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Mini-review

Translational Prospects of ultrasound-mediated tumor immunotherapy: Preclinical advances and safety considerations

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ABSTRACT

Immunotherapy is considered as a promising therapeutic approach for cancer treatment. This therapy focuses on the specificity and memory of the immune system against malignant cells to achieve a sustained cure with minimal toxicity. However, the effectiveness of immunotherapy is often limited by the insufficient delivery and low accumulation of therapeutic molecules in tumors. Ultrasound exposures with the presence of microbubbles can be used as an effective method to greatly increase cell membrane permeability and enhance tissue accessibility to drugs or genes, thereby improving the access of therapeutic molecules into non-permeable tissues and enhancing the therapeutic outcomes. In this review, we discuss challenges in current tumor immunotherapy and feasible approaches that could potentially overcome these obstacles with the help of ultrasound; thereafter, we elaborate on the recent advantages of ultrasound-mediated delivery approach in tumor immunotherapy.

1. Introduction

Cancer is the second leading cause of death globally, and it was responsible for an estimated 9.6 million deaths in 2018 [1]. Immunotherapy has been investigated worldwide as the fourth major cancer treatment modality, following surgery, chemotherapy, and radiotherapy. In recent years, immunotherapy has undergone dramatic progress [2] and has consistently improved the overall survival of patients with advanced-stage lung cancer [3] and metastatic melanoma [4,5] in clinical trials. A dendritic cell (DC) vaccine was recently approved in the US for the treatment of castration-resistant prostate cancer; however it exhibited a low response rate [6]. Moreover, adoptive T-Cell transfer (ACT) continues to progress with developments in the technology of antigen-specific T-cell receptor (TCR) gene and chimeric antigen receptor (CAR) gene transduction to lymphocytes [7]. This method has been demonstrated to mediate the objective regression of metastatic melanoma in 49%–72% of the patients, with complete durable responses observed in 40% of patients. Furthermore, checkpoint inhibitor (CI) immunotherapy, such as the *anti*-PD-1 antibody (aPD-1), has been shown to be highly effective in clinical use [8,9]. However, only 15%–20% of unselected patients with non-small cell lung cancer achieved objective responses [8]. It is believed that major hurdles still exist for immunotherapy [10], such as the insufficient delivery and low concentration of therapeutic molecules in the tumor.

Physiologically, it is difficult for drugs and genes to extravagate through non-permeable barriers, such as vascular walls, the blood-brain-barrier (BBB) and cell membranes. Evidence indicates that there are many factors that may enhance this difficulty in the setting of cancer. For example, tortuous and leaky tumor vessels may impede leukocyte infiltration and extravasation [11]. Even when extravasation is successful, immune cells like T-cells are usually accumulated in the stroma but not distributed within tumors [12]. Moreover, the high interstitial fluid pressure (IFP) in tumors inhibits convective transport [13]. On account of these barriers to treatment permeation, it is necessary to develop safe and efficient methods for delivering tumor immunotherapeutic agents.

It is perhaps well known by now that ultrasound technology, though better known for its role in clinical diagnostic imaging, can also be used for therapeutic purposes. With the presence of microbubbles, ultrasound can temporarily open "repairable holes" in cell membranes to improve their permeability, enabling more efficient delivery of the therapeutic agents across the cell membrane into the tumor or immune

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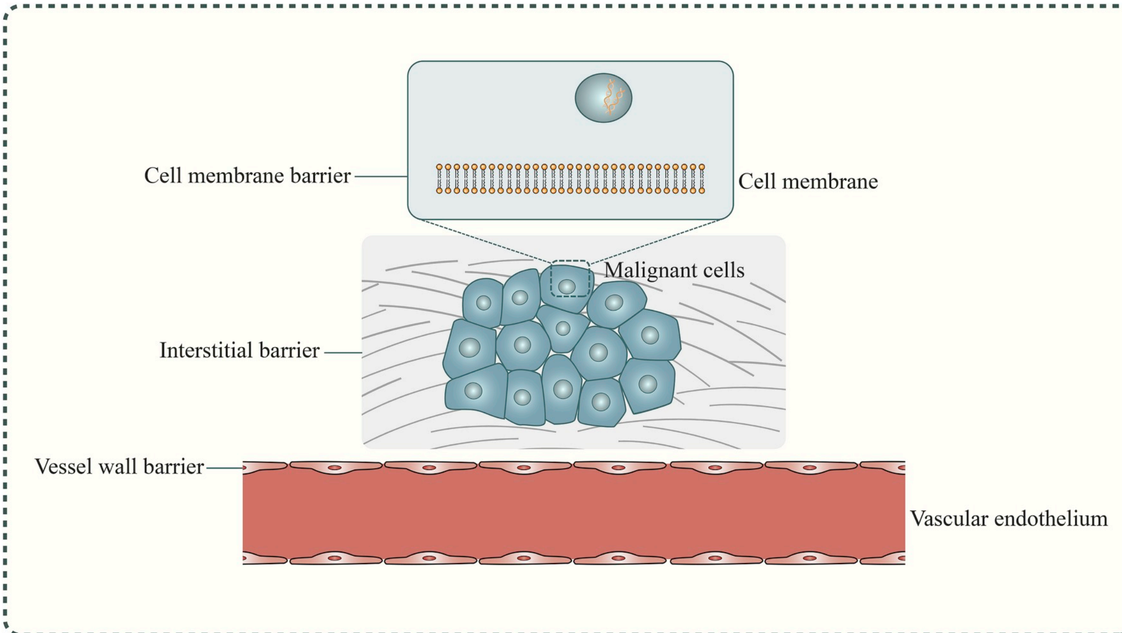

Fig. 1. Schematic illustration of the obstacles to delivery in tumor immunotherapy.

cells [14]. Similarly, microbubble-mediated ultrasound exposures can effectively enhance vessel permeability and promote drug access into extracellular spaces [15]. Recent studies have also demonstrated that treatments with ultrasound alongside microbubbles could transiently open the BBB and facilitate drug delivery into the brain [16]. Based on these bioeffects, ultrasound may provide a safe delivery approach to overcome these aforementioned obstacles to drug permeation.

