Published in final edited form as:

Clin Exp Metastasis. 2016 February ; 33(2): 115–124. doi:10.1007/s10585-015-9761-y.

Differential Growth and Responsiveness to Cancer Therapy of Tumor Cells in Different Environments

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Abstract

Tumor metastasis often confers poor prognosis for cancer patients due to lack of comprehensive strategy in dealing with cells growing in different environment. Current anticancer therapies have incomplete effectiveness because they were designed assuming metastatic tumors behave similarly in different organs. We hypothesize that tumors growing in different sites are biologically heterogeneous in growth potential, as well as in tumor response to anti-cancer therapies. To test this hypothesis, we have developed a multi-organ tumor growth model using the hydrodynamic cell delivery method to establish simultaneous and quantifiable tumor growth in the liver, lungs and kidneys of mice. We demonstrated that growth rate of melanoma tumor in the liver is higher than that of the lungs and kidneys. Tumors in the lungs and kidneys grew minimally at the early stage and aggressively thereafter. Tumors in different organs were also heterogeneous in response to chemotherapy and immune gene therapy using dacarbazine and interferon beta gene, respectively. Lung tumors responded to chemotherapy better than tumors in the liver, but showed minimal response to interferon beta gene therapy, compared to tumors in the liver and kidneys. We also confirmed differential tumor growth of the metastatic colon cancer in mice. Our results point out the importance of a better understanding of the differences in tumor growing in diverse environments. The biological heterogeneity of metastatic tumors demonstrated in this study necessitates establishing new drug screening strategies that take into account the environmental difference at the sites of tumor growth.

Keywords

Tumor Metastasis; Tumor Heterogeneity; Tumor Microenvironment; Cancer Gene Therapy; Melanoma

Introduction

While an increasing number of human cancers have become treatable, especially with early detection, the presence of tumor metastasis often confers poor prognosis because successful treatment of metastatic tumors remains as an unmet need in clinic [1]. Current preclinical

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Conflict of Interest

The authors declare that they have no conflict of interest.

evaluation of anticancer therapies is generally focused on subcutaneous or orthotopic tumor models, and incompletely addresses the differential behavior of metastatic tumors in different environments. In addition, the lack of a full understanding of biological heterogeneity of metastatic tumors, particularly tumor-stroma interactions within the tumor microenvironment, limits our ability to predict the therapeutic outcomes of a given anticancer therapies [2,3]. Therefore, appropriate tumor models are urgently needed for reliable anticancer drug screening and development, and for the assessment of tumor-environment interactions of the metastasized tumors.

Hydrodynamics-based delivery was initially established for gene transfer into mouse hepatocytes via tail vein [4,5]. This method involves a rapid injection of a large volume of DNA solution into the tail vein to generate high intravascular pressure within the inferior vena cava (IVC), causing back flow into the blood vessels with connection to IVC, and consequently, increasing the permeability of vascular endothelium, resulting in the influx of DNA into parenchymal cells in the liver, and to a lesser extent, in the kidneys [6]. Hydrodynamic gene delivery is presently among the most efficient non-viral methods for gene transfer, and it is increasingly applied for gene therapy, gene drug discovery, and animal model establishment [7]. Recently, the method was utilized for successful delivery and subsequent growth of tumor cells into mouse liver, lungs, and kidneys simultaneously [8].

In this study, a systematic approach was employed to achieve a quantitative assessment of the differential behavior of tumors growing in different organs. We aimed to investigate whether tumors grow and respond differently to anticancer therapies when seeded into different organs. We show that tumors grow differently in different organs, despite originating from the same cell population. We further demonstrate that tumors are also heterogeneous in response to selected anticancer therapies. Our results have significant clinical implications since most current anticancer approaches do not consider the heterogeneity of tumor behavior in different organs, leading to profound failure to treat cancer once metastasis has developed. In addition, our data suggest that more complex regimens are needed to treat metastatic tumors residing in different organs.

