Biomaterials 49 (2015) 1–8

Contents lists available at ScienceDirect

Biomaterials

journal homepage: www.elsevier.com/locate/biomaterials

# Radiation-/hypoxia-induced solid tumor metastasis and regrowth inhibited by hypoxia-specific upconversion nanoradiosensitizer

Yanyan Liu [a], Yong Liu [b], Wenbo Bu [a, *], Qingfeng Xiao [a], Yong Sun [b], Kuaile Zhao [c], Wenpei Fan [a], Jianan Liu [a], Jianlin Shi [a, *]

[a] State Key Laboratory of High Performance Ceramics and Superfine Microstructures, Shanghai Institute of Ceramics, Chinese Academy of Sciences, Shanghai 200050, PR China
[b] Cancer Research Institute, Fudan University Shanghai Cancer Center, Shanghai 200032, PR China
[c] Department of Radiation Oncology, Fudan University Shanghai Cancer Center, Shanghai 200032, PR China

ARTICLE INFO

*Article history:*
Received 12 January 2015
Accepted 20 January 2015
Available online

*Keywords:*
Hypoxia
Metastasis
Tumor regrowth
Nanoradiosensitizer
Radiotherapy

ABSTRACT

Tumor resistance to ionizing irradiation and cancer cell's metastasis stimulated by radiation often lead to anti-cancer failure, and can be negatively caused by a key role – cellular hypoxia. In this regard, the exploitation of hypoxia-specific cytotoxic agents which assist to potentiate the anti-tumor effect of radiotherapy (RT) as well as efficiently counteract radiation-/hypoxia-induced cancer cell metastasis, becomes especially important, but has been widely overlooked. Herein, a core/shell-structured multifunctional nanoradiosensitizer with upconversion nanoparticle (UCNP) as an inside core, mesoporous silica as the shell and a cavity in between, has been constructed, in which UCNP core serves as radiation dose amplifiers and bio-reductive pro-drug – tirapazamine (TPZ) loaded in cavity is an hypoxia-selective cytotoxin and the silica shell provides the protection and diffusion path for TPZ. Such nanoradiosensitizer has been employed to inhibit the hypoxia-reoxygenation and the subsequent replication of cancer cells that often occurs after a single unaccompanied RT at low doses, and to silence the expression of transcription factors that support the progression of malignancy in cancer. This study confirms the radiotherapeutic benefits of utilizing nanoradiosensitizer as adjuvant to low-dose RT, and the results demonstrate the highly efficient hypoxia-specific killing in oxygen-dependent anti-tumor therapies.



## 1. Introduction

Malignant tumors are characterized by uncontrolled growth, invasion and metastasis, and the latter are the major causes of death of patients, even by the primary tumors being under control. It has been known that solid tumors account for more than 85% of cancer mortality [1]. Radiotherapy (RT) as a form of local treatment used mainly for solid tumors can inhibit tumor growth, which, however, at certain doses, may activate pro-invasive and pro-metastatic cellular activities through the upregulation of key molecules and thereby actually stimulate cancer cell invasion and metastasis [2–4]. Oxygen deficiency, known as hypoxia, occurs in the core part of most solid tumors and is believed to be one of the major causes for the failure of RT. In order to survive hypoxia, cancer cells develop resistance to pro-apoptotic signals; these cells become radioresistant and intrinsically pro-metastatic [5]. To overcome the poor locoregional control rates of RT, regionally fractionated irradiation exposure [6], carbogen inhalation [7], and hyperthermia [8] have been frequently applied as adjunctive therapeutic tools in an attempt to sufficiently increase the local concentration of oxygen and augment the radiosensitivity of hypoxic cells. Such methods may have positive effects on the local control of cancer but often require advanced techniques, equipments and patient monitoring during the treatments, while the outcomes can be highly variable.

Recent experimental findings from our group suggest that the synergetic chemo-/radio-therapy [9] or thermo-/radio-therapy [10] can produce a statistically significant anti-cancer effect by increasing the radiosensitivity of hypoxic cells. However, these radiotherapeutic strategies, without exception, lack specificity towards hypoxia and do not address the chemo-resistance of hypoxic cells caused by certain biochemical and/or physiological mechanisms [11] or the heat shock response during photothermal treatment [12]. Furthermore, the biological changes induced by both

* Corresponding authors. Tel.: +86 21 52412610; fax: +86 21 52413122.
*E-mail addresses:* wbbu@mail.sic.ac.cn (W. Bu), jlshi@mail.sic.ac.cn (J. Shi).

http://dx.doi.org/10.1016/j.biomaterials.2015.01.028
0142-9612/

hypoxia and the signaling molecule activation by exposure to radiation, will undoubtedly initiate metastatic processes, lead to increased malignancy and negatively affect patient prognosis [13,14]. In spite of these sever negative effects, the cancer hypoxia has not taken into serious consideration in recent studies, probably due to the difficulties in detecting and treating hypoxia in malignant tumors. In order to develop an anti-cancer RT strategy that takes not only the hypoxia in tumors, but also the radiation- and hypoxia-induced invasion and metastasis of cancer cells, into considerations, an elaborately designed hypoxia-specific radiotherapeutic agent is highly desired to address the problems of hypoxia and hypoxia-related negative issues.