In this review, we discuss the challenges that exist in current tumor immunotherapy, and then focus on the possible solutions that may be offered by ultrasound technology, and specifically on how ultrasound-mediated delivery approaches may be capable of opening biological barriers and improving the efficacy of anti-cancer immunotherapy delivery.

2. Obstacles to delivery in tumor immunotherapy

The transportation of drugs and genes to a tumor is considered to be dysfunctional and is hampered by various obstacles, including tumor vessels, the interstitium, and the cell membrane (Fig. 1).

The cell membrane is regarded as a delivery barrier if the drug itself cannot get across the cell membrane [17]. The effectiveness of the cancer immunotherapy is dependent on how efficiently drugs are delivered into cells. It is known that tumor blood vessels are highly irregular and chaotic compared with those in normal tissue [11]. The leakiness of tumor vessels is heterogeneous in spatial distribution, leaving avascular spaces of varying sizes [18]. By using intravital microscopy, it is demonstrated that 53 ± 6% of blood vessels in melanoma do not support blood flow. Clinical observations are consistent with preclinical results [19]. When the drug/gene is intended to be delivered into the brain, the BBB that formed by endothelial cells and connected by tight junctions limits the passage of blood-borne molecules and materials [20].

An increase in interstitial fluid pressure (IFP) is seen with solid tumors indicating a very prominent barrier in treatment. This obstacle, resulting in an inefficient uptake of therapeutic drugs, is shown to be created by proliferating tumor cells [21], stromal cells [22], and the extracellular matrix (ECM) exerting mechanical stress which compresses blood vessels, which in turn results in an elevated IFP, restricting drug and lymphocyte extravasation and penetration. Others factors, such as hyperpermeable vessels and the lack of functional lymphatic vessels inside the tumors, also contribute to the elevated IFP. Evidence has indicated that the IFP in most solid tumors is increased, such as breast, colorectal tumors and metastatic melanoma [23]. IFP values have been recorded to be as high as 60 mmHg in certain tumors, while the osmotic pressure is only about 28 mmHg in human capillaries. In a study on melanoma or lymphoma, patients that exhibited progressive response to chemotherapy demonstrated a decline in IFP, while patients who did not respond well had increasing IFP measures [24].

It is necessary to develop feasible strategies to overcome the above obstacles, such as lowering tumor IFP with specific antagonists. Preclinical and clinical studies have demonstrated that vascular normalization can augment drug delivery and tumor-infiltrating lymphocytes [11,25,26]. Research into an advanced-stage epithelial ovarian cancer mouse model indicates that treatment with 3TSR could normalize the tumor vasculature and therefore enhance the tumor blood perfusion. This treatment also resulted in greater intratumoral influx of macrophages, natural killer (NK) cells, cytotoxic T-cells and T-helper cells compared with either treatment alone [26]. Other papers look into methods which modify the physicochemical properties of drugs, such as molecular weight, shape, charge, to improve the rate of delivery through tissue, most notably by manipulating nanoparticle cellular uptake [27].

Modulation of the tumor microenvironment to lower the IFP holds great value for improving the efficacy of immunotherapy [28]. An analysis performed on slices from human lung tumors exhibited a lower concentration of T-cells in the fibronectin-rich regions than in the loose-fibronectin zones. Compared with the control slices, the treatment of tumor slices with 0.5 mg/ml collagenase for 30 min decreased the content of stromal collagen, but increased the number of T-cells (two fold) in contact with the peripheral tumor cells [12]. Similarly, the adoptive transfer of CAR-redirected T lymphocytes (CAR-T-cells), which were engineered to express heparanase and degrade the polymeric heparan sulfate-molecules, was recently demonstrated to possess greater tumor infiltration potential and antitumor activity due to its improved capability to degrade ECM proteins [29].

Even though many efforts have been adopted to overcome the biological barriers biochemically to improve the efficacy of the tumor immunotherapy, the results are still not satisfactory. Many difficulties still exist for immunotherapy, such as the chemical toxicity, as well as

