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Cancer Cell Damage at Laser-Induced Plasmon-Resonant Photothermal Treatment of Transplanted Liver Tumor

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Abstract Here, we report the morphological investigation of gold nanorod (GNR)-mediated photothermal changes in transplanted liver tumors PC-1 and discuss photothermal induced therapeutic pathways of cancer cell damage. Thirty male outbred albino rats with transplanted liver cancer PC-1 are used in the experiment. The GNRs (length 41 ± 8 nm, diameter 10.2 ± 2 nm) suspended in saline with gold concentration of 400 μg/ml are injected intratumorally an hour before laser irradiation. The tumor is irradiated during 15 min with the near infrared (NIR) 808-nm laser at a power density of 2.3 W/cm^2. Temperature control of the tumor heating is provided with IR imager. The withdrawal of the animal from the experiment is performed 24 h after the laser exposure. We use the standard histological and immunohistochemical staining with antibodies for Ki-67, p53, FAS receptor, FAS ligand, and EGFR for morphological study of transplanted tumors. After plasmonic photothermal therapy (PPT), the pronounced necrotic changes in the tumor tissue are revealed. The decreasing expression of the proliferation marker Ki-67 and the increasing expression of apoptosis markers (p53, FAS receptor, and FAS ligand) are observed after PPT.

Keywords Gold nanorods · Plasmonic photothermal therapy · Laser hyperthermia · Transplanted liver tumor · Immunohistochemical staining

The lack of effectiveness of existing therapy methods of malignant tumors necessitates the search of new treatment technologies. The active use of nanotechnologies in diagnostics and treatment of different diseases and pathological processes is currently one of the promising areas of their biomedical applications [1]. Modern laser hyperthermia, which is used as antitumoral therapy, has a significant drawback: because of low spatial selectivity, the optically induced heat affects both tumor and surrounding healthy tissue [2]. A way of increasing the selectivity of the laser heating is photothermosensitization of tumor tissue by administration of gold nanoparticles with different shapes and structures [3].

The gold nanoparticles show great potential as photothermal therapy agents and as imaging agents in connection with their important properties: biocompatibility, high surface reactivity, resistance to oxidation, and plasmon resonance [4].

Numerous reports on biodistribution, toxicity, and cellular interactions of gold nanoparticles have been published over the past decade [5–7]. It was established that the biological and toxic effects of nanoparticles depended on several critical parameters such as the particle size and shape, the nature of surface functionalization, and the dose and route of administration [8].

The effects of shape and size of gold nanoparticles have been discussed in several reports for gold nanospheres and nanoshells [9, 10], gold nanorods (GNRs) [10, 11], gold-silver nanocages [12], and nanoclusters [10]. It has been shown [12] that the increase in the suspension temperature is close for gold nanoshells, nanorods, and nanocages provided

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that their optical density at the laser wavelength is identical. Nevertheless, the plasmonic photothermal therapy (PPT) with gold nanorods is preferable because of easy tuning to the near infrared (NIR) laser wavelength, high colloidal stability, and the high absorption/scattering ratio. In fact, the gold nanorods are the most effective convertors of light to heat [11], thus making them suitable for photothermal treatment of xenografted tumors in mouse [13]. For 810-nm lasers, the aspect ratio of gold nanorods should be about 4. This explains our choice of nanorods length (41 nm) and diameter (10.2 nm). Gold nanorods with close dimensions have been used by von Maltzahn et al. [13].

Photothermal induced cell death can take place via apoptosis or necrosis depending on the laser dosage, type, and irradiation time [14]. To accomplish highly effective photothermal ablation of cancer cells, the understanding of the mechanism for the cellular death induced by hyperthermic stress from gold nanostructures is very important. Here, we report the morphological investigation of GNR-mediated photothermal changes of transplanted liver cancer PC-1 and discuss photothermal induced therapeutic pathways of cancer cell damage.

1 Material and Methods

1.1 Preparation and Characterization of GNRs

For photothermal experiments, the gold nanorods were synthesized in the Laboratory of Nanobiotechnology (Institute of Biochemistry and Physiology of Plants and Microorganisms, RAS, Saratov, Russia) by previously reported method [12, 15]. To prevent nanoparticle aggregation in biological tissue and enhance biocompatibility, nanoparticles were functionalized with thiolated polyethylene glycol (MW = 5000, Nektar, USA) as reported previously [16]. Geometrical parameters of gold nanorods were determined from analysis of transmission electron microscopy (TEM) images (Libra-120, Carl Zeiss, Germany) at the Center of Collective Use in the Institute of Biochemistry and Physiology of Plants and Microorganisms, RAS. The nanorod dimensions were 41 ± 8 nm (length) and 10 ± 2 nm (diameter), and the concentration of nanorod suspension was 400 μg/ml, which corresponds to optical density of 20 at 808 nm.

