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Pan_02 murine pancreatic cancer model

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Abstract: Aim: The aim of this study was to investigate and describe basic features of Pan_02 murine pancreatic adenocarcinoma tumor model. Pan_02 has very low sensitivity to chemotherapeutics therefore it is very similar to human pancreatic cancers.
Materials and methods: Mice were subcutaneously injected with different number of cells and tumor growth was measured. Tumors were also investigated with ultrasonograph VEVO2100 in doppler mode to detect viable and functional blood vessels. Collected tumor samples were investigated to asses necrosis and blood vessel permeability.
Results: We found substantial differences in tumor growth depending on a number of inoculated tumor cells. Mice injected with 0.5×10^6 cells gave the most consistent pattern of growth. All tumors showed substantial vascularisation but bigger tumors were more likely to develop larger blood vessels instead of a more dense network.
Conclusions: Murine Pan_02 cancer model shares many features with human PDAC cancers and therefore it is a good model to study new drugs. Injection of 0.5×10^6 cells gives consistent results although it requires more time for the tumor to grow. It also allows the immune system to adapt. Owing ta good vascularisation, Pan_02 is a good model to study chemotherapy against pancreatic adenocarcinoma but it may not be the best model for immunotherapy since it does not respond to the immune stimulation (unpublished data).

Key words: coronary sinus, anatomy, corrosion casting, morphology.

Introduction

Pancreatic cancer is so called silent killer because it remains undetected until the very last stage when the prognosis is very poor [1–3]. Pancreatic ductal adenocarcinoma (PDAC) is one of the most common pancreatic cancers responsible for almost 90% endocrine tumors

with deadly median <6 months survival [4]. It makes PDAC one of the most lethal cancers with less than 5 year of overall survival [5, 6].

Cancer research is based on *in vitro* and *in vivo* studies which are designed to mimic human cancers. Depending on the goal, different models can be used, e.g. slightly immunogenic models are required for immunotherapy whereas inhibition of metastasis has to be performed in a highly metastatic model. Research on chemotherapy needs well vascularised tumors as chemotherapeutics are delivered mainly with blood flow.

We were looking for well described murine cancer models with various properties regarding vascularisation, metastasis dynamics of growth but very few information was available. Majority of publications give little information on the model and focus basically on therapeutic results. Therefore, we have decided to further characterise the possibilities of Pan_02 tumor model.

The pancreatic adenocarcinoma (PDAC) Pan_02 model has been established in 1984 by Corbet *et al.* in C57BL/6 female by 3-methylcholantherene treatment [7]. There are some controversies about the correct cell line name. Here in we decide to use Pan_02 but in literature one can also find: Pan_02, Pan-02 or Pan 02 which refer to the same cell line [8].

Materials and methods

Cell culture

Pan_02 a murine pancreatic adenocarcinoma (PDAC) was purchased from DCTD Repository at Frederic National Laboratory for Cancer Research. (Maryland, United States). Cells were cultured under standard conditions in humidified incubator at 37°C with 5% CO2 in RPMI (LONZA) with 10% FBS (LONZA). Cultures of Pan_02 were digested with 0.25% trypsin, resuspended in PBS with 5% FBS to inhibit further trypsinisation and washed twice with PBS. Cells were counted and tested for viability with trypan blue. Viability was always > 85%. For injection cells were suspended in PBS.

Laboratory animals

8 to 10 weeks old male C57BL/6 were obtained from Jagiellonian University Medical College Department of Immunology animal facility. Mice were housed in animal facility with water and food *ad libitum*. For every procedure they were moderately anesthetized in isofluranum vapours (Baxter). Tumor size was measured with calliper and volume calculated according to the formula $V = 4/3\pi \times \text{length} \times \text{width} \times \text{height}$. Tumor cells were injected subcutaneously in 100 µl PBS. Mice were treated differently in two experiments. Details are described in the 'results' paragraph. HOECHST (Hoechst 33342, InvitrogenTM, Eugene, Oregon, USA) was injected into a tail vein shortly before mice were anesthetized to visualise diffusion into the tumor. Procedures were approved by Local Ethical Committee for Animal Experimentation in Krakow: 95/2014 and 19/2013.