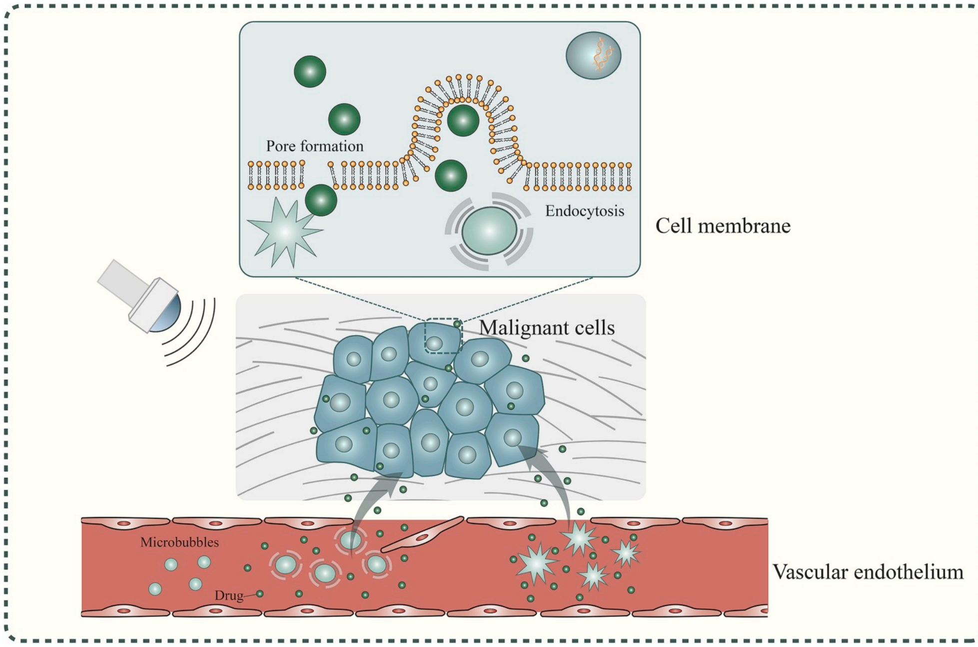

Fig. 2. Schematic illustration of ultrasound-mediated microbubble treatment can open biological barriers and improve delivery efficacy.

safe delivery methods to selectively deliver therapeutic genes or drugs into tumor cells. Several studies have exploited physical stimulis (e.g., elecporation, magnetic field, sonoporation) to open the biological barriers [30–32] thereby increasing the cellular uptake of genes and drugs. Among these, ultrasound mediated biological barrier opening has received great interests to facilitate tumor immunotherapy [20].

3. Ultrasound-mediated delivery *via* opening the biological barriers

Using ultrasound in combination with microbubbles has been shown to increase the penetration of a number of therapeutic agents into tumors, such as chemotherapeutic drugs, genes, and cell [33,34]. A suitable strategy for ultrasound-mediated delivery should be able to (1) pass through biological barriers including the vascular endothelium, interstitium, cell membrane, BBB/blood-tumor barrier (BTB) [35,36], (2) and improve the diffusion of therapeutic components in interstitial tissue [37]. The interaction of ultrasound with microbubbles can also open these biological barriers and improve delivery efficiency (Fig. 2).

3.1. Transmembrane transport

Emerging researches use ultrasound to transport non-permeable compounds (e.g., drugs [38], genes [39], peptides and proteins) into living cells. Sonoporation has been demonstrated as an alternative approach to other non-viral vector systems (such as electroporation and liposome-mediated transfection) for gene transfection [40–42]. The bioactivity of sonoporation is similar to, and in some cases has been found to be superior to, electroporation [43,44]. Evidence has demonstrated that sonoporation can achieve a significantly higher transfection efficiency (42%) than electroporation (30%) or the conventional lipofectamine method ($< 3\%$), for Ramos B-cell lines [44].

Transmembrane transport is thought to be associated with temporal cell membrane openings following ultrasound exposure. After ultrasound exposure, the pores were visualized in the cell membrane using scanning electron microscopy or atomic force microscopy [14,45]. In the presence of ultrasound contrast agents (UCAs), the pores with diameters greater than 75 nm were formed and opened for several seconds, and the internalization of molecules with diameters up to 37 nm was efficiently delivered following ultrasound exposure (2.25-MHz focused transducer, 20% duty cycle (DC), 100 Hz pulse repetition frequency (PRF), 10s sonication duration). In this study, sonoporated cells in the presence of UCAs demonstrated strong viability (70%) and only mild cytotoxicity compared with insonified cells without UCA (approximately 80%) [46]. Moreover, the pore size was demonstrated to correlate with the ultrasound driving parameters. It was observed that the sonoporation pore size increased with an increase in the ultrasound pressure, treatment time and PRF; the DNA transfection efficiency increased while the cell viability decreased with the increase in pore size [45]. According to the transmembrane current (TMC), measured with the voltage clamp, the maximum mean radius of the sonoporation pores was estimated to be $0.106 \pm 0.032\,\mu m$ for an acoustic pressure of 1.5 MPa (duration: 13.3 μs), and increased to $0.171 \pm 0.030\,\mu m$ for an acoustic pressure of 1.7 MPa and $0.182 \pm 0.052\,\mu m$ for a pulse duration of 40 μs (1.5 MPa) [47]. This implied that exogenous antitumor agents with sizes smaller than the pore size could passively diffuse into the cytoplasm *in vitro* via sonoporation pores generated in the cell membranes.

In addition to pore formation, active transport across the membrane, such as endocytosis, has been demonstrated to assist the ultrasound-mediated cell membrane permeabilization. It has been confirmed that microbubble-mediated sonication could induce the formation of hydrogen peroxide (H_2O_2) and an influx of calcium ions into the sonoporated cells [48,49], which in turn is believed to be correlated with endocytosis [36]. To further investigate the contribution of endocytosis and pore formation to intracellular delivery, the mixtures of endothelial cells and FITC-dextrans with different molecular weights (4.4–500 kDa) were exposed to ultrasound in the presence of microbubbles. It was demonstrated that endocytosis played a key role in ultrasound-mediated micrbubble destruction (UTMD), particularly for larger molecular size (> 500 kDa), and the contribution of transient pores decreased as the molecule size increased [50].