In this study, we report the successful fabrication of a multifunctional nanoradiosensitizer with high hypoxia-specific and radiosensitive cytotoxicity to substantially enhance the radiotherapeutic efficacy on solid tumors. This theranostic is featured with a rattle-type structure with an upconversion nanoparticle (UCNP) core located within the hollow core of an outer mesoporous silica (HMs) shells, hereafter referred to as UCHMs. Moreover, in order to induce oxidative and free radical damages during RT, cancer cells must be exposed to a normal level of oxygen, as a result the hypoxic cells in tumors may survive the largest doses of ionizing radiation given in clinical practice. Even if very few hypoxic but viable cells remain after RT, they are sufficient and active enough to initiate tumor metastases and regrowth. Thus, any agent designed to complement the RT-mediated killing of cancer cells must function in the distinct microenvironment of hypoxia (*e.g.*, low oxygen tension, low pH, low glucose concentration, and high reducibility) [15,16]. From this viewpoint, tirapazamine (TPZ), an agent only active under hypoxic condition [17], is adopted to encapsulate in UCHMs to overcome the oxygen dependence of RT. These TPZ-filled UCHMs (TPZ@UCHMs) exhibit a greatly enhanced cytotoxicity and anti-tumor efficacy compared with free TPZ owing to the excellent biocompatibility and bioavailability of UCHMs [18]. The experiments described herein test these hypotheses.

## 2. Experimental

### 2.1. Cell

The HeLa human cervical carcinoma cell line was obtained from Beijing Chuanglian North Carolina Biotechnology Research Institute (Beijing, China). Cell cultures under normoxic conditions ($pO_2$: 21%) were maintained in a humidified incubator at 37°C in 5% $CO_2$ and 95% air. Hypoxic conditions ($pO_2$: 2%) were produced by placing cells in a hypoxic incubator (SANYO $CO_2$ incubator, MCO-5M, Moriguchi, Japan) in a mixture of 2% $O_2$, 5% $CO_2$, and 93% $N_2$.

#### 2.1.1. UCHMs

In order to assess the bio-safety of UCHMs, HeLa cells were first seeded at $2 \times 10^3$ cells/well in 96-well plates and cultured in normal air for 24 h. A subset of the plates was transferred to hypoxic conditions for another 24 h. These normoxic or hypoxic cells were treated with various concentrations of UCHMs (37.5, 75, 150, 300, and 600 μg/mL) or vehicle control (10 μL PBS) for 24 h and then subjected to normoxic conditions for another 24 h. The total culture time was 4 days. Cell viability was measured using Cell Counting Kit-8 (CCK-8) (Dojindo Molecular Technologies, Kumamoto, Japan). O.D. at 450 nm was then determined, and the survival percentage of treated cells was calculated using the formula $[O.D._{\cdot UCHMs}/O.D._{\cdot control}] \times 100\%$.

#### 2.1.2. CCK-8 assay

Normoxic or hypoxic cells were treated with TPZ or TPZ@UCHMs for the indicated period of time, as part 2.1.1 described. Cells were treated with vehicle control (0.1% DMSO), with various concentrations of TPZ (1.25, 2.5, 5.0 and 10.0 μg/mL), or with various concentration of TPZ@UCHMs (62.5, 125, 250 and 500 μg/mL; [TPZ]: 1.25, 2.5, 5.0 and 10.0 μg/mL respectively) and were co-incubated for 48 h. The concentrations of TPZ loaded in TPZ@UCHMs were exactly matched with those of free TPZ. Statistical analysis was performed using the Student's two-tailed t-test (*$P < 0.05$; **$P < 0.001$; ***$P < 0.0001$).

#### 2.1.3. Cell apoptosis assay

For cells apoptotic assay, the incubation of HeLa cells ($3 \times 10^4$ cells/well in 6-well plates) was carried out as the above CCK-8 proliferation assay. For each 6-well plate, the cells were divided into four parts before exposure to the corresponding treatment: PBS, UCHMs (62.5 μg/mL), TPZ (1.25 μg/mL) or TPZ@UCHMs (62.5 μg/mL) in either air or hypoxia for 24 h. After 4 days, both suspended and adherent cells in each group were totally collected, washed twice with PBS, and then suspended in 1.0 mL binding buffer. Annexin V-FITC Apoptosis Detection Kit (Beyotime Institute of Biotechnology, China) was added (2 μL Annexin V-FITC and 2 μL propidium iodide per medium) and cells incubated for a further 30 min. The date analysis was performed by flow cytometry.