Immunohistochemistry

Tumor tissue was frozen and cut into 4 μm sections every 300 μm. We used primary antibodies anti-CD31 (BD Pharmigen) and anti-9F1 (Radboud University Nijmegen Medical Centre), secondary antibody with HRP and stained with DAB (Sigma).

Histochemistry

Tumor tissue was embedded in paraffin, cut into 4 μm specimens every 300 μm. Necrotic areas were visualised by H&E staining that was performed according to the protocol by Llewellyn *et al.* [9]. The percentage of necrotic areas were analysed with ImageJ.

Blood flow measurement

Imaging was performed using ultrasound imaging system VEVO 2100 (VisualSonics, Toronto, Canada) with transducer MS-550D (wave frequency 32 MHz) in a doppler mode. Measurements were carried out in about 13 weeks old mice, 4 weeks after tumor cells inoculation. Doppler mode was used to characterize the area of functional vessels in the tumor. Images were analysed with ImageJ v.1.43U (Wayne Rasband, National Institute of Health, USA) to estimate percentage of vessel area in relation to tumor area.

Statistics

All experimental data were analysed with Statistica v.10 software. $P = 0.05$, unless stated otherwise. We used one-way ANOVA and post-hoc Tukey test for different N.

Results

Dynamics of tumor growth

As shown in Fig. 1A, 1B and 1C, 0.5×10^6 cells required more than three weeks to form palpable tumors which is about six days longer comparing to 10^6 and 2×10^6 cells. On the other hand, dynamics of growth plays in favour of 0.5×10^6 cells because as we can see in Fig. 1A, tumors grew uniformly. Injection of 10^6 and 2×10^6 cells resulted in a high diversity of the size of growing tumors. At the time when some mice had palpable tumor the other did not. Injection of 0.5×10^6 cells gave equal results in all mice. Also the final result was different, as the injection of 0.5×10^6 cells resulted in bigger tumors and more aggressive growth comparing to other groups. From experimental point of view it is better to wait 2–3 weeks longer but have more uniform groups. Optimally, Pan_02 needs 2 months to develop larger tumors. It may be experimental disadvantage but since spontaneous tumors have longer incubation time, developing older experimental tumors may lead to clinically better results.