3.2. Increasing transvascular transport

Ultrasound has been documented to alter the vascular integrity, such that it can be beneficial for the agents to extravasate across the vessel wall into the tumor interstitium [51]. It is believed that the gap-junction distance between vascular endothelial cells can be increased temporarily by ultrasound exposure. Electron microscope images have

Table 1
Application of ultrasound-mediated cancer immunotherapy.

Experiment type	Tumor type	Immunological materials	Approach	Material	US parameter	Responsible immune subsets	Result	Reference
Cytokine-based immunotherapy								
in vivo	Human hepatic cancer cells SK-Hep1	IFNbeta	Intratumor injection	BR14	1 MHz,2.0 W/cm(2), 50% duty cycle, 10 min	N/A	Suppressed tumor growth	[68]
in vivo	Murine colon adenocarcinoma cell line Colon26	IFNbeta	Intratumor injection	liposomes	*in vitro*: 1 MHz, 0.5 W/cm(2), 20% duty cycle, 30 s; *in vivo*:1 MHz, 2 W/cm(2), 50% duty cycle, 10 min;	N/A	Suppressed tumor growth and increased survival	[69]
in vivo	Murine ovarian carcinoma OV-HM cells	IL-12 gene	Intratumor injection	liposomes	1 MHz, 0.7 W/cm(2), 60s	Infiltration of $CD8^+$ T cells	Suppressed tumor growth	[67]
in vivo	SCCVII tumor bearing mice	IL-12 gene	Intravenous injection	liposomes	1.5 W/cm(2), 5 min	N/A	Suppressed tumor growth	[65]
in vivo	Murine prostate cancer cell line TRAMP-C1 and TRAMP-C2	pORF-mIL-27	Intravenous injection	microbubbles	1 MHz, 50% duty cycle, 45 V, 2 Hz, 2 min, 1 W/cm(2), acoustic pressure of 0.12 MPa	Increased $CD3^+CD8^+$ cells and decreased Tregs	Suppressed tumor growth	[66]
Tumor targeting antibody								
in vivo	Human head and neck squamous carcinoma cell lines	Cetuximab	Intravenous injection	microbubbles	1.0 MHz,MI:0.5,a pulse repetition period of 5 s, 20% duty cycle,5 min	N/A	Suppressed tumor growth and increased survival; cells apoptosis	[79]
in vivo	HER2/neu-positive human breast cancer cells BT474	Trastuzumab	Intravenous injection	microbubbles	PRF:1 Hz, 1% DC, PNP:0.69 MPa, sonications were 60 s in duration and consisted of 10 m bursts	N/A	Suppressed tumor growth and increased survival	[80]
Checkpoint inhibitor (CIs)								
in vivo	Murine colorectal cancer cell line CT26	PD-1	Intravenous injection	microbubbles	1 MHz transducer, 50 0.1-ms-long pulses spaced 1 m apart, 20 s intervals, duration of 2 min Peak negative pressures 1.65 Mpa	N/A	Suppressed tumor growth and increased survival, increase the anti-tumor effects of CI therapy	[82]
Dendritic cell (DC)-based vaccines								
in vivo	CD8-OVA1.3 cells, EL4 cells,E.G7-OVA	pDNA expresses OVA	*in vitro* sonoporation	Man-PEG_{2000} bubble lipoplexes	2.062 MHz, duty cycle 50%; burst rate, 10 Hz; 4.0 W/cm(2), 20 s	N/A	Suppressed tumor growth	[86]
in vivo	B16BL6 melanoma cells	pDNA expresses gp100 and TRP-2	Intravenous injection & intradermal injection	Man-PEG_{2000} bubble lipoplexes	1.045 MHz; duty, 50%; burst rate, 10 Hz; 1.0 W/cm(2); 2 min	Increase CTLs	Prevention of metastatic and relapsed melanoma	[89]
in vitro	DC from bone marrow of C57Bl/6 mice	mRNA	*in vitro* sonoporation	mRNA-lipoplex loaded microbubbles	1 MHz, 2 W/cm(2), 50% duty cycle, 30 s	N/A	DC maturation	[90]
in vivo	Mouse melanoma cell line MO4 and T cell lymphoma E.G7-OVA	antigen mRNA and TriMix mRNA	*in vitro* sonoporation	mRNA-loaded microbubbles	1 MHz, 2 W/cm(2), peak negative pressure 800 kPa, 20% duty cycle (2 m on, 8 m off), 30 s total insonation time per OptiCell ™	Increased CD8 + T cells	Suppressed tumor growth and increased survival	[91]
Regulatory T cells								
in vivo	Tregs from patients with HCC	Foxp3-miRNA/shRNA	*in vitro* sonoporation	microbubbles	2.5 MHz, MI of 1.4, 150/181 s	Decreased ratio of Tregs/CD4 + T cells	Suppressed tumor growth	[97]
NK cells Therapy								
in vivo	Human HER2-expressing MDA-MB-231 breast tumor cells	NK-92	Intravenous injection	Definity®	551.5 kHz focused ultrasound transducer, range 0.32–0.35 Mpa	HER2-specific NK-92 cells accumulating in tumors	Suppressed tumor growth and increased median survival	[104]
in vivo	Human colorectal adenocarcinoma cells (LS-174T)	NK cells	Intravenous injection	microbubbles	510 kHz transducer,0.50 MPa peak acoustic pressure	NK cell accumulation in tumors	Increased water content and edema	[101]