1.2 In Vivo Experiments

The experimental study was performed at the Center of Collective Use of Saratov State Medical University n.a. V.I. Razumovsky (Saratov, Russia) on 30 healthy mature albino male rats weighing 180–220 g. The experiments were conducted according to the "International Guiding Principles for Biomedical Research Involving Animals" [17].

The experimental model of rat liver cancer was reproduced by transplantation of tumor cell suspension of liver cancer (cholangiocarcinoma line PC-1), obtained from the bank of tumor strains of Russian Cancer Research Center n.a. N.N. Blokhin. 0.5 ml of 25 % tumor cell suspension in Hank's solution was implanted subcutaneously in rats. When the tumor reached a diameter of 3.0 ± 0.3 cm^3, the animals were randomly divided into three groups (10 rats in a group): group 1—without exposure, group 2—with only laser irradiation of the tumor, and group 3—with intratumoral administration of gold nanorods and laser irradiation of the tumor. Prior all medical procedure or treatments, the rats were anesthetized with Zoletil 50 (Virbac, France) in a dose of 0.05 mg/kg. An hour before laser irradiation, the animals were injected intratumorally by the solution of the gold nanorods in the amount of 30 % of the tumor volume. The injection of the gold nanoparticles directly into the tumor was conducted according to the paper by Xie and co-workers [18], where the authors demonstrated that using such modification of intratumoral injection leads to prolonged retention of nanoparticles in tumor tissue. The animals were irradiated with an 808-nm CW laser LS-2-N-808-10000 (Laser Systems, Ltd., St. Petersburg, Russia) at a power density

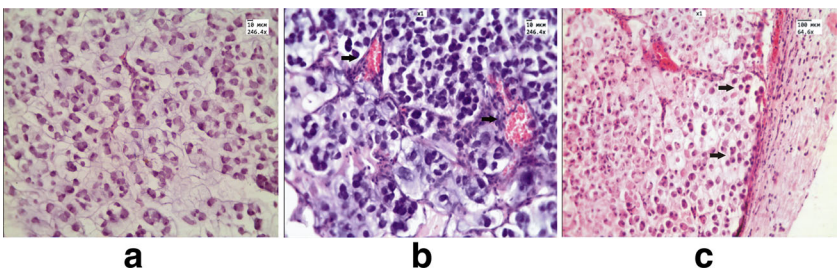

Fig. 1 **a** Liver cancer without treatment, magnification ×246.6. **b** Liver cancer after only laser treatment. The *arrows* indicate full-blooded vessels, magnification ×246.6. **c** Liver cancer after PPT with gold nanorods. The *arrows* indicate the tumor cells with degenerative changes in the subcapsular zone of the tumor. Staining with hematoxylin and eosin, magnification ×64.6

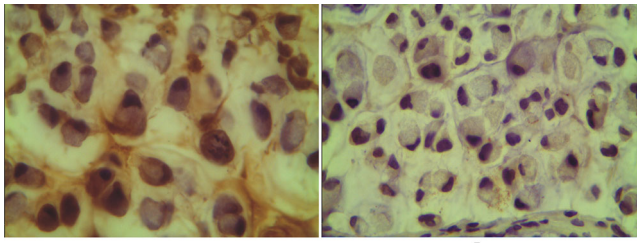

Fig. 2 **a** Liver cancer without treatment. **b** Liver cancer after PPT with gold nanorods. Immunohistochemical staining with antibodies to Ki-67. The positive reaction manifested in a brown staining of the nuclei of tumor cells, magnification ×774.0

