Irregular Solid Tumor Growth. Estimation of Electrochemotherapy Efficacy

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Our investigations show that the Lewis lung carcinoma (LLC) tumor volume estimated by commonly used formulas employing *in vivo* measured orthogonal diameters is significantly less than that of the same tumors excised and defined by weighing. For the quantitative investigation of tumor growth dynamics, a new index – tumor growth relative rate (TGRR) was proposed. Fourty C57Bl mice bearing LLC tumors were used for estimation of tumor volume *in vivo* and by weighing as well as for TGRR measurements. To estimate the anti-tumor effectiveness under elecrochemotherapeutic (ECT) treatment, a special study on 37 mice was carried out. A modified formula based on experimental measurements for estimation of tumor volume has been suggested. TGRR measurements show that tumor growth in control groups as well as under various ECT treatments is not linear and not regular. TGRR fluctuates in a wide range. Meanwhile, using TGRR a more effective anti-tumor treatment could be indicated. The findings presented in this paper suggest that the TGRR coupled with tumor volume measurements is an informative additional indicator of tumor growth dynamics and of ECT efficiency in some kinds of solid tumors.

Key words: tumor volume, tumor growth relative rate, electrochemotherapy effectiveness, Lewis lung carcinoma

INTRODUCTION

Tumor volume is an important indicator of its growth dynamics. Many investigators used this indicator for the quantitative estimation of chemotherapic and electrochemotherapic influence on tumor development (1–7). All of the mentioned authors for the estimation of tumor growth measured mutually orthogonal diameters – length, width, height (*a, b, c* respectively) and calculated tumor volume using the formula:

$$V_{t1} = 1/6\ \pi \times (a \times b \times c). \quad (1)$$

Many other authors (8–14) for the same purpose used a different formula:

$$V_{t2} = 1/6\ \pi \times (a \times b^2). \quad (2)$$

Formulas (1), (2) are similar in principle. It means that in formula (2) the height is equalized to the width ($b = c$). Actually this is not the case. Using the mentioned formulas, tumor volume was calculated and its growth dynamics in time was presented in many publications of the above-mentioned authors. Otherwise, *in vivo* it is easier to measure the tumor width than its height. This seems to be the reason for some authors to prefer formula (2) against (1).

Our investigations have shown that for some kind of tumors the volume values calculated by formulas (1) and (2) are significantly underestimated. We also found that together with volume measurements an important characteristic of tumor development is its relative growth rate.

The aims of this work were: *i*) more accurate estimation of tumor volume by weighing carefully excised Lewis lung carcinoma (LLC) tumors in mice and comparison with that calculated by formulas (1) and (2); *ii*) to investigate the tumor growth dynamics using a new quantitative indicator – tumor growth relative rate (TGRR); *iii*) to estimate the anti-tumor effectiveness of electrochemotherapy on the basis of tumor growth relative rate.

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MATERIALS AND METHODS

Animals and tumor model. Twelve-week old female C57Bl mice (Institute of Immunology, Vilnius, Lithuania) were used in this study. Animals were housed under standard conditions. The room temperature was maintained at 22 °C with a natural day–night cycle. The LLC tumor cells were injected subcutaneously in the right hind limb of the experimental mice.

Tumor size estimation by weighing (V_{tw}). This method is based on a simple idea: tumor volume is equal to the weight of water ousted by the tumor put in the flask filled with water. Therefore tumor volume was defined according to the protocol: a) when the average tumor diameter reached about 5–6 mm mice population was divided into eight experimental groups containing five animals in each group. The mutually orthogonal tumor diameters *a, b, c* were measured at two-day intervals. After measuring these values the animals were killed in a CO_2 gas chamber. Then tumors were excised as free as possible from surrounding tissues and its volumes were determined by weighing; b) a small (50 ml) flask was filled with distilled water up to the mark on the neck of the flask and its weight (P_1) was found. The density of used water (ρ_w) depending on temperature was defined in each trial; c) the weight of excised tumor (P_t) was found; d) the tumor was put into the flask, which was filled then with distilled water up to the same mark and the weight (P_2) was defined. Thereby the tumor volume was found by the formula:

$$V_{tw} = 1/\rho_w \times (P_1 - (P_2 - P_t)). \quad [3]$$

The accuracy of the weighing was within 0.1 mg and the accuracy in defining the water level at the mark on the neck of the flask due to the parallax was about 1 mm. Therefore the total error of tumor volume estimation in this way was at least 2–3 mm^3, *i.e.* less than 2% of the total tumor volume.

