INTRAVITAL MICROSCOPY FOR ASSESSMENT OF ANTI-TUMOR NANOTHERAPEUTIC DELIVERY

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The development of effective relief for cancer is one of the most urgent tasks of biomedicine. Despite the success of anti-tumor nanotherapeutics, low targeted delivery effectiveness remains a major limiting factor for widespread introduction of those into clinical practice. Tumor microenvironment is a complex, multicomponent system, the dynamic interaction of which with nanoparticles requires adequate analysis methods. Intravital microscopy presents a unique opportunity for *in vivo* assessment of drugs and body's cells in the real-time mode. The review describes the possibilities and prospects of using intravital microscopy to study the nanotherapeutic biodistribution and delivery to tumor cells in preclinical animal models.

Keywords: intravital microscopy, preclinical tumor model, nanotherapeutics

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ИНТРАВИТАЛЬНАЯ КОНФОКАЛЬНАЯ МИКРОСКОПИЯ В ИЗУЧЕНИИ ДОСТАВКИ ПРОТИВООПУХОЛЕВЫХ НАНОПРЕПАРАТОВ

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Разработка эффективных средств борьбы с онкологическими заболеваниями — одна из актуальнейших задач биомедицины. Несмотря на успех противоопухолевых нанопрепаратов, низкая эффективность целевой доставки остается основным лимитирующим фактором для их широкого внедрения в клиническую практику. Опухолевое микроокружение — сложная, многокомпонентная система, динамическое взаимодействие которой с наночастицами требует адекватных методов анализа. Интравитальная микроскопия представляет уникальную возможность для изучения препаратов и клеток организма *in vivo* в режиме реального времени. В обзоре описаны возможности и перспективы использования интравитальной микроскопии в изучении биораспределения и доставки нанопрепаратов к опухолевым клеткам на доклинических моделях на животных.

Ключевые слова: интравитальная конфокальная микроскопия, экспериментальная опухолевая модель, нанопрепараты

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Progress and challenges of anti-tumor nanotherapeutic delivery

Nanoparticles (NPs) are increasingly used in medicine, including such fields, as chemotherapy, antimicrobial therapy, radiotherapy, diagnostics (magnetic resonance imaging (MRI), computed tomography), regenerative medicine, hyperthermia [1]. In the context of fight against cancer, three major NP applications can be distinguished. First, nanoformulations of chemotherapy drugs (Doxil, Caelyx, Onivid), the use of which makes it possible to increase drug delivery specificity and reduce accumulation of drugs in healthy tissues, have been introduced into clinical practice. Second, ferumoxytol and other metal NPs are successfully used for noninvasive detection of tumor foci by MRI. Third, nanoparticles with radionuclide components have a great potential for teranostics. In this field, the most common are polymeric NPs, liposomal carriers, dendrimers, iron oxide NPs, silicon dioxide NPs, as well as carbon nanotubes.

Efficacy of all the above diagnosis and treatment methods depends directly on NP accumulation in the tissues. It is clear that an administration route plays an extremely important role in biodistribution of the drug. In both most experimental studies and clinical practice, NPs are used in the form of intratumor (local) or intravenous (systemic) injections. In the first case, high concentration of the drug in the tumor focus is achieved, and it is possible to significantly reduce accumulation of the drug in healthy tissues. Unfortunately, this administration route can be used in specific clinical situations only, specifically when there is an easily accessible and clearly visualized primary tumor focus without metastasis. In contrast, systemic administration theoretically allows nanotherapeutics to reach tumors of any localization and size with the bloodstream; however, low delivery

effectiveness associated with the existence of several biological barriers represents a major flaw of this strategy. Thus, NPs have to escape capture by organs of the reticulo-endothelial system, flow out of the bloodstream (extravasate) in the neoplastic area, and penetrate through the dense connective tissue on their way to tumor cells. It should be noted that, despite the above challenges, it is systemic administration of the drug that represents the most promising method of combating a variety of neoplasms.