Materials and Methods

Materials

The pLIVE plasmid vector was purchased from Mirus Bio (Madison, WI). Mouse IFN β 1 gene was sub-cloned into pLIVE plasmid using complementary DNA sequences. DNA sequencing was used to confirm the sequence of the constructed plasmid. Plasmid was prepared using the method of cesium chloride-ethidium bromide gradient centrifugation, and kept in saline at -80°C until use. The purity of the plasmid preparation was examined by absorbency at 260 and 280 nm and 1% agarose gel electrophoresis. Dacarbazine (DTIC) (purity 98%), and 5-fluorouracil (5-FU) (purity 99%), were purchased from Sigma-Aldrich (St. Louis, MO).

Cells

B16-F1 (murine melanoma) and C26 (murine colon carcinoma) cells were obtained from ATCC. B16-F1 cells were cultured in DMEM with 10% FBS and 1% penicillin/streptomycin, and C26 cells were cultured in RPMI with 10% FBS and 1% penicillin/streptomycin. Luciferase-tagged cells were created using Lenti viral vectors containing luciferase reporter gene. At 80% confluence, the medium was removed, and each plate was treated with 4 ml of Trypsin/EDTA solution (0.25% Trypsin, 2.21 mM EDTA) at room temperature for 4 min. Cells were washed twice with serum-free medium and filtered through a membrane filter (40 µm pore size) and centrifuged at 1,400 rpm for 5 min at room temperature. Cell concentration was determined using a hemocytometer and diluted with serum-free medium to the desirable concentration. Standard calibration curves were established for B16F1 and C26 cells correlating tumor cell number and luciferase activity.

Mice and treatment

Female Balb/c (6–8 weeks old, 18–22 g) mice were purchased from Charles River Laboratories and housed in a pathogen-free environment in the Animal Facility of the University of Georgia. The animal procedures used were approved by the Institutional Animal Care and Use Committee of the University of Georgia (protocol #: A2011 07-Y2-A3). For growth quantification, 12 mice were used and three at each time point. For treatment studies, animals were divided into control and treatment groups (5 mice each). Tumor cell suspension and plasmid solution were injected hydrodynamically via tail vein. Chemotherapy with DTIC or 5-FU was administered intra-peritoneally with vehicle as control. Tumor sensitivity to chemotherapy and immunotherapy was quantified using the equation:

$$“Sensitivity = (\#cells_C - \#cells_T) / \#cells_C”$$

where $cells_C$ and $cells_T$ refer to tumor cell number in control and treatment groups.

Hydrodynamic injection

The procedure of hydrodynamic delivery has been previously reported for gene [4,5] and cell delivery [8]. Briefly, for hydrodynamic cell delivery, a volume equivalent to 8% body weight of cell suspension in serum-free medium was injected into the tail vein over 5–8 sec. For conventional cell injection, the same number of cells was injected into tail vein in a volume of 200 µl over 10 sec. For gene delivery, saline solution of plasmid DNA was injected via tail vein following the procedure of hydrodynamic gene delivery [4].

H&E staining

Tissue samples were fixed in 10% neutrally buffered formalin and dehydrated using increasing ratios of ethanol/water (v/v). Tissue samples were embedded into paraffin for 16 hrs. Paraffin-embedded tissue samples were cut into sections at 6 µm in thickness and dried at 37 °C for 1 hr before incubation in xylene, followed by a standard H&E staining using a commercial kit (BBC Biochemical, Atlanta, GA).

Analysis of luciferase activity

After animal euthanasia, tissue samples from the selected organs were collected and immediately frozen in liquid nitrogen and kept at -80°C until use. For the luciferase assay, 1 ml of lysis buffer was added to each sample (~150 mg) and kept on ice. The thawed tissue was homogenized using a tissue homogenizer (1 min, max speed). The tissue homogenate was centrifuged in a microcentrifuge (10 min, 10,000 rpm at 4°C), and the supernatant was collected. Ten μl of supernatant was taken for luciferase and protein assay according to the previously established procedure [4].