Definition of the tumor growth relative rate (TGRR). Our previous time-related tumor volume measurements showed that tumor growth at a given moment is dependent on the size of tumor cell population at the previous time interval and that the tumor growth rate at different time intervals isn't steady. On the other hand, under the influence of anticancer drugs tumor growth could be stopped or reduced. These findings suggest proposing a new quantitative measure – tumor growth relative rate (TGRR) defined as

$$r_t = (v_t - v_{(t-1)}) / \Delta t \times 1/v_{(t-1)}, \quad [4]$$

where v_t is tumor volume at the present time interval, $v_{(t-1)}$ is tumor volume at the previous time interval, Δt is the time interval between the measurements (the discrete time interval of one day is used). Therefore r_t is the value of the tumor volume change per day expressed by part of the former volume; *e.g.*, the growth rate 0.3 means that tumor enhancement per day comprises 30% of the tumor volume that has initiated this increase.

Tumor volume values measured every two days were used for daily tumor volume estimation. Then, using formula [4] the TGRR was estimated.

Electrochemotherapy effectiveness estimation using TGRR. Strong electric pulses combined with anticancer drugs is a widely used method for cancer treatment. This kind of treatment is called electrochemotherapy (ECT) (8). Investigation was carried out on 37 mice with the aim to estimate ECT efficiency using TGRR as indicator.

The animals bearing LLC tumors with the diameter reaching at least 4–5 mm were divided into six experimental groups (8 mice in group I and 6 mice in all other groups): group I (control) no bleomycin (BLM), no electric pulses (EP); group II – only BLM (Nippon Kayaky, Japan) at a dose of 5 mg/kg was injected intraperitoneal; group III – BLM + + EP – mice of this group were treated combining intraperitoneal injection of the mentioned dose of BLM with an application 30 min after injection of eight square-wave electric pulses of 900 V/cm electric field intensity, with the pulse width 0.1 ms at a repetition frequency 1 Hz. Electric pulses were delivered by two flat, parallel stainless steel strips 8 mm wide, 20 mm long, the distance between the electrodes can be changed according to the tumor diameter (15). The electrodes were placed percutaneously at the opposite margins of the tumor. Good contact between the electrodes and the skin was assured by the removing hair from the tumor area and using a conductive gel. Square wave high voltage pulses were generated by an electroporator, which was designed and manufactured in our laboratory (15). The pulse characteristics were adjusted with a storage oscilloscope. ECT treatment was performed without anesthesia and was well tolerated by the mice. Groups IV–VI were treated according to the same protocol as for group III but with a different pulse duration: 0.25 ms, 0.5 ms and 1.0 ms, respectively. The tumor diameters were measured with a caliper every two days in order to calculate V^*_{t2} and TGRR using formulas [5] and [4], respectively.

Statistical analysis. Data are presented in the graphs by the arithmetical mean and the standard error of the mean (SE) of the values of each expe-

rimental group; a t test was used to analyze the differences among the data.

RESULTS

Tumor volume dynamics. The graphs based on 40 tumor volume values (V_{tw}) defined in eight groups by weighing and volumes of the same tumors (V_{t1}, V_{t2}) calculated by formulas [1] and [2] with respect to time are presented in Fig. 1 (curves 1, 3, 2 respectively). Estimations according to the t test show that the difference of V_{tw} and V_{t1} means for all respective groups is statistically significant ($p < 0.01$). This finding shows that the tumor volume calculated by formula [1] is significantly underestimated. However, the difference of V_{tw} and V_{t2} means for some groups isn't always statistically significant ($p > 0.05$). This notion suggests that the size of samples is too small and maybe it is conditioned by tumor shape for which formula [2] fits better. A more detailed analysis revealed a better agreement of the calculated values with those measured when tumors are more flat than those of ellipsoid shape. However the difference of at least 15% remains. The ratio coefficient of the volume mean defined by weighing to the volume mean calculated by equation [2] is 1.17 ± 0.26. These figures suggest to modify formula [2] as follows:

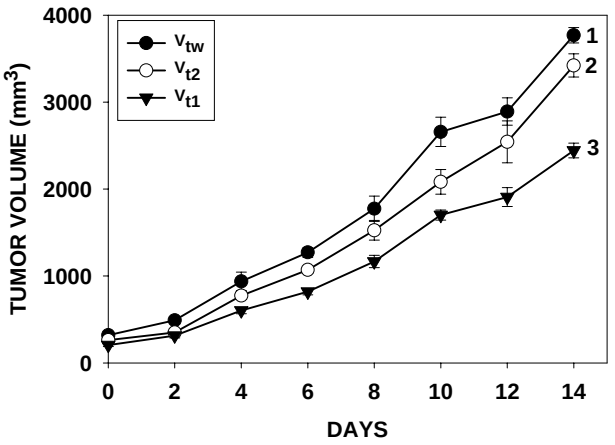

Fig. 1. Time-related LLC tumor growth. V_{tw} – excised tumor volume defined by weighing; V_{t1} – volumes of the same tumors defined by measuring orthogonal diameters *in vivo* and estimated by formula (1); V_{t2} – the same as V_{t1}, but the volumes are estimated by formula (2). Error bars represent standard error values

$$V^{*}_{t2} = 1/6\ \pi\ (a \times b^2) \times 1.17 = 0.195\ \pi\ (a \times b^2) \cong \cong 1/5\ \pi\ (a \times b^2). \quad [5]$$

