumor grows. In this study, the effects of tumor fluids that were obtained from Dunning prostate tumor-bearing Copenhagen rats on the strongly metastatic MAT-LyLu cell line were investigatedby examining the cell's migration and tumor fluid's toxicity and the kinetic parameters such as cell proliferation, mitotic index, and labelling index. In this research, tumor fluids were obtained from rats injected with 2×10^5 MAT- LyLu cells and treated with saline solution, and 200 nM tetrodotoxin (TTX), highly specific sodium channel blocker was used. Sterilized tumor fluids were added to medium of MAT-LyLu cells with the proportion of 20% *in vitro*. Consequently, it was demonstrated that Dunning rat prostate tumor fluid significantly inhibited proliferation (up to 50%), mitotic index, and labeling index of MAT-LyLu cells (up to 75%) ($p<0.05$) but stimulated the motility of the cells *in vitro*.

Keywords: Dunning prostate tumor - MAT-LyLu cells - Copenhagen rats - tumor fluid - *in vitro*

Asian Pac J Cancer Prev, **16 (2)**, 831-836

Introduction

Cancer, which begins with the generation of the primary tumor mass from a transformed cell divided in an uncontrolled way, is a multi-step process. It is seen that the generation of the secondary tumors, namely the metastases in the distant areas of the body caused by the cells bearing invasive characters in the primary tumor mass, is the main reason behind the culmination of cancer cases resulting in death (Han et al., 2013).

Fluid accumulation is observed in the peri-tumor areas or body cavities due to reasons such as the increase in the capillary permeability in parallel with the increase in the tumoral mass, the lymphatic system insufficiency, and the increase in the interstitial fluid pressure (Heldin et al., 2004; Munson and Shieh, 2014). Tumor-related fluid accumulation can be seen in the benign or malign tumors of the central nervous system as peri-tumor cysts (Lonser et al., 2005; Baggenstos et al., 2007), inside the peritoneum in liver, stomach, ovary, colon, and pancreas tumors and in the pleural space in lung cancer, breast cancer, and lymphomas (Heldin et al., 2004; Lonser et al., 2004; Inan et al., 2008).

Solid tumors generally have an increased interstitial fluid pressure on account of reasons such as their structural organizations being different from the normal tissues and failures in blood and lymph circulations. This pressure especially increases towards the outer sides of the tumors (Boucher et al., 1990) and causes an increase in the fluid flow from the tumor edge toward the peri-tumor area (Fukumura and Jain, 2007a). It is argued that the growth factors and the interstitial fluid including the cells separated from the tumor may lead to peri-tumor lymphatic hyperplasia and facilitate the tumor invasion and metastasis through the lymph system as a result of the fluid's movement from the periphery of the tumor toward the normal tissue surrounding it (Fukumura and Jain, 2007a; 2007b; Lunt et al., 2008). The abnormal microcirculation and high interstitial fluid pressure in the solid tumors can also prevent the access of the systemically applied chemotherapeutic agent to the cancer cells with an optimal concentration besides decreasing the effect of the radiation therapy by generating a hypoxic microenvironment (Milosevic et al., 2001; Salnikov et al., 2003; Heldin et al., 2004).

It was determined that the tumor fluids might influence the kinetic parameters of tumor like cell proliferation or tumor size in the own cells of the tumors, different cell lines *(in vitro)* or tumors *(in vivo)* (Donenko et al., 1992). It was detected that fluid accumulation occurred inside the cyst-shaped formations in the peri-tumor area of the primary tumor generated by the subcutaneous inoculation of the strongly metastatic MAT-LyLu cells of the Dunning model into the Copenhagen rats and the amount of the fluid increased in parallel with the tumor growth (Wilson et al., 1982).

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Tetrodotoxin (TTX), which is a strong neurotoxin, functions as a specific voltage-gated sodium channel (VGSC) blocker at the same time. It was found that TTX decreased the invasion abilities of the cells without influencing the cell proliferation in the MAT-LyLu cells expressing a high proportion of VGSC (Grimes et al., 1995; Fraser et al., 2003). It was also observed that it increased the survival by decreasing the lung metastasis in the Copenhagen rats which formed prostate cancer with MAT-LyLu cells *in vivo* (Altun and Djamgoz, 2008; Yildirim et al., 2012).

