Static Image Analysis as New Approach for the Characterization of Tumor Cell Lysate Used in Dendritic Cell Vaccine Preparation

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Summary

Background: Safety is an important consideration for the clinical application of dendritic cells (DC) loaded with autologous tumor lysate (TL). Thus, avitalization of TL from living autologous tumor tissue has to be guaranteed.

Methods: Composition of TL was investigated by static image analysis (SIA) with the Morphologi G3 device, which simultaneously measures size and shape of up to 100,000 particles within one sample run. This approach was compared with sample characterization by high-resolution automated cell counting, trypan blue staining, and ATP quantification. **Results:** Using SIA, we only detected fragmented, non-cellular structures in completely avitalized TL, indicating complete destruction of living cells. Analysis of particle size distribution by SIA as well as CASY cell counter showed that 95% of particles had a diameter of <10 µm as a sign of cell fragmentation. Complete avitalization of TL was confirmed with trypan blue staining and ATP analysis. **Conclusion:** Regarding generation of DC vaccines, the proof of avitality of TL from living tumor tissue can clearly be achieved by SIA alone or in combination with standard assays. Our data show that SIA is a highly precise method for TL characterization. The SIA device complies with FDA regulation and, therefore, might be suitable for characterization of cellular therapy medicinal products.

Introduction

Glioblastomas are extremely aggressive tumors of the central nervous system. Despite multimodal therapy consisting of surgery as well as radio- and chemotherapy, relapse rates are high (88% within 3 years), and the overall survival is discouragingly low (26% after 2 years) [1]. Therefore, new curative therapeutic options are urgently needed. Breaking the immune resistance of the tumor microenvironment with immunotherapeutic antigen-pulsed dendritic cells (DC) can prolong clinical remissions in patients with brain malignancies [2–4]. To this end, immature DC are pulsed with an autologous protein mixture from whole tissue tumor lysate (TL) to ensure that DC are exposed to a large pool of relevant tumor-associated antigens. To systematically test this strategy, 20 clinical trials on DC immunotherapy are currently registered with ‘dendritic cell vaccination for glioma’ in the EU and USA [5–10]. In 11 of these trials, autologous TL serves as antigen material for pulsing DC. The other 9 trials used mRNA, T-associated antigen (TAA), or a glioblastoma stem cell lysate to load the DC.

While autologous tumor tissue lacks the risk of disease transmission that is inherent to allogeneic or xenogeneic tissues, it is critical that only non-viable tumor cells are injected into patients during the vaccination procedure [11]. The challenge for producing avitalized TL is to maintain the structure of antigens and, at the same time, to kill all tumor cells within the lysate [12]. This includes the need to prove that all tumor cells within a given TL have been completely destroyed. Approaches that are commonly used to characterize the integrity of cells, such as dye exclusion assays, are also applied to test the viability of TL [13–16]. In fact, trypan blue staining is widely used to investigate whether a TL sample is con-

taminated with viable tumor cells that could be harmful for patients [17–19]. Other types of assays, such as the quantification of ATP as a measure of metabolically active cells or high-resolution cell counting technologies, can also be used to analyze cell viability in TL [20].

A new technology in the context of TL characterization is static image analysis (SIA), which enables high-resolution characterization of particles with regard to size (1 μm to $>1\text{ mm}$), shape and surface properties [21, 22]. The advantage of SIA over more conventional techniques lies in the possibility to identify and analyze individual TL particles. Using SIA we were able to prove avitality of TL derived from living tumor tissue. By comparing SIA with other viability assays, this study is the first investigation of SIA technology in TL characterization.

Material and Methods

Tumor Lysates and Control Samples

Lysates were prepared from frozen tumor tissue isolated from patients with high-grade glioma in accordance with a protocol approved by the local ethics committee (commission of ethics 185/2, Ärztekammer des Saarlandes). The histology of each tissue sample was confirmed by a pathologist. Thawed samples were crushed mechanically in 10 ml NaCl 0.9% followed by standardized tissue homogenization in a closed tube system, achieved through automated tissue dissociation using GentleMacs (Milteny Biotec, Bergisch Gladbach, Germany; M-tubes, device protocol 'Protein_01_01'). The tissue samples were further processed by six freeze-thaw cycles ($-130\text{ }^{\circ}\text{C}/+50\text{ }^{\circ}\text{C}$), followed by 60 Gy gamma-irradiation (OB 29/4; STS, Braunschweig, Germany) to generate completely avitalized TL (cTL). Partly avitalized TL (pTL) with residual viable cells was generated by avoiding complete dissociation of the tumor tissue. To this end, we used a gentler dissociation program (C-tubes, device protocol 'tumor brain_01_01'), left out the freeze-thaw cycles as well as the irradiation steps. Both cTL and pTL were probed for protein concentration using bicinchoninic acid and then stored at $-80\text{ }^{\circ}\text{C}$ until further use. Primary glioblastoma cells cultured in RPMI/10% fetal bovine serum were used as non-processed living tumor cells (ITC).