In vitro assessment of IFN β 1 activity

Animals were hydrodynamically injected with 20 μg of pLIVE-IFN β 1 plasmid (empty plasmid as control). Animals were euthanized 24 hr later, and serum samples were obtained, mixed with saline at different dilutions and added to cultured B16F1 cells with regular media. The cells were treated for 0 -72 hours and stained with crystal violet (0.5% w/v) as previously described [9].

Analysis of gene expression

Total mRNA was isolated from collected tissues using TRIZOL reagent purchased from Invitrogen (Carlsbad, CA). One μg of total RNA was used for first strand cDNA synthesis using a Superscript RT III enzyme kit from Invitrogen (Carlsbad, CA). Quantitative real-time PCR (qPCR) was performed using SYBR Green as the detection reagent on the ABI StepOnePlus Real-Time PCR system. The data were analyzed using the Ct method [10] and normalized to internal control of GAPDH mRNA. Primers employed were synthesized in Sigma (St. Louis, MO) and their sequences are summarized in **Supplementary Table 1**.

Statistics

All results are expressed as means $\pm$ SD, and statistical significance was determined using student t-test and analysis of variance. A value of $P < 0.05$ was considered significant difference.

Results

Establishment of tumor growth

To establish tumor growth in multi organs, luciferase-tagged B16F1 cells (1×10^6) in serum-free medium were hydrodynamically injected into a mouse via the tail vein. Tissue distribution of injected tumor cells was assessed by measuring luciferase activity in different organs six hr after cell injection using standard cell type specific standard curves (**Fig. S1**). The results showed that hydrodynamic injection efficiently delivered cells into the liver, the lungs and the kidneys, but not into the other organs (**Fig. 1A**). Among these three organs, liver was the primary recipient organ, receiving approximately one third of the injected cells. The lungs and kidneys received 21% and 8% of injected cells, respectively (**Fig. 1B**). Up to 62% of injected cells were recovered, as calculated by luciferase activity, indicating that approximately 38% of injected cells died, adopted dormancy/senescence phenotype, or both during or after cell delivery into the mice.

Hydrodynamic injection of the tumor cell suspension resulted in tumor growth in the liver, the lungs, and the kidneys (**Fig. S2A**). In contrast, conventional tail vein injection of the same number of cells in a volume of 200 μ L resulted in tumor growth solely in the lungs (**Fig. S2B**). Tumors were macroscopically visible on the surfaces of the three organs. Tumor growth was also confirmed by H&E staining of tissue sections (**Figs. S2C, S2D**). Assessment of luciferase activity after 12 days of tumor cell injection revealed that tumor growth remained confined to these three organs (**Fig. 1C**).

Differential tumor cell survival and growth in different organs

Given the key roles of organ stroma to provide the supporting niche for the initial survival of the tumor cells [11], we examined whether different organs support tumor growth differently by measuring luciferase activity in target organs one day after hydrodynamic cell delivery. The results showed that tumor cells survived differently in the targeted organs (**Fig. 2A**), suggesting that different organs have different environments for tumor survival. We also assessed tumor growth rate in different organs using the standard curve and the measured luciferase activity at days 1, 6, and 12 after tumor cell injection to quantify tumor growth. The results in **Figs. 2B** and **2C** show that the liver is most receptive to melanoma growth. The tumor grew steadily from day one and continued to grow throughout the course of experiment. However, tumors in lungs and kidneys grew very minimally initially, but aggressively thereafter. By day 12, melanoma tumors had grown massively, and macroscopic tumors were visible on the surface of the examined organs (**Fig. 2D**). Tumor growth was confirmed with additional experiments using different number of cells (1×10^6 , 2×10^6 cells/mouse) (**Fig. S3**).