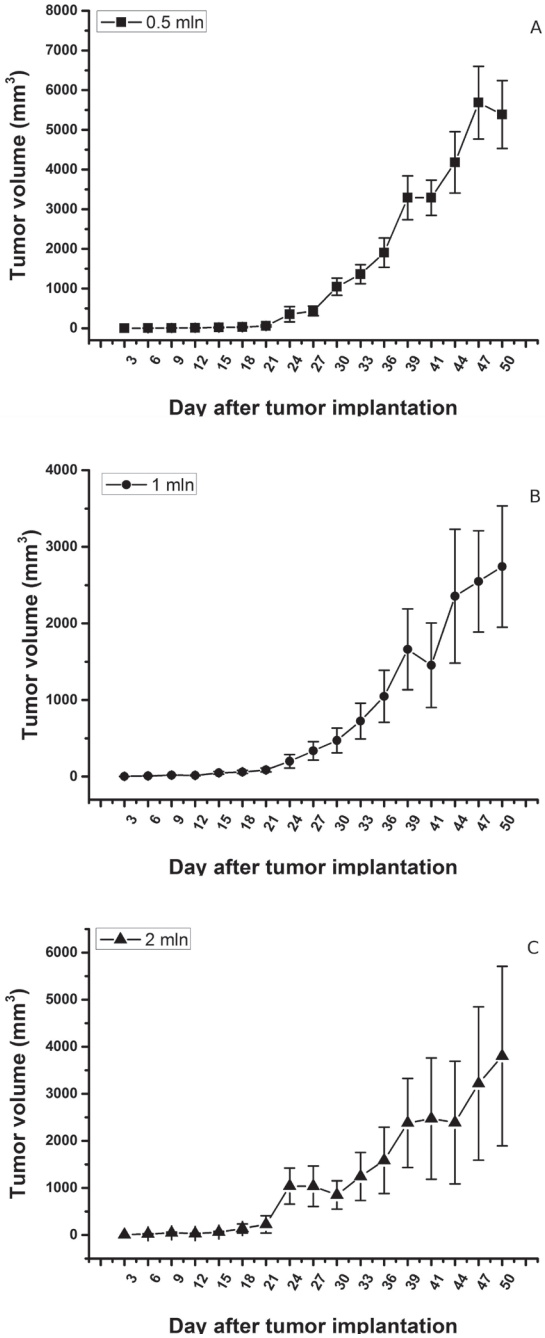

Fig. 1. Mice were subcutaneously injected above the right shoulder with different numbers of viable cells: 0.5×10^6, 1×10^6, and 2×10^6, in 100 µl of Ca^{2+} and Mg^{2+} free PBS. Six mice per group were used. Data are presented in Figures 1A, 1B and 1C respectively to the number of cells.

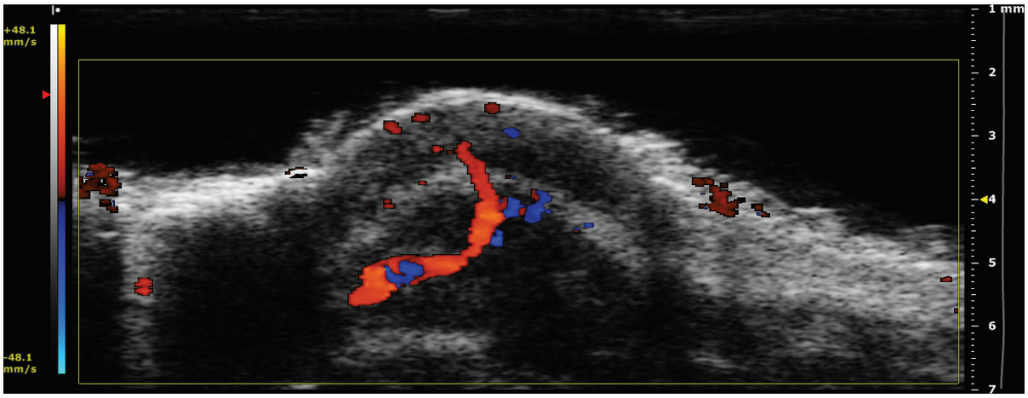

Fig. 2. Example of functional blood vessel. Mice were subcutaneously injected with 0.35×10^6 cells above the right shoulder. For this experiment we used 25 mice. Tumors were observed for over 5 weeks and when all mice reached minimal volume, tumor vascularisation was measured with Doppler ultrasonography (VEVO2100-VisualSonics).

Tumor size dependent vascularisation

Mice were divided into three groups depending on the tumor size: >50, 50–100 and <100 mm^3 in 10, 11 and 4 mice, respectively. Figures 3A and 3B show how vessel formation can be correlated with tumor size. In Fig. 3A we can see that medium size tumors have highest average area covered by vessels but in Fig. 3B we observe that the biggest tumors have highest average vessel size. Therefore, we conclude that tumor vasculature grows new blood vessels only to a certain moment. When tumor becomes big enough, the number of vessels stops growing but already existing vessels get larger, as shown in Fig. 3B. It is also important to find functional blood vessels in tumors. Because of pathological growth tumor vascullature can by dysfunctional. Thanks to doppler mode ultrasonography we were able to show functional blood vessels. It is important because these vessels contribute to tumor growth but are also the way to deliver chemotherapy.