Investigation of TGRR. Forty tumor volume values measured by weighing were used for TGRR calculations by formula [4]. Time-dependent TGRR dynamics presented in Fig. 2 (curve 1) shows that the growth rate change is neither linear nor regular. It is rather chaotic. Nevertheless, the higher rate values are observed at the beginning of tumor development. To compare the growth rate change at definite time intervals with tumor volume dynamics at the same stretch of time, curve 2 is presented (Fig. 2). Note that the mean of the growth rate at 3–6 and 9 –12 day intervals goes down, whereas tumor volume at the same time is slightly growing up. These findings show that TGRR quantification reveals some important features which are rather not observable in commonly used plots exposing the tumor's development.

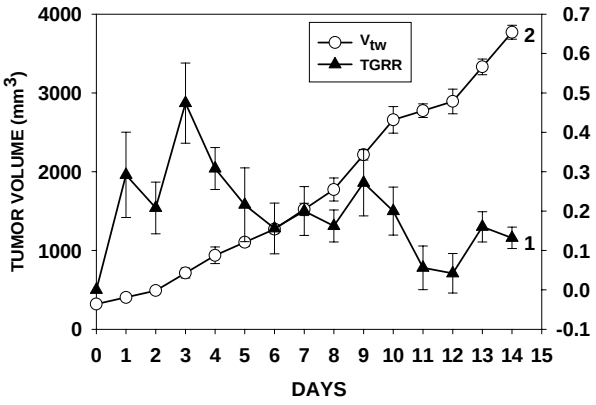

Fig. 2. LLC volume change (curve 2) and tumor growth relative rate (TGRR) dynamics (curve 1) within varying time intervals. Note the significant rate fluctuations at various time intervals. Error bars represent standard error values

TGRR and electrochemotherapy effectiveness. Tumor change under various ECT conditions couldn't be measured and watched by weighing as we did getting data presented in Fig. 1 and 2, because for this aim the animal must be killed. That's why we were forced to measure orthogonal diameters *a, b* and use formula [5] to estimate tumor size change during its development in the mice groups (Fig. 3). Graphs based on measurements carried out on six mice groups under different ECT conditions presented in Fig. 4 show that TGRR depends on the time interval after treatment: tumor change rate is neither uniform nor regular as is the case for tumor growth without any treatment (Fig. 2). The TGRR values corresponding to the zero line mean that tumor growth is stopped and the values below this line show tumor reduction *versus* the previous time interval. Note that the rate values for all time intervals in ECT group treated with 0.1 ms pulse rates are positive (curve 3), whereas for pulses lasting more than 0.1 ms TGRR becomes negative (curves 4–6). Therefore pulses of 900 V/cm and 0.1 ms

are at a subthreshold level, because tumor growth rate under this treatment remains over the zero line all the time. Figure 4 also shows that the pulse threshold is defined by combining its voltage and duration. Obviously the subthreshold pulses could be switched to overthreshold by rising their voltage or pulse duration and *vice versa*. Note that the rate rises more slightly and uniformly when BLM is combined with pulses of 1 ms *versus* pulses of less duration (Fig. 4, curve 6). Thereby TGRR could be used for identification of the more effective antitumor conditions. It is noteworthy that tumor growth inhibition could be found also using volume–time-dependent plots (Fig. 3). However, no irregular tumor growth rate and threshold effect are observed in these plots.

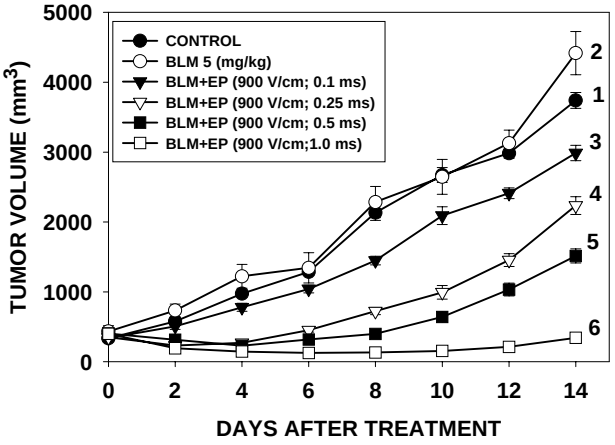

Fig. 3. LLC tumor volume change in varying time intervals under various ECT conditions. Curve 2 – bleomycin 5 mg/kg shows no observable influence; curves 3–6 – bleomycin at the same dose combined with electric 900 V/cm of various duration pulses evoke a seemingly uniform tumor growth inhibition. Error bars represent standard error values