demonstrated micron-sized endothelial gaps could be generated in the chicken embryo after 1- and 2.25-MHz ultrasound exposure. Extravasation of fluorescein isothiocyanate-labelled dextran (8.5 nm) was observed through micron-sized openings [52]. The ultrasound-mediated microbubble treatment has a direct antivascular effect in tumors, altering factors such as the capillary pore size and vessel diameter [53]. In a murine melanoma model, researchers observed an acute shutdown of blood flow measured by a power Doppler, following the intravenous administration of Definity® and low-intensity ultrasound (1-MHz at 2.2 W/cm^2 or 3-MHz at 2.4 W/cm^2; 3 min) [54]. A similar vasoconstrictive effect was observed in a brain tumor using the same method [54]. Other research has also reported increase in capillary pore size and enhanced tissue perfusion by ultrasound exposures.

3.3. Opening the blood-brain barrier (BBB)

It has been demonstrated that focused ultrasound in the presence of UCAs can cause temporary localized BBB disruption [16,55]. Ultrasound bursts (1.5 MHz) combined with UCAs was able to disassemble the molecular structure of tight junctions. The BBB disruption appeared to last up to 4 h after ultrasound exposure and permitted the paracellular passage of agents with molecular weights up to at least 40 kDa [16]. Preclinical research indicates that focused ultrasound produces, on average, a 4-fold increase in the delivery of small chemotherapeutic agents, and 3.5-fold increase in the delivery of monoclonal antibodies in brain tumors. This improvement in the delivery of anticancer agents resulted in an extended median survival time in multiple orthotopic murine tumor models [56]. One recent phase I clinical study validated the safety of MRgFUS-induced transient BBB opening with systemically administered chemotherapy in glioma patients [57]. However, brain tissue damage was induced at a pressure amplitude level of 6.3 MPa, including vascular wall damage, haemorrhage, and even necrosis [58]. It is prudent to restrict the ultrasound exposure levels in the brain to below the threshold of the BBB opening, otherwise brain tissue damage may occur.

3.4. Enhancing diffusion through the interstitium

Ultrasound is not only useful for permeablizing the biological barriers, but can also affect interstitium transport. David and his colleagues found that the application of ultrasound (1-MHz, 5 min) could increase the diffusion rate of nanoparticles injected into brain slices. The results demonstrated that 91.6% of the 100 nm particles, 20.7% of the 200 nm particles, and 13.8% of the 500 nm particles exhibited diffusive motion after ultrasound exposure. They further indicated that pulsed ultrasound could expand the extracellular and perivascular spaces of the brain without the evidence of tissue disruption [59]. It was demonstrated *in vivo* that ultrasound could facilitated the dispersion of the chemotherapeutic drug or UCAs throughout the tumor, specifically in the peripheral areas [60]. This phenomenon was potentially attributed to the heterogeneous distribution of IFP across tumors. The hyperthermia generated by ultrasound also increases the tissue perfusion [52,76], though high-temperature hyperthermia (greater than 50 °C) may produce tissue destruction [77].

It is not difficult to deduce that ultrasound-mediated microbubble treatment can enhance the drug/gene delivery by opening biological barriers. However, the reported delivery and transfection efficiency are inconsistent, owing to various tumor types, ultrasound modalities and parameters. The interactions between cavitating microbubbles and cells are very dependent on the ultrasound driving parameters, such as intensity, wavelength, frequency, pressure amplitude, pulse length (or burst duration), PRF (pulse repetition frequency), duty cycle, total treatment time, and mechanical index (MI). A wide range of ultrasound parameters have been evaluated in drug delivery both *in vitro* and *in vivo*, as discussed previously. For example, it was reported that the increased ultrasound pressure (0, 0.3, 0.75, 1.4, 2.2 or 3.0 MPa), treatment time (0, 5, 10, 20, 40 or 60 s) and PRF (0, 20, 100, 250, 500, or 1000 Hz) would enhance the DNA transfection efficiency, while decreasing the cell viability [45]. Thus far, standard parameters suitable for ultrasound-mediated microbubble drug delivery have not been well established. Further efforts are required to optimize the ultrasound treatment strategy and explicitly demonstrate the mechanisms underlying microbubble-cell interactions.

4. Ultrasound-mediated tumor immunotherapy

In ultrasound-mediated microbubble tumor immunotherapy, appropriate ultrasound delivery strategy is critical for achieving a sufficient concentration of microbubbles and drugs in target tissues, and will therefore be vital for the success of the treatment. In this section, we focus on the applications of ultrasound-mediated cytokine, antibody and immune cell therapy, and how this technology can help open biological barriers and improve the efficacy of tumor immunotherapy. A list of immunotherapeutic approaches and effectiveness of those treatments can be found in Table 1.