Cross section visualisation of: vasculature, tumor permeability and necrosis

In previous chapter we showed functional tumor vasculature changing during tumor growth. Here in, we want to show the actual existence of blood vessels by anti-CD31 and 9F1 antibody IHC staining as well its functionality by HOECHST. Non vascularised tumor part are shown by visualisation of necrotic areas with H&E. This part of the experiment has been performed on two animals only and therefore cannot be statistically significant. Nevertheless, data showing the vasculature give additional information on the tumor model. We can see that tumor growth is supported by functional vasculature but some parts of the tumor remain hypoxic and eventually necrotic.

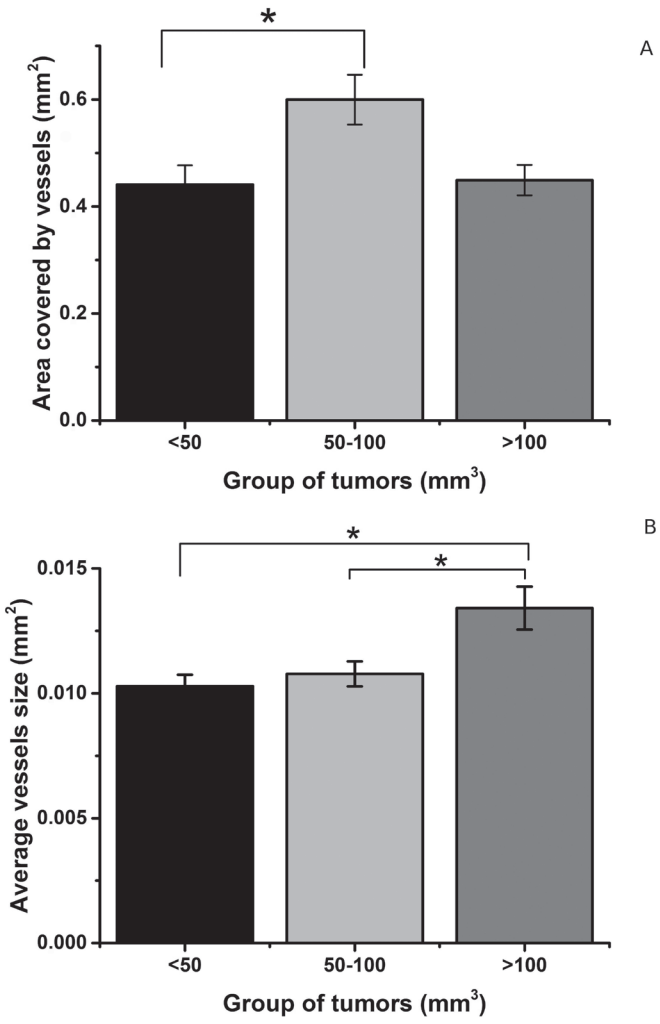

Fig. 3. Tumor blood flow divided into two measurements. Figure A shows Area covered by vessels where we see that not the biggest but the medium size tumors have the highest score. Figure B shows Average vessel size where we see that on average the biggest vessels can be found in the biggest tumors. Combining these observations we conclude that during tumor growth new blood vessels are created but at later stages blood vessels get bigger.

CD31 is a blood vessel marker found also in many immune cell types (Fig. 4B). 9F1 is a rat monoclonal antibody against mouse endothelium [10] (Fig. 4A). We show the presence of endothelial cells inside the tumor by blood vessel staining and additional evidence not only for existence but also functionality we have investigated HOECHST diffusion through the tumor (Fig. 4C). This diffusion may be accelerated due to the leaking blood vessels inside the tumor.