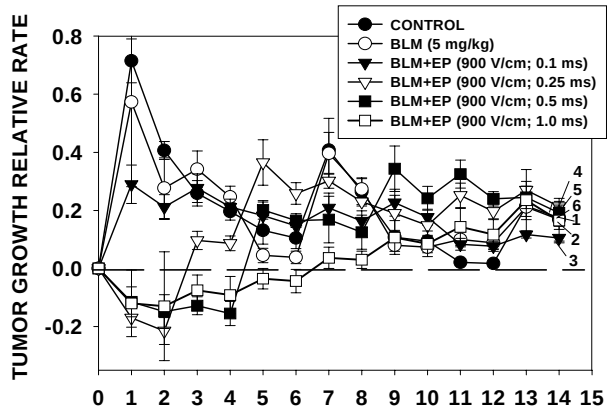

Fig. 4. Time-related TGRR dynamics under various ECT conditions. Relative rate values below the zero line show that tumor volume is reduced as compared to the previoussize. Curves 1–5 show significant relative rate fluctuations and no uniform tumor growth. Only curve 6 when pulses of 1 ms duration combined with BLM were applied reveals a slight uniform weak relative rate change. It suggests a more effective tumor inhibition. Error bars represent standard error values

DISCUSSION

Our investigations have shown that growing tumor volumes estimated by calculations using measurements of orthogonal diameters differ significantly from those defined by weighing the same excised tumors. Nevertheless, tumor volume on time-related plots based on both weighing and calculations move in a more or less parallel direction (Fig. 1). However, tumor growth rate measurement has revealed some new informative features. TGRR time-related plots indirectly testify to a non- linear and non-uniform growth of tumor cell population whose size with the time fluctuates on a large scale (Fig. 2). This phenomenon holds true also under various ECT conditions (Figure 4). The absence of stability and significant fluctuations of TGRR show that tumor growth dynamics in control group as well as under various ECT conditions couldn't be theoretically predicted using only one rate constant. Meanwhile time-related TGRR measurements disclose the treatment conditions when significantly fluctuating irregular tumor growth is transformed to a weak uniform tumor development and allow measuring the time intervals when growth is fully inhibited (TGRR under zero line in Fig. 4, curve 6). Therefore the findings presented in this paper suggest that the TGRR coupled with tumor volume measurements is an informative additional indicator of tumor growth dynamics and of ECT efficiency in some kinds of solid tumors.

CONCLUSIONS

1. Lewis lung carcinoma (LLC) tumor volume estimated by commonly used formulas employing the *in vivo* measured orthogonal diameters is significantly less than that of the same tumors excised and defined by weighing. A modified formula for the estimation of solid tumor volume is proposed.

2. Using the TGRR as a quantitative indicator it has been shown that solid tumor growth is an irregular nonlinear process. TGRR fluctuates in a wide range depending on time.

3. TGRR coupled with tumor volume measurements is an additional quantitative parameter for the estimation of tumor growth dynamics and of ECT efficiency in some kinds of solid tumors.

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NETOLYDUS STANDŽIŲ NAVIKŲ VYSTYMASIS. ELEKTROCHEMOTERAPIJOS EFEKTYVUMO VERTINIMAS

S a n t r a u k a

Šio darbo tikslas: 1) eksperimentiškai patikrinti literatūroje naudojamų naviko tūrio kitimo vertinimo formulių tikslumą; 2) ištirti standžių navikų augimo kaitą naudojant naują kiekybinį indikatorių – naviko vystymosi santykinį greitį (NVSG); 3) įvertinti elektrochemoterapijos efektyvumą panaudojant NVSG.

Navikų tūriui bei NVSG įvertinti *in vivo* buvo tirti epidermoidinės plaučių karcinomos navikai, įskiepyti C57Bl pelėms.

Mūsų tyrimais, eksperimentiškai išmatuotas epidermoidinės plaučių karcinomos tūris ženkliai skiriasi nuo tūrio, apskaičiuoto pagal literatūroje pateiktas formules. Tuo remiantis pateikiama modifikuota navikų tūrių *in vivo* įvertinimo formulė.

Panaudodami NVSG indikatorių nustatėme, kad tirtų navikų vystymasis yra netolydus, chaotiškas procesas. Šį fenomeną sunku pastebėti taikant bendrą tūrio kitimo analizę. NVSG indikatorius kartu su naviko tūrio kaitos analize gali būti naudojamas kaip kiekybinis naviko elektrochemoterapijos efektyvumo vertinimo kriterijus.

Raktažodžiai: navikų tūris, naviko vystymosi santykinis greitis, elektrochemoterapinis efektyvumas, epidermoidinė plaučių karcinoma