4.1. Ultrasound-mediated cytokine-based immunotherapy

The delivery of cytokines, such as interferons and interleukins, enables the indirect stimulation of the local immune response. IFN-beta and IL-2 are among the recombinant cytokines that have been approved for clinical use. However, clinical phase I and II trials have reported multiple serious side effects, including substantial toxicity and transient autoimmunity when these agents are administered systemically [61,62]. In contrast, preclinical studies have revealed that the local delivery of cytokine gene into tumors appears to be able to negate such side effects [63].

Cavitation by ultrasound can open cell membranes, allowing extracellular plasmid DNA to be directly delivered into the cytosol [64]. In 2000, ultrasound treatment (1.5 W/cm^2, 5 min) was applied to SCCVII murine tumors following systemic administration of DOTMA: cholesterol-based transfection complexes containing IL-12 plasmid (15 μg). The results showed that increased the CAT expression levels up to 270-fold. The IL-12 levels in the tumors with ultrasound exposure attained concentrations of 0.44 ± 0.18 ng/g (at 0.5 W/cm^2) and 0.52 ± 0.31 ng/g (at 1.5 W/cm^2), which were higher than that in the non-sonoporated tumors (0.064 ± 0.066 ng/g). The combination of systemic administration of IL-12 transfection complex with ultrasound yielded a 65% reduction in tumor growth rate as compared to the non-sonoporated group [65]. In addition to augmenting the local expression of cytokines, ultrasound treatment has also been shown to enhance accumulation of effector cells at the tumor site [64]. *In vitro* experiment on prostate cancer showed that, ultrasound enhanced delivery of Interleukin-27 gene (IL-27) increased the expression of several genes related to the IL-27 pathway, especially the gene of IL-27, EBI3, IFNc, and NF-κB. *In vivo* experiments have observed an increased accumulation of $CD3^+$ $CD8^+$ T cells and a decreased accumulation of $CD4^+$ CD25 + FoxP3 T-regulatory cell (Treg) population with the ultrasound treated group [66].

Apart from the systemic administration route, intratumoral injection has also been adopted to improve the efficiency of cytokine gene delivery. In a murine model of the subcutaneous ovarian carcinoma (OV-HM), pCMV-IL12 was intratumorally injected and delivered to tumor sites by using bubble liposomes and ultrasound exposures (1-MHz, 0.7 W/cm^2, 1 min). This delivery methodology increased local IL-12 production and stimulated the migration of $CD8^+$ T-cells to the tumor, presuming the T-cell-dependent therapeutic effect [67]. The same approach has been used to deliver IFN-beta pDNA, which achieved similar results [68,69]. Intratumor injection however, remains a relatively invasive approach, and it can be challenging if the target area is localized in a deeper region of the anatomy. Overall, ultrasound-mediated microbubble treatment shows promise for its ability to delivery cytokine genes transfer to tumor cells *via* opening cell membrane, and yield both local cytokine expression and significant suppression of tumor growth.

4.2. Ultrasound-mediated antibody-based immunotherapy

4.2.1. Anti-tumor antibodies

The use of monoclonal antibodies for cancer therapy has achieved considerable success in recent years [70]. Apart from directing toxic molecules to target cells and inhibiting target cell proliferation, mAbs can block inhibitory signals of immune cells [71], and induce immune cell destruction of target cells through antibody-dependent cellular cytotoxicity (ADCC) [72]. Recently, it has been shown that a majority of therapeutic antibodies, such as cetuximab (a monoclonal antibody targeting EGFR) [73], trastuzumab (a monoclonal antibody targeting HER2) [74], induce ADCC, that this is their main mechanism of action, and that these antibodies are dependent on NK cells for their anti-tumor effects. Antibody-based immunotherapy has been shown to be less efficient in solid tumors, owing to accumulation of immunoagents within the tumor and poor intratumor bioavailability of the antibody [17,60]. Systemic and repeated delivery of antibodies in high dose is required to reach therapeutic concentrations, an approach that will exacerbate both side effects and costs [75,76].

Ultrasound-mediated microbubble therapy may offer a method for overcoming these difficulties, by facilitating antibody delivery to tumors and thus enhancing the dose-effectiveness of tumor targeting antibodies. Cetuximab, a monoclonal antibody that targets the extracellular domain of EGFR on the tumor cells, has been shown to benefit from ultrasound-mediated delivery to tumors [77]. The target, EGFR, is a tyrosine kinase receptor that has been found to be highly expressed in head and neck squamous cell carcinoma (HNSCC), and overexpression of this receptor is associated with a less favorable prognosis [78]. It was reported that ultrasound treatment with microbubbles might improve the cetuximab cytotoxicity on HNSCC and suppress tumor growth. The *in vivo* treatment of xenografted tumors using cetuximab and adjuvant ultrasound treatment with microbubbles exhibited a 26% decrease in the tumor size compared with the group treated with cetuximab alone [79]. The results demonstrate that ultrasound-facilitated antibody delivery improve the distribution of cetuximab by increasing the vasculature barrier. Moreover, using the focused ultrasound (FUS) and microbubbles under MRI guidance, antibody therapeutics, such as trastuzumab [80], have been delivered through the BBB to treat brain metastases. These results suggest that ultrasound-mediated antibody delivery can enhance selectively accumulation of the antibody to the tumor site through the vasculature or BBB, then increase binging to the extracellular targets, thereby facilitating the antibody-based antitumor effect, including directing toxicity to target cells, blocking inhibitory signals for immune cells, and directing NK cells to kill targets through ADCC. Potential immune responses stimulated by ultrasound-mediated antibody therapy have yet to be evaluated and thus further research in this area is still much needed.

