Caliper, contrast enhanced-ultrasound (CEUS) or laser speckle contrast imaging (LSCI): techniques to follow mice melanoma growth

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October 23, 2023

Abstract

Metastatic melanoma is a cancer for which vascularization is not a diagnostic criterion. The aim of this study was to evaluate the applicabilities of laser speckle contrast imaging (LSCI) and contrast enhanced ultrasound (CEUS) in a mouse model. B16F10 cells were xenografted to C57BL/6 mice. Mice were treated with anti-PD1 or 0.9% NaCl and tumor volume was measured daily. CEUS and LSCI were performed weekly. No difference in tumor growth or median survival were observed between treated and no-treated mice. No significant difference in tumor volume measurement comparing caliper and CEUS was observed. LSCI and CEUS analyses showed a decrease in tumor perfusion in both groups of mice. Although both CEUS and LSCI are useful for measuring tumor volume, LSCI appears to be more robust and effective for monitoring tumor microcirculation. Non-invasive investigations are needed to better predict tumor vascularization: CEUS and LSCI have a good applicability in a mice model.

INTRODUCTION

Anti-PD1 immunotherapies restore anti-neoplastic immunity by limiting T cells exhaustion [1]. These new treatments have significantly modified the prognosis of metastatic or non-surgical melanoma with a 5-year survival about 50%, including side-effects and significant higher costs. For unknown reason, all patients with metastatic melanoma have not the same response to treatment.

Angiogenesis has a key role in the cancer growth. For several years, VEGF-dependent neoplastic angiogenesis has been specifically targeted to disturb neoplastic development. Despite many investigations and treatment studies, anti-angiogenic effects to prevent tumoral cells growth and metastatic development remain unsatisfactory [2]. In the early 2000’s, vasculogenic mimicry was described by Folberg et al. as a new angiogenesis-independent neoplastic vascular model [3]. Vasculogenic mimicry is an independent endothelial vascularization model, first describe in a metastatic human melanoma model by Maniotis et al[4]. At microcirculatory level, connection between endothelial and non-endothelial cells in extra-cellular matrix could increase tumoral cells exposure in blood flow (6). There are several imaging investigations to evaluate microcirculation. Among which the laser speckle contrast imaging (LSCI) and the contrast enhanced-ultrasound (CEUS). The LSCI allows a complete non-invasive screening of superficial tissue microvascularisation perfusion. It is based on the Doppler principle for measuring the speed of blood flow in tissues. By illuminating tissue with a coherent laser, it generates a pattern of light spots (speckle) whose changes in contrast are used to create two-dimensional blood perfusion maps (imaging mode) or time traces (monitoring mode), enabling analysis of blood flow in a variety of medical and scientific applications. Easy-to-use image analysis software (LDPIwin) assists in the evaluation of the results and in report generation [5]. This technic shows excellent
intra and inter-observer reproducibilities [6]. The LSCI has a good spatial resolution but have never been studied for vasculogenic mimicry evaluation.

The CEUS is a non-invasive vascular investigation, which allows a good view of neoplastic tissues and have already been validated to evaluate neoplastic microcirculation in an animal model [7] and to vascular modification due to vascular mimicry [8]. It has a high spatial and temporal resolution. Through this work, we evaluated these two imaging investigations applicabilities: laser speckle contrast imaging (LSCI) and contrast enhanced ultrasound (CEUS) in a melanoma mouse-model treated by anti-PD1.

MATERIAL AND METHODS

Cell culture

Mice melanoma cell line B16F10 were grown in Roswell Park Memorial Institute 1640 (RPMI 1640) medium (Lonza, Verviers, Belgium) supplemented with 10% FBS (Lonza, Verviers, Belgium), 10 units of penicillin, 10 mg of streptomycin, 25 μg/mL of amphotericin B (Sigma-Aldrich, Saint-Louis, USA), and 1% non-essential amino acids (Lonza, Verviers, Belgium). Cell lines were cultured and maintained at 37 °C in a humidified atmosphere with 5% CO₂.

In vivo experiments

All procedures involving animals, were conducted in accordance with protocols approved by ethical committee of the University of Angers and the regional ethics committee on animal experimentations (Authorization APAFIS#13365-2018020217041961 v3). Furthermore, animal experiments were carried out in strict accordance with recommendations in the guidelines of the Code for Methods and Welfare Considerations in Behavioral research with Animals (European directive 2010/63/UE).