Differential growth of tumor cells in different organs was also confirmed with luciferase-tagged C26 murine colon carcinoma cells. Similar to B16F1 tumors, C26 cells showed distinct survival when seeded into different organs (**Fig. 2E**). In addition, C26 cells grew in different rates in different organs (**Fig. 2F**). These results point out the heterogeneity of supportive environmental inputs among different organs, resulting in differential survival and growth potential of tumor cells in these organs.

Differential response of tumors in different organs to anticancer therapy

Beyond the pivotal role that tumor environment plays in tumor growth, it has profound effects on therapeutic efficacy. Therefore, we looked at the sensitivity of tumors in different organs to anticancer therapies by assessing the response of melanoma tumors to chemotherapy and immunotherapy using DTIC and interferon beta ($IFN\beta 1$) gene therapy, respectively. Animals were injected hydrodynamically with 10^6 cells, and received 50 mg/kg DTIC intraperitoneally. Similar to distinctive growth, melanoma tumors in different organs showed differential sensitivity to DTIC treatment, as quantified by luciferase activity (**Fig. 3A**). While significant antitumor activity was seen in the lungs and the kidneys, tumors in the liver responded modestly to DTIC in comparison to other organs (**Fig. 4B**). Differential response of metastatic tumors was also verified with the gene therapy approach. Animals were injected hydrodynamically with 10^6 cells, and three days later, received hydrodynamic injection of 10 μ g plasmid expressing mouse $IFN\beta 1$ gene. The control animals received empty plasmid. Hepatic mRNA levels of $IFN\beta 1$ gene was checked three days after gene

transfer and was more than 30-fold higher in treated animals, suggesting efficient gene transfer into mouse hepatocytes (**Fig. 3C**). IFN β 1 signaling was induced in the three organs, as evidenced by the induction of expression of Mx1 gene (**Fig. 3D**), the biomarker of IFN β 1 activity [12]. In contrast to chemotherapy, tumors in the liver and kidneys were the most responsive to IFN β 1 gene transfer, while tumors in the lungs were practically resistant to IFN β 1 (**Figs. 3E, 3F**). Tumor load reduction was obvious judging by the reduction of the number of nodules visible from organ surfaces (**Fig. 3G**), consistent with H&E staining of tissue samples from different groups (**Fig. 3H**). We also examined the response of colon cancer tumors in different organs to 5-FU and IFN β 1 gene therapy. Upon treatment, luciferase activity and tumor load were decreased in all organs (**Fig. S2**). However, tumor suppression effect varied between organs (**Figs. 3I, 3J**), albeit to a lesser extent than melanoma tumors. Therapeutic outcomes of 5-FU and IFN β 1 on C26 tumors were also visible on organ surfaces (**Fig. 3K**). These results suggest that in addition to differential growth rates, tumors in different organs are also heterogeneous in response to anticancer therapies. Since genetically identical tumor cells were seeded into different organs, the observed differential growth and sensitivity of tumor cells are largely explained by distinct environmental clues tumors receive in different organs.

We sought to confirm the differential efficacy of IFN β 1 gene therapy on melanoma tumors in different organs using various doses IFN β 1-expressing plasmid DNA. The therapeutic effect of IFN β 1 on tumor growth in the liver was dose dependent (**Fig. 4A**). In contrast, the tumor load was similar in the lungs of treated and control animals, regardless of the amount of DNA injected (**Fig. 4B**), suggesting that tumor cells are minimally responsive to IFN β 1 in spite of dose increase. Visual assessment on the kidneys could not be made because the tumor load was too low (**Fig. 4C**). Histochemistry was used to verify tumor growth and the results showed significant effect of tumors in the liver with 5 μ g of plasmid DNA, but not in the lung (**Fig. 4D**). Microscopic tumors were observed in kidneys of the control mice and animals injected with low dose of plasmid DNA at 0.5 μ g per mouse (arrows in **Fig. 4D**). To exclude the possibility that the differential response to IFN β 1 gene therapy is due to the lack of access of therapeutic protein into the lungs, B16F1 cells were treated *in vitro* with diluted sera of animals received hydrodynamic injection of IFN β 1 expressing plasmid DNA (**Fig. 4E**). Dose-dependent inhibition of melanoma cell growth was observed similar to *in vivo* experiment, suggesting that IFN β 1 was not confined to the liver, but instead, had access to the other extra-hepatic tissues, through blood circulation.