For more than 30 years it has been considered that specific NP accumulation in the tumor after systemic administration results from the so-called EPR (enhanced permeability and retention) effect, i.e. from the increased permeability of blood vessels and the decreased lymphatic drainage [2, 3]. However, in recent years, the concept of the EPR effect causes sharp criticism due to the series of failed clinical trials of nanopharmaceuticals [4, 5]. There is an opinion that this passive delivery mechanism is more typical for animal models, but it does not function in humans. In this regard, the possibility of active NP delivery, including that involving cellular carriers, attracts increasing attention of researchers [6, 7]. Neutrophils [8–11], monocytes [12,13], macrophages [14], and stem cells [15] have been proposed as potential candidates for nanopharmaceutical delivery to cancer cells.

The development of new anti-tumor therapy strategies requires thorough analysis of the processes occurring in the tumor microenvironment during carcinogenesis and in response to treatment. Tumor microenvironment is a complex system comprising both malignant and normal cells enclosed in the dense matrix of extracellular protein, as well as the chaotic blood vessel network. Due to its unique properties, tumor microenvironment can be considered a distinct tissue type. The features of the immune cell behavior in this tissue allow us to speak about tumor microenvironment as one of the cancer hallmarks [16]. More attention has been paid to new treatment methods targeting non-tumor cells of the tumor microenvironment, such as anti-angiogenic therapy and immunotherapy. However, in-depth understanding of the interplay between cells of the tumor microenvironment and nanopharmaceuticals is necessary to improve effectiveness of the above therapeutic approaches.

Exploration of the mechanisms underlying the NP delivery and antitumor activity was challenging for a long time due to the lack of adequate methods to assess the dynamic interaction between nano-objects and body's cells in situ. Until recently, the range of available tumor assessment methods was limited to microscopy of fixed specimens and biochemistry analysis, i.e. the methods not allowing one to study behavior of nanopharmaceuticals in the body in the real-time mode. The situation changed dramatically with the emergence of intravital microscopy (IVM) enabling investigation of dynamic processes in living tissues at the cellular and subcellular levels [17, 18]. This method that has proven successful in assessment of various biological processes [19–21] can be used to study the mechanisms underlying delivery and anti-tumor activity of nanopharmaceuticals at a deeper level [22].

In Russia, the use of IVM for assessment of the NP biodistribution and delivery into a tumor was embedded in pre-clinical trials of candidate anti-tumor drugs. This review presents the pilot results and prospects of using IVM to develop innovative anti-tumor therapy methods.

Intravital microscopy in studying the anti-tumor pharmaceutical delivery pathways

Interaction between NPs and cells of the immune system can have both negative and positive effects on the efficacy of therapy with nanopharmaceuticals. Thus, sequestration of NPs in resident macrophages of the liver and spleen reduces effectiveness of the therapeutic agent targeted delivery to tumors [23, 24]. In contrast, the firmly adherent leukocytes that capture NPs in the tumor microenvironment can function as a depot of the drug, the long-term gradual release of which into the tissues improves the anti-tumor response [25]. Finally, preservation of mobility by blood leukocytes after binding to NPs allows these to transport the drug over long distances and break through physiological barriers [26], which provides the basis for the concept of cell-mediated delivery. It has just recently been proposed to use neutrophils for intratumor delivery of nanopharmaceuticals; in contrast to the passive accumulation mechanism, the factors that contribute to or prevent active NP delivery are poorly understood.

The IVM method allowed us to monitor behavior of NPs of various types in the tumor. The study involved magnetite nanoparticles (MNPs) covalently bound to the Cy5 cyanine dye and liposomes, into which a lipophilic dye (DiD) was incorporated. It was shown that MNPs could break through the vascular barrier via both passive transport and the use of neutrophils as a "Trojan horse". The latter mechanism was first recorded in the real-time mode: NPs were adsorbed on the surface of the neutrophil, which, going beyond the vessel, carried these to the tumor tissue (Fig. 1A). It is interesting that transient elimination of neutrophils from the bloodstream resulted in the decreased accumulation of MNPs in the tumors. These findings confirm neutrophil involvement in delivery of MNPs to tumor cells [27].

