Chapter 26

Orthotopic Breast Cancer Model to Investigate the Therapeutic Efficacy of Nanobody-Targeted Photodynamic Therapy

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Abstract

Photodynamic therapy (PDT) is characterized by the local application of laser light, which activates a photosensitizer to lead to the formation of singlet oxygen and other toxic reactive oxygen species, to finally kill cells. Recently, photosensitizers have been conjugated to nanobodies to render PDT more selective to cancer cells. Nanobodies are the smallest naturally derived antibody fragments from heavy-chain antibodies that exist in animals of the *Camelidae* family. Indeed, we have shown that nanobody-targeted PDT can lead to extensive and selective tumor damage, and thus the subsequent step is to assess whether this damage can delay or even inhibit tumor growth in vivo. To evaluate the therapeutic efficacy of PDT, mouse models are mostly employed in which human tumors are grown subcutaneously in the flank of the animals. Although very useful, it has been suggested that these tumors are further away from their natural environment and that tumors developed in the organ or tissue of origin would be closer to the natural situation. Thus, this chapter describes the development of an orthotopic model of breast cancer and the application of nanobody-targeted PDT, for the assessment of the therapeutic efficacy.

Key words Nanobody-photosensitizer, Targeted photodynamic therapy, Orthotopic breast cancer model

1 Introduction

Photodynamic therapy (PDT) induces cell death through local light activation of a photosensitizer, which in the presence of oxygen leads to toxic reactive oxygen species [1]. Despite the local application of light, conventional photosensitizers are nonselective molecules, which leads to collateral damage to normal tissues. Recently, photosensitizers have been conjugated to nanobodies for targeted photodynamic therapy. Nanobodies are the smallest naturally derived antibody fragments from heavy-chain antibodies that exist in animals of the *Camelidae* family [2]. In vitro, these conjugates have shown to be very potent and selective [3–6]. In

Mans Broekgaarden et al. (eds.), *Photodynamic Therapy: Methods and Protocols*, Methods in Molecular Biology, vol. 2451,
https://doi.org/10.1007/978-1-0716-2099-1_26, © Springer Science+Business Media, LLC, part of Springer Nature 2022

vivo, such conjugates accumulate rapidly at the tumor site enabling illumination within 1–2 h after intravenous injection [7–9]. In an orthotopic model of oral squamous cell carcinoma, nanobody-photosensitizer conjugates targeting the epidermal growth factor receptor (EGFR) led to extensive tumor damage with minimal toxicity to the surrounding tissues [7]. Thus, subsequent research is focused on assessing whether the extensive damage observed 24 h post-nanobody-targeted PDT is sufficient to delay cancer growth or even able to inhibit tumor growth in vivo. One of these studies showed selective and significant regression of breast cancer tumors after a single treatment session [9]. To evaluate the therapeutic efficacy of PDT, mouse models are mostly employed in which human tumors are grown subcutaneously in the flank of the animals. This is because it facilitates both the application of PDT and the assessment of the response (through caliper measurements of tumor volume). For instance, a number of studies have been published in which photosensitizers have been targeted to the human epidermal growth factor receptor 2 (HER2) with antibodies, such as trastuzumab [10–12]. These studies investigated the growth inhibition in subcutaneous tumors at the flank of mice, as models of breast [10], gastric [11], or non-small cell lung cancer [12]. Although very useful, it has been suggested that the subcutaneous localization of such models is far away from their natural microenvironment. Thus, in the particular case of breast cancer, it has been advised to use orthotopic implantation of cancer cells directly into the mammary fat pad, under direct vision, to allow cancer cells to benefit from the microenvironment of the organ of origin [13, 14].

In this chapter, we describe an orthotopic model of breast cancer, used to investigate the therapeutic potential of PDT, with nanobody-photosensitizer conjugates, in the preclinical setting [9]. In this particular case, we refer to a model that overexpresses HER2, and thus the nanobody employed is specifically binding to HER2. In parallel, we described the similar procedure with a low HER2-expressing breast cancer cell line, to validate the specificity of the therapy. The photosensitizer employed is the IRDye700DX, which is a water-soluble silica-phthalocyanine derivative, that has been employed throughout our nanobody-targeted PDT studies [3–9, 15], and thus the settings employed for illumination (i.e., fluence and fluence rate) are the same [7–9]. The model described here is suitable for the development and evaluation of treatment of different breast cancers. Although this chapter is focused on nanobody-targeted PDT, the basic components of the PDT and illumination can be adapted to other photosensitizers or other types of conjugates.