4.2.2. Checkpoint inhibitors (CIs)

In addition to antibodies that directly target molecules expressed by tumors, the development of antibodies that prevent the checkpoint-mediated inhibition of T-cell responses has reinvigorated the field of tumor immunotherapy [8,9]. CI immunotherapy, particular the *anti*-PD-1 antibody (aPD-1), is playing an increasingly prominent role in cancer treatment. However, objective tumor responses are achieved in only a minority of patients [81]. It is believed that the presence of immunosuppressive tumor microenvironment limits the clinical benefits of CI immunotherapy. The combination of a CI (aPD-1 antibody) with microbubble-mediated low-intensity ultrasound treatment has exhibited potential for significantly enhancing tumor inhibition and prolonging animal survival time relative to mono-therapies. It was found that the ultrasound-mediated microbubble treatment resulted in a shutdown of blood flow within the tumors, with peak enhancement intensity reducing by 88 ± 3.6% relative to the control treatment of microbubbles and ultrasound ($p < 0.05$). Moreover, one mouse of six in the treatment group, which received the combined ultrasound-mediated microbubble and aPD-1 treatment, exhibited complete regression of its tumor, and underwent a re-challenge experiment. There was no significant difference in the count of tumor-draining lymph nodes (TDLN) cells or IFN-γ expression among the groups. It was proposed that the enhanced antitumor effect was associated with the anti-vascular effect induced by ultrasound-mediated microbubble therapy, rather than by the T-cell dependent mechanism alone [82]. The mechanism of development of an adaptive immune response, however, still remains unclear.

4.3. Ultrasound-mediated immune cell-based immunotherapy

4.3.1. Dendritic cell (DC)-based vaccines

Dendritic cell (DC) based vaccines are one of the most commonly used strategies in tumor immunotherapy [83,84]. DCs are the most potent antigen-presenting cells (APCs), and they have been used as vaccine carriers for tumor immunotherapy. Various kinds of macromolecules can be delivered into DCs, including Ags, peptides, proteins, pDNA and mRNA [85,86]. Following exposure to tumor antigen, DCs migrate to peripheral lymph nodes and induce activation of cytotoxic T lymphocytes (CTLs), triggering the immune response and enhancing immunological surveillance [87,88]. The transfection efficiency of molecules into APCs determines their effect on the prevention of tumor metastasis and the likelihood of relapse.

Ultrasound exposures can improve delivery of poorly permeant molecules not only into tumor cells but also into APCs. Ultrasound used in combination with responsive mannose-modified gene carriers Man-PEG_{2000} bubble lipoplexes caused approximately 500 to 800-fold higher gene expression in APCs *in vivo* than when Man-PEG_{2000} lipoplexes were administered alone. Applying this method to DNA vaccination using OVA-encoding and melanoma pDNA also obtained effective vaccine effects against OVA-expressing cancer cells [86], and for metastatic and relapsed melanoma [89]. Specifically, after immunization was performed by ultrasound-mediated microbubble, the secretion of Th1 cytokines (IFN-γ and TNF-R) and the activities of cytotoxic T lymphocytes (CTLs) were specifically enhanced in the presence of B16BL6 melanoma antigens. mRNA encoding with a tumor-associated antigen was also transiently transfected to DCs by means of mRNA-lipoplexes bound to ultrasound responsive microbubbles. As determined by flow cytometry, over 50% of the DCs contained fluorescently labelled mRNA-complexes. These labelled cells exhibited a significant up-regulation of CD40 expression, in the presence of the activation stimulus (LPS), with only a minor shift in the CD40 and CD86 in the absence of LPS [90]. Other research has also been performed which intended to design antigen-loading and stimulation of maturation in a single step of sonoporation. Microbubbles loaded with both antigen mRNA and TriMix mRNA (a mixture of three mRNAs, required for effective CD8 + T-cell priming) were transfected into DC with the aid of ultrasound using the mouse melanoma cell line MO4 and the T-cell lymphoma cell line E.G7-OVA. Aside from their capacity to stimulate antigen-specific T cells to proliferate, mRNA sonoporated DCs were also shown to activate $CD8^+$ T cells, leading to antigen-specific lysis of tumor cells. In this group, 30% of animals observed complete tumor regression and displayed long-term antigen-specific immunological memory [91]. Other researches have also reported delivery of melanoma-derived Ag proteins to cytosol of APCs via opening of the cell membrane and achieved similar promising prophylactic immunization [92], though tumor Ag exposure poses a risk of metastasis.

4.3.2. Regulatory T cells (Treg)

Regulatory T-cells (Tregs) play an important role in maintaining immune homeostasis [93]. Tregs suppress the immune response against cancer and mediate immune tolerance to self-antigens by suppressing auto-reactive immune cells. Targeting Tregs to reduce the immunosuppressive effect is an attractive therapeutic strategy for antitumor therapy [94,95].