The mechanisms underlying the intratumor delivery of liposomes were dramatically different from that underlying the delivery of MNPs [28]. Local leaks into the perivascular space (microleaks) were most often detected (Fig. 1B). This extravasation type is characterized by the limited area of the leak and penetration depth not exceeding 20 μm from the vessel. Fluorescence intensity was even inside the microleak and considerably exceeded that in the blood vessel lumen. Microleaks usually occurred rapidly (within minutes), and later the microleak zone remained almost unchanged.

Another, less frequent type of the leak (Fig. 1B) covered a vast area of the interstitium penetrating the tissues to the depth of several hundred microns. Such macroleaks were spatially and temporally unstable showing the diffusion gradient and dynamic changes in signal intensity, which, however, never exceeded fluorescence intensity of the circulating liposomes. Furthermore, we observed repeated waves of liposome extravasation from the same macroleak site. In contrast to microleaks, this extravasation type is likely to reflect NP diffusion in the interstitial space. The liposome diffusion waves always spread from the center of the tumor to the periphery, probably due to higher intra-tissue pressure inside the tumor.

As with MNPs, newtrophils also contributed to the release of liposomes from the vascular bed. When assessing the dynamics of microleak occurrence in the real-time mode, it was reported that in some cases these occurred in the neutrophil extravasation sites. Likewise, microleaks were reported occurring after the neutropil release from the blood vessel (Fig. 1B).

It should be noted that, in contrast to MNPs, liposomes were not captured by neutrophils, so the reported examples of the neutropil-mediated leaks represent a fundamentally new mechanism underlying NP delivery: liposomes exit the blood vessel not on the neutrophil, but through the vascular barrier pores opening temporarily at the time of cell transmigration. As a result, we can speak about four mechanisms of liposome delivery into the tumor: spontaneously occurring and

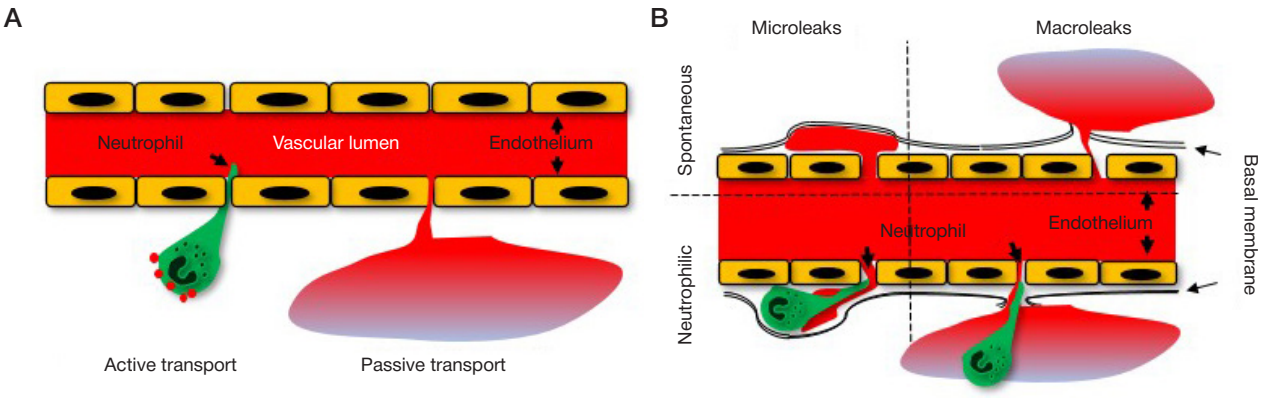

Fig. 1. Role of leukocytes in delivery of anti-tumor nanopharmaceuticals. **A**. Mechanisms underlying extravasation of magnetite nanoparticles in the tumor microvascular bed. **B**. Mechanisms underlying extravasation of liposomes in the tumor microvascular bed

neutrophil-dependent micro- and macroleaks (Fig. 1B). In the context of neutrophil depletion the effectiveness of liposome accumulation in the tumor decreased by 20–30%, which allows for a rough estimation of the contribution of neutropils to delivery of liposomal drugs to the tumor focus.