Besides direct anti-proliferative activity, type 1 interferons like IFN β 1 exert antitumor immune response primarily through natural killer cell-mediated production of perforin and granzyme b, and immune-activating cytokines. Therefore, we looked at the expression levels of these target genes in tumor-loaded organs upon IFN β 1 gene transfer. Results showed that the treatment markedly induced the expression of granzyme b (**Fig. 4F**) and perforin (**Fig. 4G**) in the three organs. However, the level of induction in the lungs was lower than that of the liver and kidneys, in parallel with the treatment efficacy in these organs. IFN γ expression was also induced in the three organs upon treatment with IFN β 1 (**Fig. 4H**), but to a lesser degree than the effector cytolytic molecules. Together, these results suggest that the environment in the liver is highly conducive to IFN β 1 activity against tumor growth.

Similarly, minimal efficacy against lung tumors indicates that the environment in the lungs is not favorable for IFN β 1-mediated tumor suppression, since effector molecules were not sufficiently induced, indicating an environment of immune suppression, in spite of IFN γ induction.

Discussion

Modeling tumor metastasis for reliable assessment of anticancer therapies has been a major challenge for the development of efficient anticancer therapies. In this study, we took advantage of the hydrodynamic delivery method to establish a multi-organ tumor growth in mice. We demonstrated that tail vein injection of tumor cell suspension in a volume of 8% of body weight over 5-8 seconds results in simultaneous delivery, and subsequent growth of tumor cells in the liver, lungs, and kidneys. Extravasation of tumor cells upon conventional injection is often inefficient due to limited vascular permeability [13]. However, upon hydrodynamic injection, the permeability of vasculature in the liver and kidneys is dramatically increased due to retrograde dynamic pressure [8], resulting in extravasation of tumor cells into the milieu of these organs. This mechanism has been recently verified using fluoroscopic imaging of injected phase contrast medium to visualize the back flow of injected phase contrast medium from the inferior vena cava to the liver and kidneys in real time [14]. Upon restoring normal circulation, cells remaining in blood vessels will move through the heart and then to the lungs, where cells are trapped and filtered out in the lung vasculature [15] to establish tumor growth. This would explain the confined cell delivery and tumor growth in the liver, lungs, and kidneys, but not in the other organs. Given the liver plasticity and the fenestrated vasculature, the liver is the primary organ impacted by hydrodynamic procedure [6]. In contrast, the lack of intra-vascular pressure in conventional injection resulted in entrapment and sole growth of tumor cells in the lungs because cells had no access to the other organs due to embolic effect of the tumor cells passing through the lung endothelium with blood flow. Similar to other transplantable tumor models involving direct injection of tumor cells into organ parenchyma, our model differs from the process of natural tumor metastasis, and only recapitulates the last step in metastatic cascade, *i.e.* the colonization into secondary organs. Hydrodynamic cell injection is advantageous over other orthotopic injections in being more convenient and non-invasive. In addition, tumor cell distribution and subsequent tumor growth is fully reproducible using hydrodynamic injection. We believe that this procedure is convenient and appropriate for examining tumor cell survival and growth in different organs, exploring tumor-stroma interaction, and assessing therapeutic activity of anticancer regimens.