2 Materials

2.1 Mice

NU/NU nude female mice aged 4–6 weeks are used (e.g., from Charles River Laboratories, L'Arbresle, France). Mice should be housed for 1–2 weeks to acclimatize before experiments commence.

Hold the mice in individually ventilated cages (e.g., Green Line IVC, Tecniplast, USA) with environmental enrichment like nesting material and provide them with autoclaved pallet food (e.g., RM3 diet, pelleted, Special Diets Services, UK, *see* **Note 1**) and sterilized water ad libitum.

2.2 Cell Lines

Different cell lines can be employed; here we report on the usage of HCC1954 and MCF7 cell lines, which were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA) (*see* **Note 2**).

2.3 Equipment and Solutions for the Inoculation of Cells Under Direct Vision and for Monitoring Tumor Growth

1. Tumor cell suspension or material (which should be collected before starting the operation and maintained on ice).
2. Chamber with calibrated isoflurane vaporizer.
3. Isoflurane gas (*see* Subheading 2.6).
4. Buprenorphine (*see* Subheading 2.6).
5. Electric heating pad.
6. Isoflurane face mask.
7. 0.5 mL Sterile insulin syringe (30G).
8. Sterile cotton swabs.
9. Alcohol.
10. Sterile surgical instruments including scissors, small anatomical forceps, and a clamping needle holder.
11. PDS resorbable suture (5-0, 17 mm).
12. Digital caliper.

2.4 Equipment for Photodynamic Therapy

1. Heat lamp.
2. Warm water bath.
3. Mouse restrainer.
4. 1 cc Syringe and 27-gauge needle or 0.5 mL insulin syringe (30G).
5. Dark room.
6. Chamber with calibrated isoflurane vaporizer.
7. Isoflurane gas (*see* Subheading 2.6).
8. Buprenorphine (*see* Subheading 2.6).
9. Electric heating pad.

10. Eye ointment.
11. Nanobody-photosensitizer conjugates (*see* Subheading 2.5).
12. Electric heating pad.
13. Isoflurane face mask.
14. Anatomical forceps with curved tip.
15. Black paper.
16. Laser, optic fiber, and power meter (*see* Subheading 2.7).

2.5 Nanobody-Photosensitizer Conjugates

Here, HER2-targeted nanobodies conjugated to the photosensitizer IRDye700DX were prepared, as described in [9].

2.6 Medication

1. Isoflurane, for general anesthesia (*see* **Note 3**).
2. Ophthalmic ointment, to apply during anesthesia (e.g., Lacri-Lube).
3. Painkiller: Before illumination administer the painkiller buprenorphine (0.05–0.1 mg/kg per 12 h) by subcutaneous injection.

2.7 Laser

For illumination of the tumors in the mammary glands, we used a 690 nm laser (e.g., Modulight, Tampere, Finland) with the setting as in [9]. The power at the end of the optic fiber should be calibrated with a power meter (e.g., Gigahertz Optik, Türkenfeld, Germany).

3 Methods

3.1 Culturing Cell Line

3.1.1 Thawing Cryopreserved HCC1954 and MCF7 cells

1. If the HCC1954 or MCF7 cells are stored in liquid nitrogen, transport the vial from liquid nitrogen to the laboratory on dry ice.
2. Prior to thawing, open and close the screw cap slightly to release the pressure and allow the nitrogen to escape during thawing.
3. Thaw the vial with HCC1954 or MCF7 cells in a 37 °C water bath until a small amount of ice remains. Use 70% ethanol to clean the outside of the vial to prevent contamination and transfer the suspension with a sterile Pasteur pipette into a 15 mL tube. Quick thawing is essential to preserve cell viability.
4. Dilute the HCC1954 or MCF7 cell suspension in cold culture medium described in Subheading 2.2; the first few mL should be added drop by drop.

5. Transfer the 15 mL tube with HCC1954 or MCF7 cell suspension to a centrifuge and spin the cell suspension gently at 300 × *g* for 5 min.
6. Carefully remove the supernatant without disturbing the pellet of cells. Resuspend the pellet in 6 mL of culture medium with a sterile pipette and place in a small T25 flask.
7. Place the flask in a 37 °C, 5% CO_2 incubator with the cap loosened to allow gaseous exchange to occur (or fully closed if the caps have a filter top).
8. Refresh medium the next day.