Trypan Blue Staining

Trypan blue staining was performed on 7 cTL samples and 1 pTL sample as control. TL aliquots were diluted 1:1 with 0.4% trypan blue solution (#93595; Sigma-Aldrich, Steinheim, Germany), incubated for 3 min at room temperature and analyzed in a hemocytometer. Unstained (viable) and blue stained (non-viable) cells/particles were counted by three independent observers, and an average value was taken. In average, an absolute number of 3,380 blue particles in the 7 cTL samples and 6,443 blue/white particles in the pTL sample were counted.

Automated Cell Counter

10 μl of cTL or ITC as control sample were suspended in 10 ml system dilution liquid and measured with the CASY TT cell counting device (Roche, Mannheim, Germany) according to manufacturer instructions. The CASY technology combines particle identification using resistance measurement with pulse area analysis based on digital pulse processing technology [23]. This allows for discrimination of dead and living cells. Cell count and viability is given as cells per ml and percentage of all cells, respectively.

ATP Assay

The CellTiter-Glo assay (#G7570; Promega, Mannheim, Germany) was performed in 96-well micro plates (#655094; Greiner bio-one, Frickenhausen, Germany) according to manufacturer instructions. The reaction was allowed to

stabilize for 15 min and read in a luminometer (Genios Pro; Tecan, Mänendorf, Switzerland). The indication of the linear range by manufacturer is between 5 and 50,000 cells per sample. A titration curve was measured ($n = 7$) with freshly isolated peripheral blood mononuclear cells (PBMC) (10–100,000), or PBMC spiked with cTL (0.5 ng to 5 μg cTL; 5 μg cTL per 100,000 cells). As positive control, one pTL sample was tested. Independent zero valuations were carried out (NaCl as empty value of TL, and RPMI medium as empty value for PBMC suspension). The 7 tested cTL samples were incubated for 4 h at room temperature before measurement to ensure a complete hydrolysis of ATP delivered by destroyed cells.

Static Image Analysis

Automated imaging by using the Morphologi G3 device (Malvern Instruments, Herrenberg, Germany) is a high-resolution direct technique for characterizing particles from around 1 μm to $>1\text{ mm}$ in size. In contrast to classical light microscopy, statistically representative distributions are achieved by analyzing up to hundreds of thousands of particles per measurement. This instrument measures the size, the size distribution, and the shape of particles [22]. The calculated size is represented by the circle equivalent diameter (CE diameter). The form factor of the particles is expressed as aspect ratio result (width/length). To evaluate how much the shape of a particle differs from a perfect sphere, the parameter of circularity is used. Circularity is defined as the perimeter of the particle divided by the perimeter of an equivalent area circle. Spherical particles show aspect ratios and circularity values close to 1. In addition to particle size and form analysis, images of each particle are taken during analysis and stored for later examination. Five cTL samples were scanned to test for avitality, and 2 ITC samples served as controls. To this end, a 10 μl sample volume was tested for particle screening in a defined area of a glass cover slip. As cTL composition varies highly, a descriptive statistical analysis based on individual result data was performed.

Statistics

Statistical calculations were performed using the software package Microsoft Excel by applying the two-tailed Student's t-test. Data from independent experiments are shown as mean values $\pm$ standard deviation (SD) as indicated. Statistical probability is expressed as * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$. Values were considered significant when $p < 0.05$.

Results

Trypan blue staining is an established method to validate avitalization in autologous TLs [24–25]. Here we used trypan blue to screen TLs from clinical glioma tissues for viable cells. Evaluation of cTL samples by trypan blue assay resulted in the detection of mainly small cell fragments and some larger pieces of tissue (fig. 1A). Systematic analysis of cTL from 7 patients showed that 100% of the fragments and the large particles stained positively with trypan blue, demonstrating that complete avitalization was achieved (fig. 1C). Gently homogenized pTL control samples, on the other hand, contained up to 25% viable cells, which are excluded from trypan blue staining (fig. 1B, C). Together our results show that trypan blue staining is a tool to differentiate viable from non-viable particles within TL.