Tumor metastasis is a highly inefficient process because, among millions of disseminated tumor cells, very few cells successfully engraft, survive and proliferate to form macro-metastatic tumors at distant sites [15,16]. It is well recognized that efficient tumor metastasis and growth competency are not random, rather, there is an emerging pattern of organotropism, *i.e.* organ specificity [17]. Although the blood flow pattern contributes to organ specific metastasis, the propensity of tumor cells to metastasize to, and grow in specific organs is largely controlled by local homing mechanisms that involve coordinated interactions between tumor cells and stromal components of the organ environment. Consistent with these theories, we have demonstrated that different organs have different

supportive potential for initial tumor survival and subsequent growth upon hydrodynamic delivery. In spite of initial significant cell death in the liver, melanoma cells recovered and proliferated aggressively to form macroscopic tumors as early as six days after injection. This would suggest a supportive environment for tumor growth in the liver, which can be attributed to the abundance of growth factors and active growth signaling, along with high vasculature and nutrients availability, making it the second most common target for metastasized tumor cells [18]. Despite tumor growth in the liver, there was a trend of decreased growth rates with time, largely due to increased tumor size. Recently, it has been hypothesized that the reduction in growth rate with increased tumor size is due to systemic inhibition of angiogenesis [19]. In contrast, tumor cells in the kidneys and lungs had minimal growth in the first six days, *i.e.* no apparent increase in tumor load in these organs. The lung is a very common metastatic site, largely because it is the first capillary bed encountered by circulating tumor cells after passing the vena cava and heart. However, mounting evidence suggests that not all physically entrapped cells will successfully establish tumor growth in the lungs because of the need for cell adhesion and vascular remodeling molecules to mediate extravasation and interaction with lung stroma [20,21]. In current study, we show that in spite of a significant number of tumor cells delivered into the lungs, approximately only 20% of the cells survived and initiated tumor growth. Significant cell death is broadly due to the failure to extravasate, and consequent vulnerability to the shear stress of blood flow, immune checkup and anoikis (lack of adhesion apoptosis) [22]. However, tumor cells may acquire a protective shield through pulmonary tumor embolism by co-opting blood platelets, using them as shields to protect from shear stress, as well as immune cells [2], permitting survival and adaptation of tumor cells to establish macroscopic tumors at later stages. Overt metastatic melanoma in the kidneys, on the other hand, is clinically uncommon. However, microscopic metastases of melanoma have been detected in up to 50% of patients [23]. This suggests that the local environment in the kidneys is not favorable for tumor growth and explain the infrequency of metastasis of most human cancers in the kidneys. In this study, we demonstrated that initial melanoma growth in the kidneys is inefficient, and a significant increase in the cell number was seen only at later stage of tumor progression. Given the minimal initial tumor cell death in the kidneys, it would indicate that the apparent lack of increased tumor cells is likely attributed to the adoption of dormancy phenotype, rather than an active proliferative status that is counter-balanced by active apoptotic events. Indeed, it has been reported that melanoma cells remained solitary with modest aggressiveness, even long after treatment [23,24]. The marked increase in growth at later stage may suggest remodeling and the activation of local stroma in the kidneys and lungs, boosting tumor cell growth. Together, these findings conclude that tumor cell behavior varies in different organs, and thus, treatment regimens targeting cell proliferation machinery should accordingly be modified. Notably, differential growth behavior is not cell line-specific, because this trend was also shown with colon carcinoma cells, in which tumors grew at various rates, with more aggressive growth in liver; the primary target organ for colon cancer metastasis.

The environmental factors that dictate the tumor growth profile in different organs are also critical determinants of tumor response to anticancer therapy. Given the heterogeneity of environmental components among different organs, the outcomes of a given anticancer