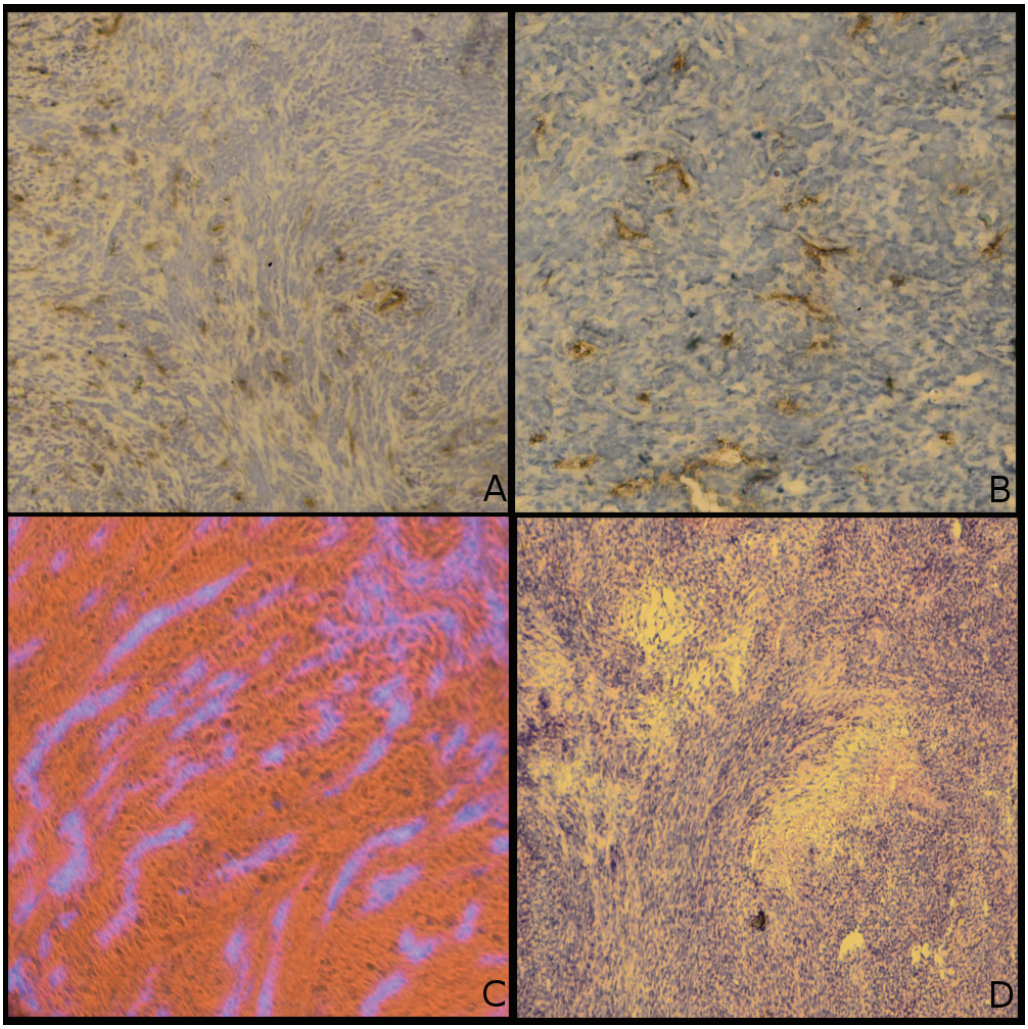

Fig. 4. Pan_02 tumors cut into 4µm slides. Figures A, B and C were cut as frozen section. Figure D is a paraffin embeded tissue. Figure A shows a staining of 9F1, figure B shows a staining of CD31, figure C shows a diffusion of HOECHST and figure D shows a H&E staining.

HOECHST diffused throughout as shown in Fig. 4C but vasculature supports not more that 20% of the tumor mass (Fig. 5). Indeed large hypoxic and necrotic areas were found by H&E staining as shown in Fig. 4D. Our data show tumor necrosis and in Fig. 5 we can see the distribution of necrosis in these two examples. To visualise blood vessels post mortem we have stained tissue slices for two blood vessels markers: 9F1 and CD31, shown in Fig. 4A and 4B.