Necrosis leads to cell fragments which can be distinguished from viable cells based on their reduced size and loss of ATP. To explore additional methods for assessing avitalization in cTL, we performed cell size analysis in cTL compared to intact, viable tumor cells (ITC) from a primary glioblastoma cell line (fig. 2A) using the CASY TT cell counter. Based on this method, we found that trypsinated ITC

Fig. 1. Light microscope image of TL after trypan blue staining (hemocytometer) and average vitality **A:** Sample of cTL with 100 % of dead blue-colored cells. **B:** Sample of gentle homogenized pTL showing remaining viable (non-colored) cells. Scale bars (100 μm) are indicated. **C:** Trypan blue cell counting results by hemocytometer in cTL ($n = 7$) and pTL samples by three independent investigators.

Fig. 2. Light microscope images of ITC. ITC in culture (**A**), or in suspension before use as control sample for living cells (**B**). Scale bars (100 μm (**A**); 100/10 μm (**B**)) are indicated.

(fig. 2B) (median diameter 18–20 μm ; 87.5% of cells > 10 μm were significantly larger than particles detected in cTL (78.7% of particles < 10 μm) (fig. 3). Events < 10 μm were detected by CASY TT as cell debris and dead cells after device calibration. In addition, we used a luminescence-based assay to assess intracellular ATP, which dissipates from necrotic cells following their permeabilization [20]. In agreement with this, we only detected a small amount of 5–5,000 ng of ATP in cTL samples, which was lower than the ATP content of 10 intact control cells (PBMC) and close to the detection limit of

the assay (fig. 4A). In contrast, the ATP signal of pTL samples was positive and could clearly be discriminated from the cTL sample signal. The resulting ATP signal in pTL was 10 times higher compared to cTL samples (fig. 4A). When spiking cTL samples (0.5 ng to 5 μg) with increasing numbers of PBMC (10–100,000), a reproducible increase of the luminescence signal proportional to the number of PBMC could be detected (fig. 4B). Together, our results show that the ATP release assay reliably detects the absence of viable, metabolically active cells.

Fig. 3. Screening of cTL and ITC by CASY TT cell counter. **A, B:** Dashed lines are used to adjust the delineation of debris (left of line) and dead cells (right side of the line). Solid lines serve to adjust the delineation of dead cells (left side of the line) and living cells (right side of the line). **C, D:** Corresponding microscopic images of ITC sample (**C**) or cTL sample (**D**) are shown in inlays. Scale bars (10 μ m) are indicated.

Fig. 4. ATP assay. Titration of luminescence rise by ATP release in viable PBMC, and ATP signal in both cTL ($n = 7$) and pTL ($n = 1$). The signal of the cTL alone is below the detection limit and has almost no ATP signal ($\square$). An ATP signal of pTL (5 μ g, $\diamond$) was detectable (**A**). $n = 5$ pTL; logarithmic representation of the Y-axis. The spiking of PBMC (10–100,000 cells, $\triangle$) with increasing amount of cTL (0.5 ng to 5 μ g) leads to a reproducible increase of the luminescence signal parallel to the PBMC curve (not shown) (**B**). Data shown are mean $\pm$ SD, P of 100,000 cells < 0.05 (*) compared to cTL (5 μ g).

Fig. 5. Results of particle analysis of cTL and LTC samples by SIA technology. Test result of cTL (**A, B**) and LTC samples (**C, D**) are given: Size distribution of identified particles in measured area (**A, C**), and a selection of pictures of identified particle (**B, D**).

The composition of cTL was further analyzed by SIA, and results are presented as size distribution (fig. 5). Paralleling our results with CASY TT, we found that over 95% of the particles in cTL had a mean diameter of $\leq 10 \mu\text{m}$ with a maximal diameter of 62.08 μm (fig. 5A). The circularity and aspect ratio values were < 0.5 indicating non-circular particles (fig. 5B). The LTC particles, in contrast, were significantly larger with diameters up to 138 μm and circularity values between 0.5 and 1.0, indicating circular cells (fig. 5C). SIA technology also allowed us to perform a detailed morphological analysis of large cTL particles, which exhibited a highly irregular shape distinct from the round shape of the LTC (fig. 5B, D). Moreover, when we screened cTL samples with a filter that was based on circularity and diameter of LTC samples, we only visualized artifacts such as air bubbles but no cell-like structures. Therefore, we conclude that SIA is an effective tool to confirm complete destruction of cells within cTL based on size, shape as well as morphology.