treatment will vary among different organs. In agreement, we brought to light two cases of differential tumor response to DTIC chemotherapy and IFN β 1 gene therapy, when grown in different organs. IFN β 1 gene therapy approach was chosen based on the pleiotropic activities of interferons regulating direct antitumor activities, such as apoptosis induction, and regulating cell immune responses, allowing depiction of cell-intrinsic, and environment-mediated antitumor response. In addition, gene therapy approach will provide sustained levels of IFN β 1 in mice to maximize therapeutic outcomes, given the very short half-life of IFN β 1 protein. The differential efficacy profile among different organs was more prominent with IFN β 1 treatment, rather than DTIC. While the efficacy of IFN β 1 was limited to tumors in the liver and kidneys, tumors in all organs responded to DTIC, albeit to different degrees. The discrepancy between the two therapies is largely attributed to their mode of actions. DTIC works directly on tumor cells as an alkylating agent [25], and therefore, reflects the intrinsic resistance of tumor cells. Since tumors in all organs evolved from a genetically identical cell population, it is expected to see less variation in response to therapy. On the other hand, IFN β 1 treatment reflects both intrinsic and acquired mechanisms of resistance because it mediates antitumor effects through direct cytotoxicity on melanoma cells [26], activation of natural killer cells and cytotoxic T cells [27], and antiangiogenic effects [28]. Thus, the heterogeneity of IFN β 1 efficacy among different organs is attributed, at least in part, to differences in local immune cell infiltration and activation, and/or the extent of dependence on active angiogenesis in these organs. The higher expression levels of perforin and granzyme B in the liver and kidneys may suggest a superior induction of antitumor immune response in these organs in comparison to the lungs. In addition, pulmonary embolization with tumor cells mentioned earlier may further contribute to the resistance of lung tumors because it protects tumor cells from cytotoxic drugs and effector immune cells. Consistent results were obtained in the dose-response study, which further supports our idea of heterogeneous response to IFN β 1 among different organs. These results indicate that the environment in the lungs is generally immunosuppressive and in favor of tumor survival, whereas liver and kidneys are better candidates to be considered for immunotherapy for melanoma. Therefore, alternative and more potent regimens are needed to overcome immunosuppressive events and to eradicate tumors in lungs.

In summary, we demonstrated in this study that the hydrodynamic cell delivery is efficient for reliable establishment of multi-organ tumor growth in mice. This model provides a convenient tool for examination of tumor growth, tumor-stroma interactions in different anatomical locations, and for therapeutic screening of anticancer regimens. The observed diverse tumor growth and sensitivity to anticancer therapies in different organs highlights the impacts of the tissue environment on tumor cell behavior, and the need for a serious consideration of environmental factors for proper design of anticancer regimens. Functional dissection of the tumor microenvironment may reveal an organ-specific signature of stromal components that dictate distinct cell behavior in different organs. Such profiling will certainly help predict progression and sensitivity of tumors in different organs to selected anticancer therapies. To date, targeting single pathway with monotherapy has been insufficient in spite of initial response, and tumor resistance is often developed with subsequent tumor relapse and regrowth. Therefore, combined therapies tackling multiple targets and pathways potentially maximize the efficacy against tumors in different organs.

Paralleling the progress in “personalized” medicine for cancer patients, we now have the tools for the development of therapeutic strategies for the treatment of tumors residing in different organs and for maximal therapeutic outcomes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgement

The study was supported in part by grant from National Institutes of Health (RO1HL098295). We thank Miss Francisca Carlson for proofreading this manuscript.

Abbreviations

DTIC	Dimethyl-(triazenyl)-imidazole-carboxamide
IFNβ	Interferon beta
5-FU	5- Fluorouracil
Mx1	Myxovirus gene 1

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Fig. 1. Distribution of hydrodynamically injected tumor cells in the liver, lungs, and kidneys 1×10^6 cells/mouse (serum-free medium as control) were injected via tail vein by hydrodynamic injection. Animals were euthanized six hr after injection, and tissues were collected and analyzed for luciferase activity. (A) Tissue distribution of injected tumor cells. Dotted line represents background level. (***) $P < 0.001$, calculated by student t-test (B) Proportion of cells distributed in the liver, lungs, and kidneys. UR: Unrecovered fraction. (**) $P < 0.01$, calculated by ANOVA test). (C) Relative luciferase activity in different tissues collected from animals received 1×10^6 cells/mouse hydrodynamically via tail vein, 12 days

after tumor cell injection. Dotted line represents background level. (***) $P < 0.001$, student t-test). Data represents mean ($\pm$ SD) (n= 5).