It was the purpose of our study to investigate the effects of the tumor fluids which were obtained from Dunning prostate tumor bearing Copenhagen rats (which received and did not receive TTX treatment), on the strongly metastatic MAT-LyLu cell line.

Materials and Methods

The MAT-LyLu cells were placed in the RPMI-1640 (Gibco) culture medium supplemented by 1% Foetal Bovine Serum (Sigma) and 2 mM L-glutamine (Gibco) (Grimes et al., 1995). The 2x105 MAT-LyLu cell was subcutaneously inoculated into the upper side of the right-front extremities of the 2.5 and 3-month early Copenhagen rats to generate a primary tumor (All of the procedures involving animals were carried out under the ethical regulations of Istanbul University, Faculty of Veterinary Medicine). In the 25-day period following the inoculation, physiological saline (SAL) was injected to the 1st experimental group and TTX injection was applied to the 2nd experimental group. Injections were applied to the inoculation area until the tumor became palpable, and afterwards, they were made in an intratumoral way. Tumors were removed by sacrificing the rats on the 25th day and the collected tumor fluids were added into the MAT-Lylu cell medium by 20% after being sterilized following centrifugation at 5000g. Apart from these, a control group was formed without tumor fluid addition.

The effects of the tumor fluid on the MAT-LyLu cells were determined by researching the changes in the cell kinetic parameters, toxicity, and lateral movement. The toxic effect was detected through the trypan blue method, and the lateral cell movement (motility index) through the wound heal method (Fraser et al., 2003). While the proliferation of the MAT-LyLu cells was evaluated with the colorimetric MTT spectrophotometer method of Mosmann (1983), the mitotic index of the cells was found with the Feulgen technique after the acetic acid/alcohol fixation, and the labelling index was determined at the end of the autoradiography process conducted after staining the ^{3}H-thimidine (Amersham) applied cells with the Feulgen technique.

The data were provided as the arithmetic average of the groups and the standard error of each average value, multi-comparison was made by using the student-t test for the motility index and toxicity and by using the Dunnet test for the proliferation, and tests related to the comparison of the percentage values were applied for the mitotic index and labelling index data (Zar, 1999).

Results

Morphological Appearance

After the tumor fluid was added to the media by 20%, some of the cells became smaller and round by losing their extensions and had round protrusions in the form of small blebs on the cell membrane. Although the general appearances of the cells turned back to normal with the progress in the incubation period, accumulations of granular formations were observed in their cytoplasms (Figure 1).

In the samples prepared from the cells to which the tumor fluids of the animals given both SAL and TTX were applied, cells, which had nuclei similar to apoptotic morphology characterized by the chromatin material densifying as pieces in the periphery of the nucleus and that had not lost its integrity yet, were found (Figure 2). It was detected that the cells of this type, whose number was higher in the 48th hour compared to the 24th, were present in each tumor fluid sample applied.

Toxicity

While the percentage of the dead cells among the MAT-LyLu cells was 0.09±0.03% in the control group, it was respectively found as 0.06±0.22% and 1.06±0.53% after the application of the tumor fluids obtained from the animals included in the SAL and TTX given experiment groups. It was seen through the assessment that the tumor fluids of the SAL and TTX groups did not have a toxic influence on the cells ($p>0.05$).

Motility

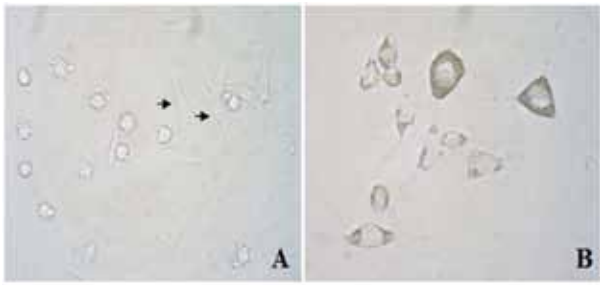

Figure 1. Morphological Change in the MAT-LyLu Cells with Tumor Fluid Addition. A). Shortly after, **B)**. 48 hours later (→cell with a normal appearance) (x400).