It was impossible to differentiate between the interendothelial and transendothelial pathways of liposome extravasation in thetumor vessels due to limited IVM resolving power [29]. However, *in vivo* monitoring of neutropils and liposomes in the tumor can shed light on the fundamental mechanisms underlying the NP transport from the blood vessel lumen to the tissues. Despite obvious differences, it can be assumed that there are some similarities in the extravasation behavior of neutropils and liposomes. Thus, after crossing the layer of endothelial cells, neutropils show adhesion or crawling behavior in the confined subendothelial space [30], and accumulation of neutropils in the perivascular compartment resembles the liposomal microleak. Then neutropils migrate through the non-dense areas of the basal membrane and are released from the perivascular space, similar to the NP breakthrough and diffusion in the macroleak site. It is noteworthy that some macroleaks emerge from the pre-existing microleaks, which further supports the idea that two patterns of liposome accumulation represent the consecutive stages of extravasation corresponding to transport of liposomes through the endothelial and subendothelial barriers.

It can be assumed that the described extravasation patterns play unequal parts in delivery of anti-tumor drugs. First, microleaks are found not only in tumors, but also in healthy tissues, which can explain the liposomal doxorubicin skin toxicity. Second, despite the fact that microleaks contribute to accumulation of liposomes around the tumor vessels, these do not provide access to tumor cells for nanopharmaceuticals. In contrast, macroleaks allow liposomes to penetrate deep into tumor tissues, promoting the therapeutic agent delivery to the target cells. This extravasation type that is specific for tumors shows differences depending on the tumor type. This suggests that passive delivery of liposomes to tumor cells is mediated primarily by macroleaks. Third, it is well known that, despite the fact that liposomal doxorubicin is better accumulated in the tumors, than free doxorubicin, there is little improvement of the anti-tumor response. Insufficient therapeutic efficacy can partially result from predominance of microleaks over macroleaks, which leads to the increased accumulation of liposomes at the macroscopic level, but in fact does not provide access to cancer cells for drugs.

Intravital microscopy in studying renal NP excretion

Assessment of biodistribution represents an essential phase of pre-clinical trials of pharmaceuticals. It allows one to determine such important parameters, as the drug excretion rate, accumulation dynamics, and preferential target organs.

According to modern concepts, the NP capability of being excreted by the kidneys is determined by the glomerular filter pore size, which is about 6 nm. Particles with the diameter above the specified threshold value cannot be released into urine. However, in recent years, more and more evidence has accumulated in the literature on the paradoxical renal filtration of large NPs. We observed a similar pattern when assessing biodistribution of the MNPs with the size (140 nm) significantly above the renal filtration threshold [31]. The transient increase in renal iron levels accompanied by negative contrast in the renal parenchyma on MRI was reported 2 h after intravenous administration of MNPs. These unexpected results were confirmed by confocal microscopy using the fluorescence-labeled MNPs. Furthermore, MNP administration was associated with the increased urinary iron levels, and ultrastructural analysis revealed intact NPs in the urine sediment.

In order to understand the cause of renal excretion of the NPs more than 20-fold exceeding the glomerular filter threshold, we performed IVM of superficial renal cortex at the time of MNP-Cy5 administration. Contrast enhancement of peritubular capillaries with the particles was observed immediately after the injection, and as early as 25 min later the fluorescence signal was localized mainly in the renal tubules. It is noteworthy that at the early stages after administration of the drug, accumulation occurred not in the lumen, but in the basal compartment of the tubular epithelium, indicating that MNPs were not filtered through the glomeruli, but reached the epithelium from the tubular interstitium. Further monitoring of the fate of MNP-Cy5 in the kidneys revealed the transient increase in fluorescence signal intensity in the renal tubular lumen.

Considering the results obtained, it can be assumed that translocation from blood to urine via peritubular endothelial and renal epithelial cells represents an alternative excretion pathway for synthetic NPs with the size above the glomerular filtration threshold (Fig. 2). It can be assumed that this is an underestimated mechanism that can explain some previously reported examples of paradoxical renal excretion of large NPs.