3.1.2 Culture HCC1954 or MCF7 cells

1. Take a T75 flask with HCC1954 or MCF7 cells from the 37 °C, 5% CO_2 incubator and remove culture medium with a sterile pipette.
2. Wash/rinse the adherent monolayer of HCC1954 or MCF7 cells with PBS twice to remove serum because trypsin is deactivated by serum.
3. Dispense 1 mL of trypsin-EDTA (0.05% trypsin/0.02% EDTA) in the flask and spread evenly over the bottom. Incubate for 2–5 min at 37 °C until cells visibly detach and check detachment of cells under the microscope.
4. Resuspend cells in 10 mL of culture medium (contains FCS and therefore neutralizes trypsin) and add 0.5 mL to a new T75 flask. It is recommended to dilute cells 1:20 (*see* **Note 4**).
5. Add 14.5 mL of medium to the 0.5 mL cell suspension and put the flask in the 37 °C, 5% CO_2 incubator.

3.1.3 Preparing HCC1954 or MCF7 Cells for Inoculations in 30 μL PBS

1. Take a T75 flask that is 70–80% confluent with HCC1954 or MCF7 cells from the 37 °C, 5% CO_2 incubator.
2. To start harvesting cells, follow **steps 2** and **3** from Subheading 3.1.2.
3. Resuspend cells in 10 mL culture medium and add the cell suspension to a 15 mL tube.
4. Count cells using a hemocytometer and microscope. Calculate the total amount of cells in the 10 mL suspension. Trypan blue staining can be used to exclude dead cells during counting.
5. Depending on the amount of mice that will be used in the experiment, take the correct volume of the MCF7 or HCC1954 cell suspension and pipette into a 15 mL tube. Place the tube in a centrifuge and spin cells down (300 × *g* for 5 min). Remove the medium and resuspend the pellet of cells with the correct amount of PBS, so that 500,000 MCF7 cells or 3,000,000 HCC1954 cells are diluted in 30 μL PBS.

3.2 Inoculation of Tumor Cells in the Mammary Fat Pad

1. During inoculation, mice need to be under general anesthesia. Bring the mice to a chamber that is connected to the calibrated isoflurane vaporizer (*see* **Note 3**).
2. When the mouse is under general anesthesia, perform a subcutaneous injection of buprenorphine (0.05–0.1 mg/kg per 12 h).
3. Because of the risk for hypothermia all experiments should be performed by using an electric heating pad or other supplemental heat during anesthesia. Place the mouse on an electric heating pad with isoflurane face mask. Adjust the anesthesia as suggested (*see* **Note 3**).
4. Monitor mice under anesthesia by looking at the respiratory rate and pattern, mucous membrane color, and toe pinch (to confirm the success of anesthesia).
5. Apply ophthalmic ointment on the eyes of the mice.
6. Clean the inguinal area using a cotton swab dipped into alcohol (or an alternative disinfectant).
7. Use the tip of a surgical scissor to make a small incision (preferably max 5 mm) between the fourth and fifth nipple (of the inguinal group), lateral of the midline. Make a small pocket by inserting closed forceps.
8. Use small anatomical forceps to expose the mammary fat pad (white color). Pull the mammary gland out to expose the fat pad (*see* **Note 5**).
9. The MCF7 or HCC1954 cells, diluted in 30 μL PBS, are aspirated from the vial that is prepared in Subheading 3.1.3 by using a 0.5 mL insulin syringe (30G). Make sure that cells are evenly distributed in the 30 μL PBS.
10. Inject the MCF7 or HCC1954 cells into the mammary gland. Initially the mammary tissue gets swollen, and then release the fat pad to its position.
11. Suture the incision by using a clamping needle holder and a PDS resorbable suture (5-0, 17 mm), with two tight knots (to prevent opening of the sutures).
12. After surgery watch the mice until they gain consciousness. After the mice are fully recovered, they are brought back to their cages.
13. Check the mice the following day, to see if the sutures are still present. If not, bring the mice under general anesthesia for new sutures (described as above).

3.3 Monitoring Mice and Tumor Growth

1. After the operation, inspect the animals once a day, as a routine inspection for emergencies (*see* **Note 6**).

2. Assess tumor growth and animal welfare individually three times a week by inspection of behavior, weight measurement, visual inspection, and measurement of the tumor. Four to six weeks after inoculation, tumors grow to ~5–6 mm in diameter.
3. Visual inspection and measurement of the tumor have to be done under general anesthesia. Tumor diameter can be measured with digital calipers. Tumor volume (mm^3) is measured with the formula length × width^2 x 0.52 (*see* **Note 7**).