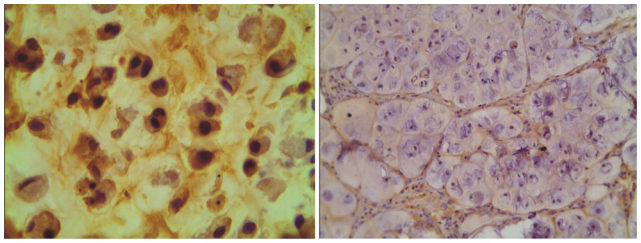

Fig. 4 **a** Liver cancer without treatment. Immunohistochemical staining with antibodies to EGFR. The positive reaction manifested in a brown staining of the membranes of tumor cells, magnification ×774.0. **b** Liver cancer after PPT with gold nanorods. Immunohistochemical staining with antibodies to EGFR, magnification ×246.4

of 2.3 W/cm^2. Irradiation was carried out percutaneously within the area of a tumor during 15 min. Temperature control of the tumor heating was provided by IR imager IRI4010, Infrared Integrated System (IRYSYS, UK). The withdrawal of the animal from the experiment was performed 24 h after the laser exposure.

1.3 The Morphological Studies

The tissue samples of tumors for morphological studies were collected. The standard histological and immunohistochemical staining with antibodies for Ki-67, p53, FAS receptor, FAS ligand, and EGFR was used for morphological study of transplanted tumors. Morphometric analysis of histological preparations was performed with a digital image analysis system "Medical Microvizor" μVizo-101 (LOMO, Russia). For the statistical analysis of the results, "SPSS 17.0" program was used.

2 Results

2.1 Histological Examination

From morphological analysis, the tumors in the first group of rats had a lobed structure where segments were separated by thin layers of connective tissue. Tumor cells had an oval-rounded shape with eccentrically located nuclei. A significant portion of cytoplasm was occupied by large vacuoles containing mucus. Some clusters of mucous structure were observed in the intracellular space (Fig. 1a).

For the second group of the rats (laser treatment only), the tumor temperature increased up to 42 °C. The tumors of this group kept a lobed structure (Fig. 1b). There were small foci of necrosis (5–10 % of the total area of tissue), and the tumor cells with necrotic changes were noted. The single mitosis was identified. The vessels were full-blooded, and there was thickening of the connective tissue septa and infiltration of leukocytes. Additionally, small foci of hemorrhage were noted.

For the third group of the rats (laser irradiation and gold nanorods as nanosensitizers), we observed a significant increase of tumor temperature (up to 60 °C). After PPT with intratumoral administration of GNRs, the pronounced necrotic changes were revealed in the tumor tissue (Fig. 1c). The tumor cells with degenerative changes persisted only in the subcapsular zone, and tumor necrosis occupied up to 80–90 % of the area of the slice.

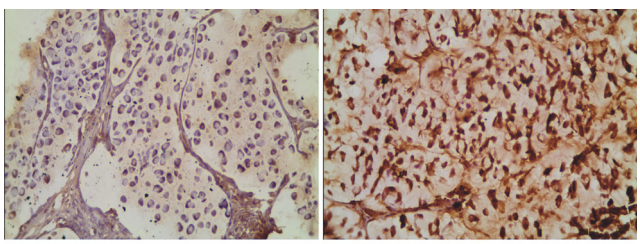

Fig. 3 **a** Liver cancer without treatment. **b** Liver cancer after PPT with gold nanorods. Immunohistochemical staining with antibodies to p53. The positive reaction manifested in a brown staining of the nuclei of tumor cells, magnification ×246.4

2.2 Immunohistochemical Examination

Decreasing of the proliferation marker Ki-67 expression and increasing of the apoptosis marker expression p53 were observed after PPT (Figs. 2 and 3).

Prior to the treatment in the majority of cases, there is moderate (up to 70 %) or severe (30 %) expression of the epidermal growth factor in tumor cells. After PPT treatment, a weak expression of this marker in intact tumor cells was observed, thus indicating the retardation of tumor growth (Fig. 4).

The apoptosis markers—FAS ligand and FAS receptor—indicate weakly pronounced expression in 20–30 % of cells prior to the therapy (Fig. 5). After the combined action of the laser hyperthermia and gold nanoparticles, expression of these markers becomes more pronounced and occurs in 40–50 % of the tumor cells.