Seven-week-old male C57BL/6 mice were housed at the university animal facility (Service Commun d’Animalerie Hospitalo-Universitaire - Université d’Angers, France). Syngenic allograft model of melanoma was obtained by injecting subcutaneously a suspension of 10⁵ B16F10 melanoma cells in 100 μL of PBS 1X (Lonza, Verviers, Belgium) into the right flank of mice. For optimal measurement of the tumor and its vascularisation, body hair was removed manually. The mice were shaved with a razor and the remaining hair was removed with depilatory cream. Tumor volume was monitored.

Mice were randomized and were treated when tumor volume was approximatively 100 mm³. Mice were treated twice a week (days 1, 3, 8, 10, 15 and 18) with 3 mg/kg/day mice anti-PD1 (BioXcell, Lebanon, USA) or NaCl 0.9%. Tumor volume was monitored according to different techniques such as (i) caliper with the formula: \( V = \pi / 6 \times L \times W^2 \) \((V \ll \text{volume}, L \ll \text{length} \text{ and } W \ll \text{width})\) and (ii) ultrasonography. In accordance with ethical rules, animals were sacrificed when the tumor volume was greater than 2500 mm³ or when mice showed signs of suffering.

Contrast enhanced-ultrasound

This analysis was performed at the beginning of the protocol (W0) and after one week (W1). Mice were anesthetized with 2% isoflurane. Pre-warmed echo transmission gel was applied to the tumor mice. Acquisition time did not exceed 80 seconds. For each tumor, at least 3 measures corresponding to length (L), width (w) and height (h), were made by ultrasonography imaging with an Arietta v70 equipment (Hitachi Medical Systems, Saint-Priest, France). The volume (V) in mm³ was calculated considering tumors as semi-ellipsoids, with the formula \( V = (4 / 3 \pi \times L \times w \times d) / 2 \) (w, L and d, are the tumor width, length and depth, respectively).

Laser speckle contrast analysis

Measurements were performed twice at W0 and W1 in an air-conditioned and temperature-controlled laboratory. Scanning LDPI measurements were performed by using a PeriScan PIM 3 System 14 and proprietary acquisition LDPIwin software (both by Perimed-Instruments, Järfalla, Sweden). The wavelength with most limited penetration depths into the skin and which is prevaunised for healthy skin was choosen, which is
the 670 nm laser diode [9]. The distance between laser head and skin chosen by the operator was 12 cm. Acquisition time did not exceed 80 seconds. Mice were anesthetized with 2% isoflurane. Tumoral perfusion was evaluated in different ROI (region of interest) – one on an aluminum patch to avoid artefacts (ROI 1), one in normal skin (ROI 2) and one in tumoral site (ROI 3).

The perfusion was determined with the following equation: (ROI1-ROI3)-(ROI2-ROI3)

Statistical analysis
Data were presented as means ± SEM. Statistical differences were determined using unpaired Mann-Whitney test when comparing between two independent groups, and Kruskall-Wallis test followed by a Dunn’s post-hoc test when comparing across three or more independent groups. p value < 0.05 was considered significant. The tumoral growth analysis was performed thanks to a linear mixt model (longitudinal data), including the time influence (intra and inter day variability) and random intercept (intra and inter individual variability). In order to obtain a reliable statistical model, the tumoral volume was converted into a decimal logarithmic scale. Survival analysis was done using a log rang model to compare survival curves and to calculate survival medians.

RESULTS

Anti-PD1 antibody does not alter median or survival rate
No significant difference in mice survival was observed in anti-PD1 treated mice in comparison with control mice (Figure 1.A). Moreover, no significant difference was observed in median survival: it was 12 days for control mice while 13 days for anti-PD1 treated mice (Figure 1.B).

Measurement of tumor volume using a calliper or CEUS
Monitoring of tumor volume with caliper showed no significant difference between anti-PD1 treated mice and control mice (Figure 2.A). Moreover, the measurement of tumor volume through CEUS did not show any difference between control and anti-PD1 treated mice at day 0 and after 7 days (Figure 2.B). In control mice, analysis of tumor volume did not show any significant difference between caliper or CEUS techniques. Likewise, no significant difference was observed in anti-PD1 treated mice. However, although these data are not significant, the distribution of the data appeared to be different. Indeed, the group of mice treated with anti-PD1 and whose tumour volume was measured by caliper showed a wider dispersion of points, indicating greater variability and lower reproducibility for this condition. When tumour volume was measured by ultrasound, the distribution of points was tighter and more consistent, suggesting better reproducibility of the data (Figure 2.C).

Better detection of microcirculation by LSCI than CEUS
From CEUS data, a difference in tumor size was observed at the beginning of the treatment in anti-PD1 treated mice in comparison with control mice. No difference was found after one week of treatment between these two groups (Figure 3.A). LSCI analyses revealed a decrease in tumor perfusion after 7 days in anti-PD1 treated mice compared to control mice (Figure 3.B).