3.4 Photodynamic Therapy

3.4.1 Intravenous Injection of Nanobody-PS Conjugates

1. If tumors are visible by the human eye and the tumor measures around 100–130 mm^3, photodynamic therapy can be applied. The tumor should not be larger than 130 mm^3.
2. Place the cage with mice under a heat lamp to increase blood flow to the tail vein.
3. Prepare a warm water bath but do not exceed 45 °C to prevent overheating. Test the temperature of the water by finger dipping before transporting the mouse.
4. Place the mouse into the restrainer. Put the tail of the mouse into the warm water bath for 1–2 min until the vein is expanding.
5. Use a 1 cc syringe and 27-gauge needle (or 0.5 mL insulin syringe, 30G) and hold the needle parallel to the tail vein with the bevel side up. Start the injection as distal as possible. When a second injection site is needed you can perform an injection more proximal than the first attempt.
6. Insert the needle into the vein. If the needle is correctly placed into the vein you can see the tip into the vein and injection of 100 μL will be easy without any resistance. Press the injection site for some time until bleeding has stopped.

3.4.2 Illumination

1. Illumination of the tumor must take place in a dark room.
2. Based on the quantitative fluorescence spectroscopy measurements, as performed in [7, 9], the time point for illumination should be determined, which in this study is 2 h postinjection of the conjugates.
3. During illumination of the tumor, mice need to be under general anesthesia. Bring the mice to a chamber that is connected to the calibrated isoflurane vaporizer (*see* **Note 3**).
4. Perform **steps 2–5** as described under Subheading 3.2.
5. Pull the breast tumor using a small anatomical forceps with curved tip.

6. Cover the rest of the body, except the breast tumor, with black paper to protect the animal from any scattering laser light (*see* **Note 8**).
7. Illuminate the tumor with the laser, at the appropriate fluence rate and for the desired fluence, here as described in [9].

3.5 Follow-Up and Tumor Measurements

1. Visual inspection and measurement of the tumor have to be done under general anesthesia. Tumor diameter can be measured with digital calipers. Tumor volume (mm^3) is measured with the formula length $\times$ width^2 $\times$ 0.52 (*see* **Note 7**).
2. The follow-up is desirable for at least 30 days after PDT, or until the tumor reached the maximum size, mostly a maximum of 500 mm^3 (*see* **Note 9**). Plots of tumor volume over time will then indicate the effect of the therapy on the tumor growth (i.e., slower growth or partial/complete regression), compared to the control group(s).

3.6 Histological Assessment Post-PDT

If of interest, sacrifice mice (*see* **Note 10**), collect the breast tumors and possible surrounding tissue, and freeze for cryosections or process for paraffin sections, for subsequent processing for histological analysis of the phototoxic effect.

4 Notes

1. If it is intended to perform fluorescence imaging to detect the photosensitizer, mice should be fed with a complete but chlorophyll-free diet or alfalfa diet, as a breakdown product of chlorophyll is fluorescent and can interfere with the fluorescence detection.
2. This chapter describes the development of a high HER2-expressing model with the cell line HCC1954, though it is in fact advised to perform these studies with two models that vary in expression level of the target (e.g., high and low, where MCF7 can be used as low), in order to validate the selectivity of the therapy. The HCC1954 cell line is established with cells of a female patient with a ductal carcinoma (TNM stage IIA, grade 3) of the mammary gland. The low HER2-expressing cell line MCF7 is established in Detroit with cells of a Caucasian woman from metastases of adenocarcinoma of the breast. The recommended culture medium for the HCC1954 and MCF7 cell lines is Roswell Park Memorial Institute medium, RPMI 1640 (Thermo Fischer Scientific), supplemented with 10% FBS (fetal bovine serum, Gibco® by Life Technologies) and 1% penicillin-streptomycin (Gibco® by Life Technologies).

3. For general anesthesia during the experiments, place the mice in a chamber that is connected to a properly calibrated isoflurane vaporizer. Perform induction of isoflurane gas anesthesia by 4% isoflurane in oxygen with a flow of 0.8 L/min. During experiments, maintain anesthesia by 2% of isoflurane in oxygen with a flow of 0.8 L/min.
4. Each cell line has a different growth rate; thus the dilution made to keep the cell line in culture should be appropriate for each cell type.
5. For more details on the inoculation of cells into the mammary fat pad, see [16, 17].
6. Inspect the animals at least once a day, as a routine inspection for emergencies, as described in the "code of practice, animal experiments in cancer research." Check the animals individually at least once a week. An animal should be euthanized when it loses 15% of body weight in a period of 1–2 days, when the tumor mass has become too large (i.e., it hampers normal behavior) or causes clinical symptoms, when serious clinical symptoms are present, when the animal is no longer eating or drinking, when the behavior becomes seriously abnormal, or when the endpoint of the experiment has been reached.
7. Other formulas can be used, if used for all tumor measurements. An example is (length × width × width)/2 or (length × width × height)/2.
8. Preferably cover the body with black paper, instead of aluminum foil.
9. Depending on the effect of PDT, the tumors can be assessed longer, up to 90 days, which is the period that is generally accepted to determine whether the mice are cured.
10. Sacrifice mice by induction of 4% isoflurane gas anesthesia in oxygen with a flow of 0.8 L/min followed by cervical dislocation. Cervical dislocation requires technical skills and alternatives like carbon dioxide or sodium pentobarbital are available.