Discussion

When it comes to the preparation of DC vaccines, proof of avitality in TL is an important safety issue [11]. Commonly used approaches to characterize the viability of cells are broadly based on techniques to assess the integrity of the cell membrane as well as the overall cell/particle size since necrotic cells tend to burst and form fragments [13–16]. Here, we used for the first time a novel computer-assisted imaging strategy, SIA, to test avitalization of TL from patients with glioblastoma.

In principle, it is difficult to detect a small number of viable cells in a suspension containing a high concentration of dead cells or cell fragments. To this end, we compared SIA, which measures particle size, size distribution and morphology, with established techniques such as trypan blue uptake, ATP secretion, and cell fragmentation to assess cell viability. Our study showed that all these assays including SIA measured complete absence of viable cells in

cTL. One of the strengths of SIA is that it allows for simultaneously characterizing and visualizing particles in TL. By scanning thousands of particles in a very short amount of time, SIA detects residual viable tumor cells with high reproducibility and reliability. Moreover, SIA provides the opportunity to trace back individual particles for detailed morphological inspection. This combination of high throughput capability with individual analysis is a unique feature of SIA and, as such, could be very useful for in-depth process validation procedures. While our tests provide proof of concept for SIA as a means to demonstrate TL avitalization, additional studies will be necessary to fully validate this technology for clinical application. Meanwhile, the trypan blue dye exclusion assay is validated in many centers for routine quality control of TL and is applied in most studies as the default method for the detection of avitality. Under these circumstances, SIA could be a valuable addition for routine diagnostics of TL with a comparable hands-on time for technicians compared to the trypan blue assay [26].

In addition to SIA, we analyzed TL avitality with a high-resolution cell counter based on the Coulter principle, which enabled us to determine tissue viability as a function of cell size. Consequently, we were able to detect cell and tissue fragments with high precision and, therefore, to conclude that the vast majority of tissues within a cTL sample has been avitalized. However, the shortcoming of simple size measurements is that it does not allow for interpretation of the few larger particles ($>10\ \mu\text{m}$), which could represent viable cells. This lack of specificity can neither be alleviated with flow cytometry-based assays using propidium iodide, which exhibited a high degree of variability in inter-laboratory studies that compared reproducibility and precision of viability assays with cryopreserved hematopoietic cells [27, 28]. Additionally, measuring dead cells with 7-AAD has its own problems due to a high background signal.

Viability of cTL and pTL was further analyzed by measuring the ATP content with a luminescence-based assay. Determination of ATP using firefly luciferase is one of the standard technologies for estimating the number of viable cells due to its high sensitivity since ATP assay chemistry can typically detect fewer than 10 cells in a given sample [20]. Consequently, we found the ATP assay to be very sensitive with regard to detecting viable cells in the examined cTL. However, the stability of ATP has to be taken into account when determining the loss of intracellular ATP. In our experience, TL must be incubated for at least 4 h prior to measurement to ensure that the ATP released from destroyed cells is completely hydrolyzed.

Additional problems result from the fact that the ATP assay requires large amounts of tissue, which together with the elaborate testing method limit its use for routine quality control applications.

Characterization of the cTL composition by SIA technology clearly identified fragmented, non-cellular structures and assured a complete absence of intact living cells as confirmed by trypan blue staining as well as probing for ATP. Thus we showed that an established methodology of homogenization of tumor tissue samples followed by freeze/thaw cycles and gamma-irradiation reliably kills all tumor cells. Such treated lysate was used by our laboratory for generation of mature DC vaccines as part of a successfully validated process. The availability of a sufficient amount of autologous tumor tissue is one of the limiting factors of such a DC preparation process. Therefore, it is crucial that quality control testing during the TL processing procedure consumes as little samples as possible. As only a very small amount of cTL is needed for a powerful and objective particle analysis by SIA, this could be of significant advantage compared to other methods. Finally, the instrument complies with the FDA regulation hence the SIA technology might be accepted by regulatory authorities also for TL characterization [29].

In conclusion, the assessment of bioinactivity of TL can clearly be achieved by SIA alone or in combination with standard assays. Beyond the issue of TL quality control assuring the absence of living tumor cells, a better knowledge of particle size and composition might offer new insights into the immunogenic role of TL for DC vaccination. Moreover, the structural composition of biological therapeutics is rarely characterized in detail once effectiveness has been proven [30]. For some blood products, e.g. autologous serum eye drops, particle screening by a SIA-based analyzer could therefore be a suitable tool.

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Disclosure Statement

The authors have nothing to disclose.

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