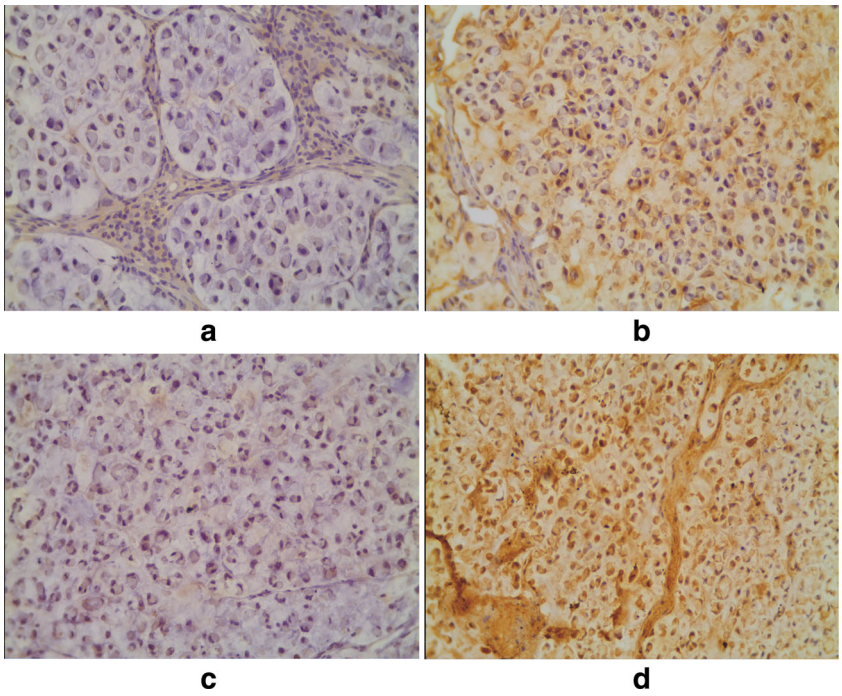

Fig. 5 **a** Liver cancer without treatment. **b** Liver cancer after PPT with gold nanorods. Immunohistochemical staining with antibodies to FAS ligand. The positive reaction manifested in a brown staining of the cytoplasm of tumor cells. **c** Liver cancer without treatment. **d** Liver cancer after PPT with gold nanorods. Immunohistochemical staining with antibodies to FAS receptor. The positive reaction manifested in a brown staining of the cytoplasm of tumor cells, magnification ×246.4

3 Discussion

In our study, we investigated the pathways of cancer cell damage in response to GNR-mediated photothermal therapy to understand the role of apoptosis and necrosis during PPT. The results indicate that intratumoral administration of gold nanorods and followed by PPT caused pronounced necrotic changes in transplanted liver tumors.

After photothermal therapy, the survival tumor cells with degenerative changes were revealed only in the subcapsular zone of tumors. Decreasing expression of the proliferation marker and increasing expression of the apoptosis markers in survival cancer cells after PPT demonstrate that apoptosis is the potential route of cell destruction by the laser heating of gold nanorods and there is some activation of apoptotic processes in the surviving tumor cells after treatment.

Our findings are in accordance with the work of Tong et al. [19], in which the tumor cell death after NIR PPT with GNRs was initiated by the disruption of the plasma membrane and subsequent influx of calcium ions induces membrane blebbing and damage of actin filaments, i.e., by the apoptosis route.

Recent studies have shown that PPT can be modulated to induce apoptosis rather than necrosis, which is appealing since apoptosis discourages an inflammatory response [20]. The further investigations are needed to optimize GNR-mediated photothermal therapy to treat cancer effectively without causing damage of the surrounding tissue.

4 Conclusion

In this work, we have shown that the combined action of gold nanorods and laser hyperthermia on transplanted liver tumors in laboratory animals has a significant damaging effect expressed in pronounced necrotic and degenerative changes of cancer cells. The decreasing expression of the proliferation marker Ki-67 and the increasing expression of the apoptosis markers—p53, FAS receptor, and FAS ligand—were observed after PPT and thus demonstrate that apoptosis is the potential route of cell destruction by GNR-mediated photothermal therapy. The further investigations can help to optimize PPT protocol to treat cancer effectively without causing any damage to the surrounding tissue.

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References

1. Boehm, F. J. (2013). *Nanomedical Device and Systems Design: Challenges, possibilities, visions*. USA: CRC Press.
2. Habash, R. W., Bansal, R., Krewski, D., Alhafid, H. T. (2007). Thermal therapy. part 3: Ablation techniques. *Critical Reviews in Biomedical Engineering, 35*, 37–121.
3. Terentyuk, G. S., Maslyakova, G. N., Suleymanova, L. V., Khlebtsov, N. G., Khlebtsov, B. N., Akchurin, G. G., et al. (2009). Laser-induced

tissue hyperthermia mediated by gold nanoparticles: Toward cancer. *Journal of Biomedical Optics, 14*(2), 021016.