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Fig. 2. Differential survival and growth rates of tumor cells in different organs

Luciferase-tagged B16F1 cells (2×10^6) were hydrodynamically injected into mice. Animals were euthanized at different time points, and organs were collected and analyzed for luciferase activity. (A) Fractions of reduced luciferase activity from the injected cells one day after injection in different organs. (B) Luciferase activity per organ at different time points. (C) Fold increase in cell number per day at early (day 1 to day 6) and late (day 6 to day 12) phases. (D) Upper, macroscopic tumor visible on organ surfaces. Lower panel, photo images of organ surfaces at 20X. (E) Differential survival of C26 cells in different organs one day after hydrodynamic cell injection (1×10^6). (F) Fold increase in C26 tumor cell number per day at early (day 1 to day 6), middle (day 6 to day 12) and later (day 12 to day 18) phase. (* $P < 0.05$, ** $P < 0.01$, ANOVA test). Data represents mean ($\pm$ SD) ($n=3$).

Fig. 3. Differential response of tumors in different organs to anticancer therapies

B16F1 cells (10^6 cells/mouse) were hydrodynamically injected via tail vein. Three days after the injection, animals received 5 intraperitoneal injections of 50 mg/kg DTIC (vehicle as control), or hydrodynamic transfer of 10 μg IFNβ1 plasmid (empty plasmid as control). Animals were euthanized on day 12. (A) Luciferase activity per organ with or without DTIC treatment. (B) Quantified B16F1 tumor sensitivity to DTIC in each organ. (C) and (D) Relative mRNA levels of *IFNβ1* and *Mx1* genes in each organ after hydrodynamic transfer of IFNβ1 plasmid, compared with control group. (E) Luciferase activity per organ with or without IFNβ1 treatment. (F) Quantified B16F1 tumor sensitivity to IFNβ1 gene transfer in each organ. (G) Images of the organs with B16F1 tumor, with or without treatment. (H) H&E staining of tissue sections from liver, lungs, and kidneys in all groups. Bars represent 50 μm. (I) Quantified C26 tumor sensitivity to 5-FU treatment in each organ. C26 cells (10^6 cells/mouse) were hydrodynamically injected via tail vein. Five days after the injection,

animals received 5 intraperitoneal injections of 20 mg/kg 5-FU (vehicle as control), or hydrodynamic transfer of 10 µg IFNβ1 plasmid (empty plasmid as control). Animals were euthanized at day 15. **(J)** C26 tumor sensitivity to IFNβ1 in each organ. **(K)** Images of the organs with C26 tumors, with or without treatment. (* $P<0.05$, ** $P<0.01$, *** $P<0.001$, calculated by student t-test and ANOVA test). Data represents mean ($\pm$ SD) (n=5).

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Fig. 4. Liver, but not lung environment, is in favor for IFNβ1 antitumor activity

B16F1 cells (10^6 cells/mouse) were injected hydrodynamically via tail. On day 3, animals received hydrodynamic transfer of various doses of pLIVE-IFNβ1 plasmid (0.5, 1.0, 2.5, and 5 μg DNA/mouse), or empty plasmid as control. Animals were sacrificed 9 days after tumor cell injection. (A) Tumor load in the livers of animals injected with same number of tumor cells but different amount of pLIVE-IFNβ1 plasmid DNA. (B). Tumor load in the lungs of same groups of mice. (C). Nonvisible tumor load in kidneys of the same groups of animals. (D) H&E staining of tissue sections from the liver, lungs, and kidneys of animals received increasing doses of IFN-β1 plasmid. Arrows in kidney sections show microscopic tumor growth. Bars represent 50 μm. (E) Effect of IFNβ1-containing serum on B16F1 cell growth *in vitro*. (F), (G), (H) Relative mRNA levels of *Granzyme B*, *Perforin*, and *IFNγ* genes in each organ after hydrodynamic transfer of IFNβ1 plasmid, compared to control group. (** $P < 0.01$, *** $P < 0.001$, calculated by t-test). Data represents mean ($\pm$ SD) (n=5).