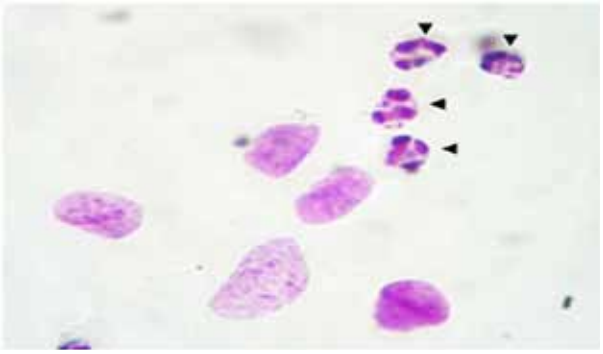

Figure 2. MAT-LyLu cells with 24-hour Tumor Fluid Application (→Shows the Cells with Apoptotic Nuclei) (x1000).

When the motility index values in the control group and groups with the addition of tumor fluids belonging to the animals treated with SAL and TTX were compared (Table 1), it was detected that none of the tumor fluids constituted a significant difference ($p>0.05$) regarding the motility indexes of the MAT-LyLu cells in 24th and 48th hours.

Kinetic parameters

Proliferation: It was observed following the addition of the tumor fluid obtained from the SAL-given animals that the proliferation of the MAT-LyLu cells decreased by 39% in the 24th hour compared to the control group ($p<0.05$) and this inhibition ratio increased to 46% in the 48th hour. Similarly, it was seen that the cell proliferation decreased by 28% and 50% respectively in the 24th and 48th hours after the tumor fluid obtained from the animals of the TTX group was added ($p<0.05$). (Figure 3) According to the data obtained, tumor fluids caused inhibition on the proliferation and this inhibition increased depending on the application period.

Mitotic Index: Upon comparison with the mitotic index of the control group's MAT-LyLu cells, it was seen that both of the tumor fluid groups led to a significant inhibition ($p<0.05$) at proportions changing between 40% and 75%, and the proportion of this inhibition increased in parallel with the application period of the tumor fluids (Figure 4, Table 2).

Labelling Index: The two groups of the applied tumor fluids caused a statistically significant inhibition on the labelling index of the MAT-LyLu cells by 50%-75% compared to the data of the control group in the 24th and 48th hours ($p<0.05$) (Figure 4, Table 2).

Discussion

Table 1. Motility Index (mean±SE) and % Change after the Application of the Tumor Fluids belonging to the Animals of the Control Group and Groups with SAL and TTX

	Motility Index		Change (%)	
	24h	48h	24h	48h
Control	0.46±0.06	0.81±0.07		
SAL	0.44±0.02	0.83±0.04	4.35(↓)	2.47 (↑)
TTX	0.46±0.03	0.92±0.01	0	13.6 (↑)

*(↓): decrease; (↑): increase

Table 2. Mitotic Index, Labeling Index (mean ± SE) and Inhibition (%) values of the MAT-LyLu Cells Resulting from the Application of the Tumor Fluids, which belong to the Animals of the Control Group and the Groups with SAL and TTX

		Control (n=6)	SAL (n=12)	Inhibition (%)	TTX (n=16)	Inhibition (%)
Mitosis Index	24h	3.02±0.08	1.61±0.16	46.7	1.81±0.22	40.1
	48h	3.19±0.14	1.04±0.18	67.4	0.80±0.17	75.0
Labelling Index	24h	36.5±0.96	14.7±0.92	59.8	17.6±1.14	51.8
	48h	34.5±1.24	8.57±0.68	75.2	16.96±2.19	50.9