4. Bellucci, S. (2008). *Lecture notes in nanoscale science and technology #7: Nanoparticles and nanodevices in biological applications. The Infn Lectures*. Berlin: Springer.
5. Dreaden, E. C., Alkilany, A. M., Huang, X., Murphy, C. J., El-Sayed, M. A. (2012). The golden age: Gold nanoparticles for biomedicine. *Chemical Society Reviews, 41*, 2740–2779.
6. Alkilany, A. M., & Murphy, C. J. (2010). Toxicity and cellular uptake of gold nanoparticles: What we have learned so far? *Journal of Nanoparticle Research, 12*, 2313–2333.
7. Zhang, X.-D., Wu, D., Shen, X., Liu, P.-X., Yang, N., Zhao, B., et al. (2012). Size-dependent in vivo toxicity of PEG-coated gold nanoparticles. *International Journal of Nanomedicine, 6*, 2071–2081.
8. Khlebtsov, N. G., & Dykman, L. A. (2011). Biodistribution and toxicity of engineered gold nanoparticles: A review of in vitro and in vivo studies. *Chemical Society Reviews, 40*, 1647–1671.
9. Harris, N., Ford, M. J., Cortie, M. B. (2006). Optimization of plasmonic heating by gold nanospheres and nanoshells. *Journal of Physical Chemistry B, 110*, 10701–10707.
10. Khlebtsov, B. N., Zharov, V. P., Melnikov, A. G., Tuchin, V. V., Khlebtsov, N. G. (2006). Optical amplification of photothermal therapy with gold nanoparticles and nanoclusters. *Nanotechnology, 17*, 5167–5179.
11. Mackey, M. A., Ali, M. R. K., Austin, L. A., Near, R. D., El-Sayed, M. A. (2014). The most effective gold nanorod size for plasmonic photothermal therapy: Theory and in vitro experiments. *Journal of Physical Chemistry B, 118*, 1319–1326.
12. Khlebtsov, B. N., Khanadeev, V. A., Maksimova, I. L., Terentyuk, G. S., Khlebtsov, N. G. (2010). Silver nanocubes and gold nanocages: Fabrication and optical and photothermal properties. *Nanotechnologies in Russia, 5*, 454–468.
13. von Maltzahn, G., Park, J.-H., Agrawal, A., Bandaru, N. K., Das, S. K., Sailor, M. J., et al. (2009). Computationally guided photothermal tumor therapy using long-circulating gold nanorod antennas. *Cancer Research, 69*(9), 3892–900.
14. Huang, X., & El-Sayed, M. A. (2011). Plasmonic photo-thermal therapy (PPTT). *Alexandria Journal of Medicine, 47*, 1–9.
15. Alekseeva, A. V., Bogatyrev, V. A., Khlebtsov, B. N., Melnikov, A. G., Dykman, L. A., Khlebtsov, N. G. (2006). Gold nanorods: synthesis and optical properties. *Colloid Journal, 68*(6), 661–678.
16. Khlebtsov, B. N., Tuchina, E. S., Khanadeev, V. A., Panfilova, E. V., Petrov, P. O., Tuchin, V. V., et al. (2013). Enhanced photoinactivation of Staphylococcus aureus with nanocomposites containing plasmon particles and hematoporphyrin. *Journal of Biophotonics, 6*(4), 338–351.
17. International Guiding Principles for Biomedical Research Involving Animals (2012). CIOMS & ICLAS. http://www.cioms.ch/index.php/12-newsflash/227-cioms-and-iclas-release-the-new-international-guiding-principles-for-biomedical-research-involving-animals.
18. Xie, H., Goins, B., Bao, A., Wang, Z. J., Philips, W. T. (2012). Effect of intratumoral administration on biodistribution of 64 Cu-labeled nanoshells. *International Journal of Nanomedicine, 7*, 2227–2238.
19. Tong, L., Zhao, Y., Huff, T. B., Hansen, M. N., Wei, A., Cheng, J. X. (2007). Gold nanorods mediate tumor cell death by compromising membrane integrity. *Advanced Materials, 19*, 3136–3141.
20. Melamed, J. R., Edelstein, R. S., Day, E. S. (2015). Elucidating the fundamental mechanisms of cell death triggered by photothermal therapy. *ACS Nano, 9*(1), 6–11.