Actually, renal clearance of large NPs usually explained by the NP degradation is often reported in the literature [32–39]. At the same time, there is growing evidence for urinary excretion of intact NPs, which has remained unexplained until now. Thus, the recent study revealed renal clearance of the 20 nm pegylated magnetic NPs [40]. The authors explained this phenomenon by potential flexibility of NPs allowing these to pass through the glomerular filter membrane. One more case of unexpected renal filtration was reported for carbon nanotubes [41]. The authors assumed that certain orientation of the nanorods sized

200–300 nm with the aspect ratio between 100:1 and 500:1 within the flow makes these capable of passing through the pores. As in our experiments, both studies cited report peak excretion 30–60 min after administration and accumulation of NPs in the proximal tubules. Despite the fact that the authors explain this by NP re-absorption from the tubular lumen by the epithelial cells, an alternative hypothesis is that NPs penetrate into the renal epithelium through peritubular capillaries. In some studies, renal filtration of graphene oxide nanosheets (1 × 1000 nm, 5 × 200 nm) was explained by morphological deformation of particles (sliding, squeezing or folding) [42, 43], while the other group assumed that urinary excretion of intact silicate NPs (sized 22 × 185 nm and 65 × 720 nm) was caused by the glomerular filter membrane barrier function impairment [44].

Significant accumulation in the proximal tubules was unexpectedly reported for ferumoxytol, the FDA-approved preparation of iron oxide NPs covered with dextran (size 17–30 nm), while the dextran NPs sized 13 nm were found mostly in the glomeruli [45]. The authors assumed that this was due to the broad range of ferumoxytol diameters, such that a certain share of NPs was below the threshold glomerular filter membrane size. In this case, one would expect that the majority of NPs would still accumulate in the glomeruli, but this was not reported. Despite the fact that ferumoxytol localization in the tubules was similar to the distribution of the 5 nm dextran NPs excreted with urine, in contrast to the latter, ferumoxytol distribution had no effect on albumin endocytosis, as well as on the expression of megalin and clathrin in the proximal tubules. These data are indirect evidence suggesting that ferumoxytol gets into the epithelium from the basolateral side without involvement of absorption mechanisms in the tubules. Although the glomerular filter membrane morphological deformation and dysfunction for each distinct type of NPs cannot be ruled out, we assume that translocation through the endothelium and tubular epithelium is a more common phenomenon that can at least partially explain the earlier reported data on paradoxical filtration.

Disclosure of the alternative mechanism underlying NP translocation in the peritubular capillaries represents a paradigm shift in bio-nanotechnology, since it allows one to assume the existence of new criteria for renal clearance. Perhaps, this fact will have important clinical implications in nephrology and oncology.

Prospects of using intravital microscopy in clinical practice

In clinical practice, anti-tumor therapy with nanopharmaceuticals usually represents a series of consecutive systemic injections. In this regard, the question arises, whether the behavior of the second and subsequent doses would be different from the behavior of the first dose. Potential effect of the first NP dose on the subsequent doses can be associated with both systemic factors (change in the extent of NP capture by cells of the reticulo-endothelial system) and the tumor microenvironment modulation.

To answer these questions, specifics of biodistribution of the repeated dose of liposomes was assessed by IVM [46]. The non-labeled liposomes were administered as the first dose; the fluorescence-labeled liposomes were administered 24 h later. It was shown that the half-life and accumulation profiles of the first and second doses of liposomes in organs and tumors were the same. Quantitative analysis revealed no differences in the rate of liposome capture by blood leukocytes: both the first and second liposome doses bound mainly to monocytes, less often to neutrophils and CD4 lymphocytes, but showed almost no interaction with CD8 lymphocytes and B cells. The pattern of capture of two doses by cells of the tumor microenvironment was also the same: in both cases, the association of liposomes