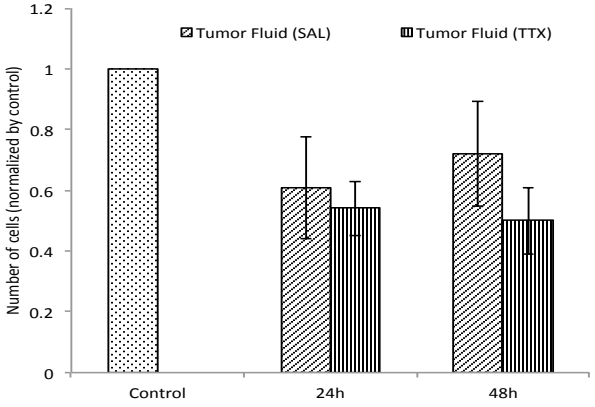

Figure 3. Effect of the Tumor Fluids on the Proliferation of the MAT-LyLu Cells

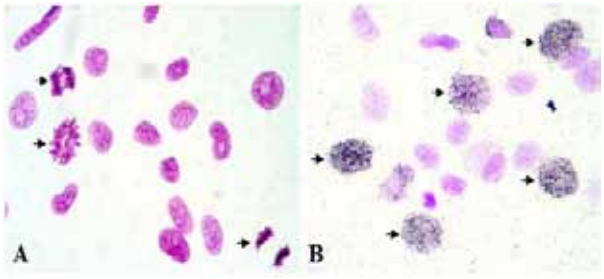

Figure 4. MAT-LyLu Cells going through Mitotic Division (A) and Synthesizing DNA (B)

In our study, the tumor fluids obtained from the rats with Dunning prostate tumor and treated with SAL and TTX were applied to the MAT-LyLu cells *in vitro*, and toxicity, kinetic parameters (proliferation, mitotic index and labeling index) and lateral movements of the cells were assessed. The tumor fluids with SAL and TTX caused inhibition on the kinetic parameters of the MAT-LyLu cells. However, no influence was observed on the motility index of the cells. The motility index of the cells is influenced by both the cell proliferation and the lateral movement. Therefore, the observation of no effects and/or changes in the motility index during the inhibition of the cell proliferation can be achieved only with the stimulation of the lateral movement. According to the results obtained from the study, the inhibition generated in the cell proliferation by the tumor fluid prevented the occurrence of the influence by balancing itself with the stimulation in the motility index of the cells. Moreover, the morphologies of the MAT-LyLu cells were deformed and cells similar to apoptotic cell morphology began to be seen as a result of the tumor fluid application. This situation is a factor influencing the proliferation of the MAT-LyLu cells.

A limited number of studies, which were conducted with the peri-tumor fluid generation in the Dunning model, are related to the content of the fluid in the literature. Wilson et al. (1982) found at the end of the biochemical and immunocytochemical analysis that approximately 30%-40% of the total protein in the Dunning prostate tumor fluid consisted of transferrin and its 24% - of albumin, and that the transferrin in the tumor fluid was 10 times higher than that of the serum and albumin had

10 times less concentration. In another study conducted with tumor fluids, Shaw et al. (1985) compared the tumor fluids of the prostate tumors generated with the weak metastatic R-3327H and R-3327G and strongly metastatic MAT-LyLu sub lines in terms of prostaglandin E2 (PGE2) level. They put forth in the tumor effusion of the MAT-LyLu cells that the PGE2 level was clearly higher than the others (Shaw et al. 1985). It was stated that PGE2, which is produced by some malignant cells and has a regulatory role in the cancer development and progress of the breast cancer, colon cancer, and prostate cancer (Badawi., 2000; Harizi et al., 2008), can cause the stimulation of the cell proliferation, angiogenesis and cellular invasion, apoptosis reduction and suppression of the immune surveillance mechanism (Rishikesh and Sadhana, 2003; Harizi et al., 2008).

The autocrine motility factor (AMF), which is also known to be released by the weak metastatic AT2.1 cells belonging to the Dunning prostate tumor model (Evans et al., 1991), is a cytokine type that can be produced by the tumor cells. Besides its angiogenic and mitogenic effect, it stimulates migration and invasion in tumor cells and can lead to tumor-dependent fluid accumulation by increasing the permeabilities of the endothelial and mesothelial cells (Funasaka et al., 2002; Huang et al., 2014). It is assumed that the tumor cell movement, metastasis, and tumor-dependent angiogenesis can be inhibited, ascitic fluid accumulation can be reduced by up to 25% and the treatment can be supported by decreasing the apoptotic resistance with the suppression of the AMF activity (Funasaka et al., 2001; Funasaka et al., 2005). According to the data that we obtained from the motility index, it is possible that AMF or a factor similar to AMF is released by the MAT-LyLu cells as well.