DISCUSSION

Noninvasive imaging techniques are being developed and improved for identifying and evaluating pathophysiological features of tumors in order to assist in the planning of individual patient treatment protocols [10, 11]. Particularly, new imaging approaches that assess tumor vascularization have improved diagnosis and treatment prediction. In this study, we evaluated (i) caliper and contrast enhanced ultrasonography (CEUS) to define tumor dimensions and (ii) contrast enhanced ultrasound (CEUS) and laser speckle contrast imaging (LSCI) to appreciate tumor vascularization.

The murine melanoma model is based on B16F10 cells known for their aggressiveness, high metastatic potential and high expression of the PD-1 receptor [12]. Taken together, these data confirm the value of evaluating the impact of anti-PD1 antibodies in limiting tumor growth. Although the survival data did not
allow us to conclude that there was a significant benefit from anti-PD1, they showed that before 7 days of treatment, no mortality was observed, whatever the group of animals, justifying the use of this time frame to evaluate the different imaging techniques.

To determine tumor dimensions, caliper and CEUS were used in this study. Although tumor volumes are similar for both methods, as recently described in non-melanoma skin cancer [13], our data suggested an interest in CEUS because of various biases described with caliper. Firstly, it has been described that caliper often overestimated tumor volume. Furthermore, the caliper measurements were smaller for small tumors compared to greater tumors also relatively seen. Consequently, volume changes measured with caliper in small and large tumors are not comparable and effects of anti-cancer drugs can easily be missed as tumors will tend towards being determined with a greater bias as they grow larger [14].

The value of CEUS has recently been confirmed by Makouei et al. [15]. In a pilot study conducted on mice with a soft tissue tumor, they confirmed that ultrasonography is a feasible and accurate imaging method to assess the tumor volume. However, these authors pointed out a number of limitations. Among these is the failure to take account of vascularization, which is an important factor in the diagnosis and treatment of metastatic tumors. To overcome these limitations, our study was supplemented by microcirculation analyses through LSCI in comparison with CEUS technique.

While CEUS analysis showed a reduction in microcirculation at treatment initiation, no difference was observed after 7 days of treatment suggesting that CEUS is not sufficiently robust for long-term studies. On the other hand, LSCI analysis confirmed a decrease in tumor perfusion over time in mice treated with anti-PD1 compared with control mice. Compared to CEUS, LSCI measurement did not need a direct skin contact which is an advantage in these types of analyses. Indeed, a strongly hold of the ultrasound probe can create a local ischemia or reduce the microcirculatory vascularization and create biased results. Besides, LSCI measure results from local microvascular analysis without a blank analysis, which minimize artefacts. Last, if using the same experimental conditions, LSCI showed a good reliability and reproducibility. These data suggest the potential clinical value of LSCI in improving the diagnosis of metastatic melanoma.

Acknowledgments

The authors would like to thank the « Ligue contre le Cancer » (CD49, CD72, CD53) for its grant. Authors also thank university hospital animal care from University of Angers for its expertise in the animal studies.

Figure legends

Figure 1
Survival of mice bearing B16F10-xenografted melanoma after treatment with mice anti-PD1 antibody or PBS. Treatment was initiated when the tumor volume was approximatively 100 mm³. (A) Kaplan-Meier survival curve of treated or not mice. (B) Evaluation of mice survival after 7 days treatment, and the survival median after tumor xenograft (n=7).

Figure 2
Evaluation of tumor volume from different methods in B16F10-xenografted mice treated with mice anti-PD1 or PBS. (A) Daily monitoring of tumor volume with caliper. (B) Evaluation of tumor volume through contrast enhanced ultrasonography (CEUS) at the beginning of the treatment and after one week of treatment. (C) Comparison of tumor volume monitoring by using caliper or through contrast enhanced ultrasonography (CEUS) (n=5-7). Data are expressed as the mean ± SEM. Kruskal–Wallis test followed by a Dunn’s post hoc test with a Hochberg correction and Mann-Whitney test was used for statistical analysis (ns p>0.05).

Figure 3
Evaluation of both tumor volume and perfusion from B16F10-xenografted mice treated with mice anti-PD1 or PBS. (A) Evaluation of tumor perfusion through contrast enhanced ultrasonography (CEUS) at the beginning of treatment and one week after. (B) Tumor perfusion was measured by laser speckle contrast
imaging at the beginning of treatment and one week after. Data are expressed as the mean ± SEM. Kruskal–Wallis test followed by a Dunn’s post hoc test (ns p>0.05).

Bibliography


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