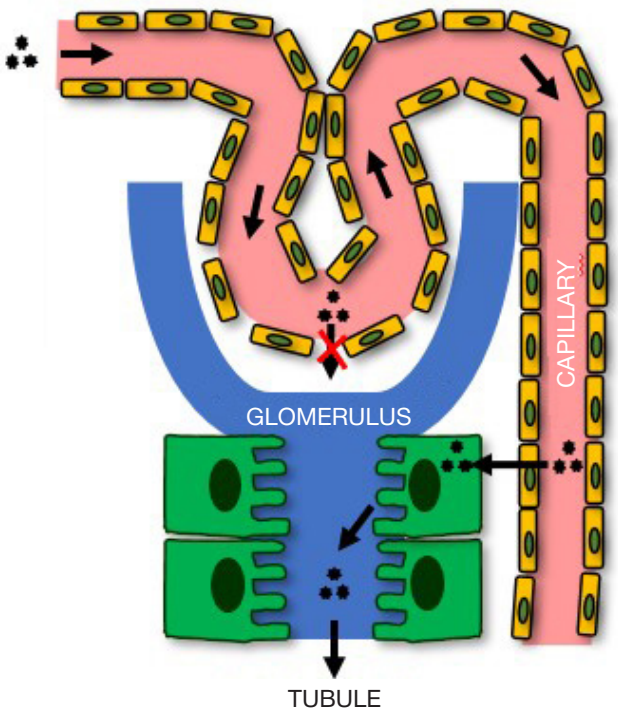

Fig. 2. Mechanism underlying renal excretion of nanoparticles with the size above the glomerular filtration threshold

with neutrophils and macrophages, and less often with other leukocytes and tumor cells was revealed. Interaction of NPs with immune cells can in some cases alter the composition of white blood cell population, which results in the fact that the subsequent drug dose faces potentially different microenvironment. However, the quantitative composition of blood and tumor leukocytes remained the same at the time of administration of the first and second doses.

As with single administration of the drug, the second dose of liposomes got into the tumor due to micro- and macroleaks. To directly assess spatial accumulation of two doses in the tumor, the experiments were conducted, in which the first and second doses of liposomes were bound to different dyes. High degree of co-localization of two fluorescence signals was reported 48 h/24 h after administration of the first/second dose of liposomes.

No difference in behavior of two liposome doses in the body opens up the possibility of using the first dose as the diagnostic one for targeted selection of the tumors showing good NP uptake, which shall also accumulate well the second (therapeutic) dose of liposomes. To test the hypothesis, the liposomes were used comprising maghemite NPs sized 5 nm that could be detected in the tumor by MRI; liposomal doxorubicin (Caelyx) was used as a therapeutic agent. IVM showed that neither loading magnetic contrast diagnostic agents into liposomes, nor the presence of the therapeutic agent in the liposomes does not disrupt the high degree of co-localization of two liposome doses in the tumor.

Validation of the algorithm for personalized diagnosis and treatment of tumors was performed in the pre-clinical model. The animals intravenously administered the dose of diagnostic liposomes were divided into groups with high and low accumulation of the drug using MRI. Then each group was divided into two subgroups, in which the animals received either Caelyx, or free doxorubicin. It was found that in the group with high accumulation of diagnostic liposomes a more pronounced decrease in the tumor growth rate and an increase in survival rate were observed compared to animals with low diagnostic

agent accumulation. It should be noted that no differences in the rate of tumor progression between groups with high and low accumulation of magnetic liposomes were revealed during treatment with free doxorubicin.

These findings suggest that estimation of accumulation of magnetic liposomes in the tumor makes it possible to predict therapeutic efficacy of liposomal drugs, but not of their free analogues.

It should be noted that the IVM technique not only allows us to solve fundamental medical and biological problems, but also has potential for practical application. Thus, in 2016, the first report was published showing the possibility of conducting IVM of tumors in patients [47].

CONCLUSION

Introduction of the IVM technique makes it possible to study biodistribution of nanopharmaceuticals and the mechanisms underlying delivery of those to tumor cells in preclinical animal models at a deeper level. Currently, the use of IVM in clinical practice is limited inter alia by the narrow range of fluorescence dyes approved for use in humans. However, the vector of development of modern microscopy methods is aimed at using the cell autofluorescence spectra for visualization of cells without any additional dyes. The emergence of commercially available microscopes using this detection principle will significantly expand the IVM diagnostic capabilities.

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