In the cells of the breast adenocarcinoma Ehrlich Ascites Tumor (EAT) inoculated into the peritoneal cavity of the mice, it was noticed that the amount of the fluid, which accumulated in the peritoneum and is called ascitic fluid, reached the maximum level together with the transition of the proliferation to the plateau phase (Funasaka et al., 2002). Lazebnik et al. (1991) revealed that the Ehrlich ascitic fluid (EAF) obtained from the EAT had an inhibitory influence on the proliferation of the EAT cells. EAF leads to a decrease in both the EAT size and the Lewis carcinoma tumor volume *in vivo* (Donenko et al., 1992).

It was determined that the effusion supernatants, which were obtained from patients with ovary cancer, included some matrix metalloproteinases and caused an increase in the migration of A2058 human melanoma cells by 400-1200% (Kohn et al., 2005).

Some molecular factors were found in malignant pleural or peritoneal effusions, which might have functions in the diagnosis and prognosis of the cancer. One of them is HLA-G, which is human leukocyte antigen (HLA) class-1 molecule protecting the embryonic formations from the possible attack of the maternal immune system in the fetal phase (Amirghofran et al., 2002; Rouas-Freiss et al., 2005). It was observed that the levels of this antigen (HLA-G5 and HLA-G6), which can be expressed by many carcinomas and has soluble forms, increased in the malignant acids obtained from the ovary and breast carcinomas (Singer et al., 2003; Davidson et al., 2005). Similarly, the soluble Fas protein, which is able to inhibit the immunogenic cells that will kill the tumor cells in its tumoral area, exists at a higher level in the malignant pleural effusions resulting from the lung cancer than the pleural effusions that emerged due to a reason other than cancer. Moreover, it was found that the soluble Fas was expressed by many lung cancer cell lines, such as H69, N291, RERF-LC-OK (Kagi et al., 1994; Mitani et al., 2003). It was determined that expression of the vascular endothelial growth factor (VEGF) was 10 times higher in the effusions of the patients with ovary, breast, and gastrointestinal carcinoma than that of the paired serum (Kraft et al.,1999).

The increased interstitial fluid pressure, which is seen in most of the solid tumors, leads to an increase in the fluid flow from the outer side of the tumor towards the peri-tumor area and the prevention of the transcapillary transport (Boucher et al., 1990; Fukumura and Jain, 2007a). The clinical importance of this situation is the fact that the intercellular fluid causes a decrease in the effectiveness of the treatment without being given at the targeted therapeutic dose into the tumor tissue of the applied antitumoral agents, besides the possibility of that fluid movement to facilitate the lymphatic invasion and metastasis. In the meantime, the hypoxic microenvironment caused by the insufficient circulation has a negative influence on the effectiveness of the radiation therapy (Milosevic et al, 2001; Salnikov et al., 2003; Heldin et al., 2004; Thongchot et al., 2014).

Therefore, it is seen that much research has been conducted on developing agents, which can be used in cancer treatment, focuses on the compounds (VEGF antagonists, platelet-derived growth factor (PDGF) antagonists, TGF-β, PGE1, TNFα etc.) known to decrease the interstitial fluid pressure in different ways to mainly normalize the blood vessels and the microenvironment (Salnikov et al., 2003; Heldin et al., 2004; Fukumura and Jain, 2007b; Hompland et al., 2012; Du et al., 2014; Rofstad et al., 2014).

Consequently, it was found in the study that the tumor fluid of the Dunning prostate tumor inhibited the proliferation of the MAT-LyLu cells and increased cell movement. The tumor fluid generated during the tumor growth must be evaluated and researched as a factor influencing the tumor/cancer progress and treatment.

Acknowledgements

The authors thank Prof. Dr. M.B.A. Djamgoz of Imperial College London for his invaluable support and encouragement during the preparation of the manuscript. This was supported by TUBITAK (The Scientific and Technological Research Council of Turkey), (project number: 104T031) and the Research Fund of the Istanbul University (project number: T-892). The authors declare no conflict of interest with regard to publication of the present study.

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