Acknowledgment

The authors received funding from the European Research Council (ERC) under the European Union's Horizon 2020 research and innovation program (grant agreement No. 677582 and No. 323150).

References

1. van Straten D, Mashayekhi V, de Bruijn HS et al (2017) Oncologic photodynamic therapy: basic principles, current clinical status and future directions. Cancers 9:19
2. Hamers-Casterman C, Atarhouch T, Muyldermans S et al (1993) Naturally occurring antibodies devoid of light chains. Nature 363: 446–448
3. Heukers R, van Bergen en Henegouwen PM, Oliveira S (2014) Nanobody-photosensitizer conjugates for targeted photodynamic therapy. Nanomedicine 10:1441–1451
4. De Groof TWM, Mashayekhi V, Fan TS et al (2019) Nanobody-targeted photodynamic therapy selectively kills viral GPCR-expressing glioblastoma cells. Mol Pharm 16:3145–3156
5. Driehuis E, Spelier S, Beltran Hernandez I et al (2019) Patient-derived head and neck cancer organoids recapitulate EGFR expression levels of respective tissues and are responsive to egfr-targeted photodynamic therapy. J Clin Med 8: 1880
6. Heukers R, Mashayekhi V, Ramirez-Escudero M et al (2019) VHH-photosensitizer conjugates for targeted photodynamic therapy of met-overexpressing tumor cells. Antibodies 8: 26
7. van Driel P, Boonstra MC, Slooter MD et al (2016) EGFR targeted nanobody-photosensitizer conjugates for photodynamic therapy in a pre-clinical model of head and neck cancer. J Control Release 229:93–105
8. de Bruijn HS, Mashayekhi V, Schreurs TJL et al (2020) Acute cellular and vascular responses to photodynamic therapy using EGFR-targeted nanobody-photosensitizer conjugates studied with intravital optical imaging and magnetic resonance imaging. Theranostics 10: 2436–2452
9. Deken MM, Kijanka MM, Beltran Hernandez I et al (2020) Nanobody-targeted photodynamic therapy induces significant tumor regression of trastuzumab-resistant HER2-positive breast cancer, after a single treatment session. J Control Release 323:269–281
10. Ito K, Mitsunaga M, Nishimura T et al (2017) Near-infrared photochemoimmunotherapy by photoactivatable bifunctional antibody-drug conjugates targeting human epidermal growth factor receptor 2 positive cancer. Bioconjug Chem 28:1458–1469
11. Korsak B, Almeida GM, Rocha S et al (2017) Porphyrin modified trastuzumab improves efficacy of HER2 targeted photodynamic therapy of gastric cancer. Int J Cancer 141:1478–1489
12. Sato K, Nagaya T, Choyke PL et al (2015) Near infrared photoimmunotherapy in the treatment of pleural disseminated NSCLC: preclinical experience. Theranostics 5:698–709
13. Talmadge JE, Singh RK, Fidler IJ et al (2007) Murine models to evaluate novel and conventional therapeutic strategies for cancer. Am J Pathol 170:793–804
14. Rashid OM, Takabe K (2015) Animal models for exploring the pharmacokinetics of breast cancer therapies. Expert Opin Drug Metab Toxicol 11:221–230
15. Beltran Hernandez I, Angelier ML, Del Buono D'OT et al (2020) The potential of nanobody-targeted photodynamic therapy to trigger immune responses. Cancers (Basel) 12:978
16. Kocaturk B, Versteeg HH (2015) Orthotopic injection of breast cancer cells into the mammary fat pad of mice to study tumor growth. J Vis Exp (96):51967
17. Tavera-Mendoza LE, Brown M (2017) A less invasive method for orthotopic injection of breast cancer cells into the mouse mammary gland. Lab Anim 51:85–88