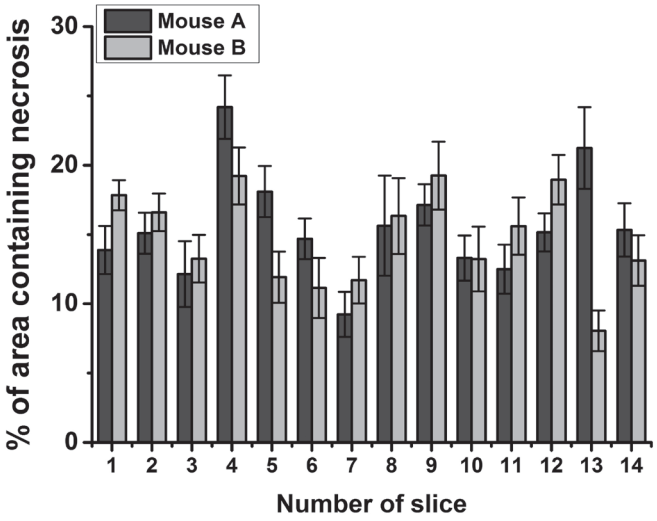

Fig. 5. This experiment has been performed on two mice only and therefore it is not statistically significant. This data shows that about 10–20% of the Pan_02 tumor volume can be necrotic.

Discussion and conclusions

Animal models of cancer are one of the main tools in cancer research. It is impossible to create an ultimate tumor model useful in all types on research. Reliable characterization of the model is a crucial factor for a good analysis of therapeutic outcome. Every model despite certain limitations offers new possibilities. A good example is a model of NUDE athymic mice carrying xenogeneic tumors, very useful in research on chemotherapy as well as in testing oncolytic viruses. On the other hand, NUDE based models are useless in research on immunotherapy as murine immune system is profoundly altered. For example, NP-18 is a human pancreatic cancer cell line used for oncolytic adenoviral research. It is very useful in showing the effectiveness of oncolytic viruses but since these mice have weakened immune system, this model cannot give relevant information on how the virus works in fully efficient immunological surrounding [11]. Therefore, research on newly designed drugs needs to be based on models which fit the specific requirements.

The aim of this study was to verify the value of Pan_02 tumor model for cancer research. We described tumor features relevant in cancer research, such as tumor growth, vascularisation and progression. Analysing the data on tumor vascularisation we conclude that Pan_02 is a good model to study chemotherapy because low molecular anticancer drugs that need to penetrate through the tissue can reach the whole mass of tumor. Majority of the tumor volume is well vascularised and permeable for drugs. Blood vessels inside the tumor are functional and support continuous tumor growth. Even at the very beginning of tumor growth, tumors are well vascularised. PDACs, including Pan_02, are very often resistant to chemotherapy, therefore, Pan_02 is a very good model for research on drugs

against pancreatic cancer [6]. Following our results one can obtain controlled tumor model with a known and predictable level of vascularisation useful not only in drug research but also in other studies such as modification of vascularisation or tumor progression. Pan_02 is a slowly growing tumor. To have experimentally suitable tumor bearing animals, one must wait up to four weeks but as show in the results section proposed protocol gives very repeatable tumors with similar growth curve.

Similarities between Pan_02 and PDAC. Pan_02 stimulated with IFNγ expresses B7-H1 which means poor prognosis in clinics [12]. Additionally, Pan_02 cells are very resistant to hypoxia induced apoptosis (unpublished data) and IFNγ induced apoptosis [13]. Working with this tumor model we have observed frequent metastases, mainly to the lungs and lymph nodes, with much smaller frequency to other organs (unpublished data). It makes this model harder to work with but also gives better correlation with clinical situations. It is especially important in case of extremely consumptive and terminal diseases like PDAC.

Despite several limitations, this study offers information about Pan_02 tumor model regarding its growth and vascularisation. In the future our study will focus on evaluation of Pan_02 model in research on metastatic properties as well as immunological cells infiltration [14]. We hope it may be helpful in cancer research and will contribute to further tumor model development.

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Conflict of interest

None declared.

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