Short interfering RNA (siRNA) is one of the promising options for knocking down the expression of target genes of Tregs, thereby reducing the immunosuppressive activity of Tregs. siRNA of Forkhead box P3 (Foxp3) can be delivered into Tregs by cavitating microbubbles, producing interference with Foxp3. It was reported that ultrasound alone could promote Tregs proliferation. Tregs proliferation rate, however, was shown to decrease with extension of exposure time, and cell viability decreased with an increasing concentration of microbubbles. The optimal transfection condition for Foxp3 siRNA in Tregs was obtained with treatment of 10% microbubbles and an ultrasound exposure time of 150–180 s under an ultrasound MI of 1.4 [96]. It was further revealed that Foxp3 knockdown was capable of suppressing tumor growth of HCC in mice, possibly by reducing the immunosuppressive function of Tregs. A decreased ratio of Tregs/CD4+ T-cells, IL-10, TGF-β, and VEGF, and an increased level of IFN-γ and IL-2 was observed following ultrasound treatment [97]. The long-term effects of UTMD-mediated Foxp3 siRNA treatment on both immunologic function and tumor growth remain unclear and require further investigation.

4.3.3. NK cell therapy

NK cells play a vital antitumor role in host innate immunity [98]. Indeed, killing efficiency of the NK cells increases proportionally with the ratio of effector-to-target cells. *Ex vivo* activated and expanded autologous NK cells adoptively transferred back to patients exhibit a certain amount of success in their targeted accumulation in tumors and in the killing effect of these NK cells on target tumor cells [99,100]. This suggests that the efficacy of the adoptive transfer of NK cells will determine their ability to recognize and target tumors [101].

Opening the biological barriers by ultrasound provides an approach to facilitate NK cells delivery to tumor sites. NK-92 is a human NK cell line that can be modified to target tumor associated antigens, such as HER-2 [102]. However, the clinical utility of this approach for treating brain tumors is limited due to NK-92 cell inability to cross the blood-brain barrier (BBB). In an intracranial model of HER2 positive breast cancer metastasis, focused ultrasound was shown to temporarily open the BBB and produce a 10-fold increase in HER2-specific NK-92 cell populations in the target region following intravenous injection of NK-92 cells, as compared with the control group [103]. In xenograft HER2-amplified brain metastasis models, it was further demonstrated that increased delivery of NK-92 occurs with the application of focused ultrasound, leading to selective tumor cell death by NK cells and an improved long-term survival over the control group [104]. Moreover, by labeling NK cells with contrast agents, such as iron supplement, the tumor homing of NK cells can be monitored and evaluated in a non-invasive manner with MRI [101].

5. Safety considerations of clinical translation

Several studies have reported on the adverse effects of ultrasound-mediated microbubble treatment [105,106]. These adverse effects include localized haemorrhage [107], microvascular leakage [108], apoptotic cell death and inflammation [109]. These side effects have also been observed in brain tissue after BBB opening using ultrasound combined with UCAs [58]. There are several other challenges and limitations seen with ultrasound-mediated microbubble treatment, in terms of both the transfection efficiency and the safety profiles, and these issues need to be addressed before this technique proceeds to clinical trials. The solution to these problems will be dependent on the future development of safe and efficient vectors. Another safety concern is that ultrasound increases temperature in the focal area of the beam and therefore has the potential to cause thermal changes in tissues. Thermal exposure has the potential to produce adverse changes in myelination and cause cell damage in neuronal tissue [110]. Due to the heterogeneity among cancer cells, careful regulation of tumor immunotherapy is also necessary to avoid the most severe of these potential adverse effects. For example, in treatment of solid cancers with CAR-T cell therapy, the transferred T cells may disappear rapidly if the tumor burden is too large, and this can cause a range of serious problems, such as cytokine release syndrome, neurologic toxicity, “on target/off tumor” recognition, and even, and most worrisome, metastasis of previously localized tumors [7,111]. In order to optimize efficacy and safety during clinical translation, it will be of vital importance to develop a better understanding of the underlying mechanism of ultrasound-cell interactions, especially the potential adverse effects of the aforementioned treatments on cancer cells, the immune system, and healthy cell populations.

6. Conclusions and perspective

The future of tumor immunotherapy will be dependent on the development of noninvasive delivery methods that can efficiently and selectively deliver tumor antigen to lymphocytes and generate sufficient numbers of reactive T-cells, and that the delivery of a sufficient number of T-cells to tumor site to destroy cancer cells, yielding high concentrations of drugs or genes in tumors. Ultrasound-mediated microbubble treatment can open the biological barriers and thereby offers an alternative approach to improve cancer immunotherapeutic effect [112]. However, this field is still in its initial stage and many of the underlying mechanisms of these bioeffects are not fully understood, and so further research is indicated [113]. Further studies are required to elucidate the mechanisms; new ultrasound contrast agents must also be engineered, and a focusable ultrasound apparatus that can precisely mediated drug delivery in tumor immunotherapy must be developed. Many factors need to be taken into account in ultrasound-mediated tumor immunotherapy, such as cancer types, genetic background, gender, age, the indications and mechanisms of different therapeutic drugs, as well as appropriate design of an ultrasound treatment strategy. Studies must also be done in order to elucidate which type of immunomodulatory adjuvants synergizes best with each type of contrast agent in different cancer types, and to elucidate the mechanism of how ultrasound enhances immune responses. It is undeniable that ultrasound-mediated permeabilization of biological barriers provides a promising delivery approach for anti-cancer immunoagents, which may prove to be a major asset in the treatment of certain types of cancer that are currently difficult to treat with such methods, but before translating to clinical trials we must develop a more thorough understanding of the therapeutic mechanisms and the underlying ultrasound bioeffects.

Conflicts of interest

The authors have no conflict of interest to declare.

Acknowledgments

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