#### 2.1.4. Clonogenic survival assay

The effect of oxygen on the radiosensitivity of HeLa cells was assessed by a clonogenic assay. 100, 100, 200, or 500 cells per flask were seeded in 25 $cm^2$ flasks and cultured in normoxia for 24 h. Flasks were divided into two groups; one group was transferred to hypoxic conditions for another 24 h, whereas the other group was kept in normoxia. After 48 h total incubation time, the corresponding cultures, from normoxic or hypoxic conditions, were exposed to 0, 1.5, 3, or 6 Gy of radiation in sealed flasks containing 5 mL of complete medium. To allow formation of colonies, after radiation, the cells in hypoxia were continuously cultured, under low oxygen pressure, for 24 h, and were then incubated in normoxia for another 10 days, without changing the media, whereas, after irradiation, normoxic cells were cultured in normal air for 11 days. To determine the clonogenic survival rate, cultures were first fixed with paraformaldehyde, and then stained with trypan blue. Colonies with greater than 50 cells were counted under the microscope, and the survival fractions (SF) were calculated using the formula SF = [colonies counted/cells seeded] × [PE/100], where PE stands for the individual plating efficiency [19].

In order to measure the radiosensitive effects of UCHMs, TPZ, or TPZ@UCHMs on hypoxic cells, 500, 500, 500, 1000, 2000, or 5000 cells per flask were seeded to 25 $cm^2$ flasks and cultured as described above. Flasks were labeled as UCHMs, UCHMs + RT, TPZ, TPZ + RT, TPZ@UCHMs, or TPZ@UCHMs + RT, successively. At 48 h after plating, media in the flasks were replaced with complete medium containing UCHMs (62.5 μg/mL), TPZ (1.25 μg/mL), or TPZ@UCHMs (62.5 μg/mL). Cells were incubated for 1 additional day prior to treatment with an X-ray source at a dose of 3 Gy. Colony forming assays were conducted as described previously.

#### 2.1.5. Confocal microscopy

HeLa cells were seeded on glass coverslips (at $10^4$ cells per slip) and incubated for 24 h under normoxic conditions. A portion of the cover slips were transferred to hypoxic conditions and incubated for another 24 h. Both cells cultured in normoxia and those cultured in hypoxia were then co-incubated with UCHMs (300 μg/mL) for 12 h, followed by four washes with PBS to remove excess nanoparticles. After nuclear staining, fluorescence imaging of cells was conducted using confocal laser scanning microscopy (CLSM) with an Olympus IX81 microscope equipped with a continuous wave NIR laser operating at a 980 nm wavelength.

#### 2.1.6. Western blotting

HeLa cells were treated with UCHMs (1.25 μg/mL) or TPZ@UCHMs (1.25 μg/mL) for indicated periods of time, similarly to the clonogenic survival assay. After 4 days, cells were harvested and lyzed, and the lysates were collected for the analysis of proteins. Equivalent amounts of protein were resolved by SDS-polyacrylamide gel electrophoresis. The final signal was detected after incubation in anti-Snail or anti-GLUT1 primary antibody, or in anti-actin antibody in order to use actin levels as a loading control, and in secondary antibody.

#### 2.1.7. The matrigel-based transwell migration assay

Transwells (8-μm pore size, 6.5-mm diameter; BD Biosciences) were coated with extracellular matrix (ECMatrix gel; BD Biosciences) on the surface of the upper chambers. The upper chambers of the wells, containing the different experimental treatments, were seeded with $1 \times 10^5$ HeLa cells in 60 μL 2% FBS culture medium. As migration-inducing medium, 1.2 mL of 10% FBS was added to each of the lower chambers of the wells. For normoxic groups, the number of cells that migrated into the lower chamber was observed after 48 h of incubation under normoxic conditions. The migration assay for hypoxic cells was conducted after cells were cultured in hypoxia and then in normoxia for 24 h each. Statistical analysis was performed using the Student's two-tailed t-test (*$P < 0.05$; **$P < 0.001$; ***$P < 0.0001$) [20].

### 2.2. Mice

Six to eight-week-old female BALB/c nude mice were purchased from the Laboratory Animal Center, Shanghai Medical College of Fudan University (Shanghai, China). Animal procedures were in agreement with the guidelines of the Institutional Animal Care and Use Committee.

#### 2.2.1. Solid tumor study in vivo

Tumors were grown from $5 \times 10^6$ cells, in 0.15 mL PBS, implanted subcutaneously in the right side near the armpit of each mouse. Experiments were started when the tumors reached a mean diameter of 7–10 mm. Tumor-bearing mice were divided into eight treatment groups, and mice in each group were administered with 150 μL volume of the corresponding potential adjuvant (or PBS) by intratumoral injection: (1) PBS alone; (2) UCHMs alone (16 mg/mL); (3) TPZ alone