Video Article

Selective Cell Elimination from Mixed 3D Culture Using a Near Infrared Photoimmunotherapy Technique

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Abstract

Recent developments in tissue engineering offer innovative solutions for many diseases. For example, tissue engineering using induced pluripotent stem cell (iPS) emerged as a new method in regenerative medicine. Although this tissue regeneration is promising, contamination with unwanted cells during tissue cultures is a major concern. Moreover, there is a safety concern regarding tumorigenicity after transplantation. Therefore, there is an urgent need for eliminating specific cells without damaging other cells that need to be protected, especially in established tissue. Here, we present a method for specific cell elimination from a mixed 3D cell culture *in vitro* with near infrared photoimmunotherapy (NIR-PIT) without damaging non-targeted cells. This technique enables the elimination of specific cells from mixed cell cultures or tissues.

Video Link

The video component of this article can be found at https://www.jove.com/video/53633/

Introduction

Eliminating specific cells without damaging other cells is extremely difficult, especially in established tissue, and there is an urgent need for a cell elimination method in the tissue engineering field. Nowadays in the field of regenerative medicine, tissue cultures using embryonic stem cells (ES), pluripotent stem cells (PSCs), or induced pluripotent stem cell (iPS) are promising materials[1-3].

Although this tissue regeneration is promising, contamination with unwanted cells is a major concern. Moreover, there is a safety concern of tumorigenicity after transplantation[4,5]. Although many studies have focused on these issues to eliminate specific cells, especially in regenerative medicine[6-8], no practical method has been developed.

Near infrared photoimmunotherapy (NIR-PIT) is a treatment based on an antibody-photoabsorber conjugate (APC). An APC consists of a cell-specific monoclonal antibody (mAb) and a photoabsorber, IR700. IR700 is a hydrophilic silica-phthalocyanine derivative and does not induce phototoxicity by itself[9]. IR700 is covalently conjugated to the antibody via amide residues on the side chain of lysine molecules. The APC binds target molecules on the cell membrane and then induces nearly immediate cell necrosis after exposure to NIR light at 690 nm. During the exposure to NIR-light, the cellular membrane ruptures leading to cell death[9-14]. NIR-PIT has proven to be effective with multiple antibodies or antibody fragments, including anti-EGFR, anti-HER2, anti-PSMA, anti-CD25, anti-mesothelin, anti-GPC3, and anti-CEA[15-21]. Therefore, NIR-PIT can be used against a wide variety of target molecules. Moreover, NIR-PIT is a well-controlled treatment that allows selective treatment of specific regions by restricting the NIR-light irradiation[18,22].

Here, we present a method of specific cell elimination using NIR-PIT from mixed 3D cultures.

Protocol

Note: The following protocol describes the necessary steps to eliminate specific cells using NIR-PIT. Controls and other details about NIR-PIT and cell viability can be found elsewhere[18].

1. Conjugation of IR700 to Monoclonal Antibodies (mAb)

1. Prepare mAb of interest at 2-5 mg/ml in 0.1 M Na_2HPO_4 (pH 8.6) solution.
2. Mix 6.8 nmol of mAb with 30.8 nmol of 10 mM IR700 in 0.1 M Na_2HPO_4 solution (pH 8.6) in a microcentrifuge tube and incubate at RT for 1 hr, covered with aluminum foil.
3. Wash the PD-10 column (see Table of Materials/Equipment) with 15 ml PBS, twice. Load the sample from step 1.2.
4. Purify the mixture via the PD-10 column by PBS elution according to manufacturer's instructions.

Note: Here, the elution was along the band color of IR700. For a 300 µl sample, PBS elution fraction is typically between 2.5 ml to 4.4 ml.

5. Determine the protein concentration with Coomassie staining by measuring the absorption at 595 nm with a spectrophotometer[9]. Determine the concentration of IR700 with absorption at 689 nm to confirm the number of fluorophore molecules conjugated to each mAb molecule[9].
   Note: It is important to determine an optimal conjugation number of IR700 molecules in 1 mAb with a spectrophotometer. Generally, around 3 IR700 molecules in 1 mAb molecule is optimal for both *in vitro* and *in vivo* work. HPLC and SDS-PAGE methods can be used to confirm whether the mAb and IR700 are bound or not.
6. Store at 4 °C after determining the protein concentration.

2. Preparation of Mixed 3D Cell Culture (Mixed Spheroid)

1. Apply sterile water (around 1 ml) into the plate reservoir section of hanging drop plates.
2. Prepare various ratios of the cell types of interest — A431-luc-GFP cells and 3T3-RFP cells — with each sample containing 5,000 cells total, suspended in 50 µl of culture media.
   Note: Prepare 1:100, 10: 100, 25:100, 50:100, *etc.* ratios of cells types of interest in culture media depending on the cell-type.
3. Incubate the mix for 5-7 days in the 96 well hanging drop plate in a humidified incubator at 37 °C and 5% carbon dioxide. Change the culture media every 2 days. Note: To make the 3D spheroid precisely, handle the plate gently, as drops containing cells fall off easily before forming 3D shapes.
4. Observe the morphology and size of the spheroids using an inverted brightfield microscope at 10X - 40X magnification. Note: Although it depends on cell types and the size of hanging-drop, ensure that the diameter of the spheroid is around 400-600 µm after 7 days of incubation in the 96 well hanging drop plate.

3. *In Vitro* NIR-PIT for Mixed 3D Cell Culture

1. Change the media of the hanging drop plates to 10 µg/ml antibody-photoabsorber conjugate (APC) containing media, and incubate for 6 hr in a humidified incubator at 37 °C and 5% carbon dioxide.
2. After 6 hr incubation, wash the spheroid twice with fresh culture media (phenol red free). Gently transfer the spheroid to a glass-bottomed 50 mm dish with 100 µl fresh phenol red free culture media using a sterile 200 µl pipette tip with the tip cut off. Place one spheroid in each dish.
3. Observe the spheroid with an inverted brightfield microscope to detect the change of morphology. To observe the optical reporters (*e.g.,* GFP and RFP) use fluorescence microscope with the following filter settings: GFP — 469 nm excitation filter, and 525 nm emission filter; RFP — 559 nm excitation filter, and 630 nm emission filter.
4. Place the light-emitting diode (LED) above the glass-bottomed dish for irradiation.
   Note: Spheroids can be exposed to NIR light either on the microscope or in the laminar hood.
   1. Measure the power density of the NIR-light with an optical power meter[9]. According to this measurement, irradiate NIR-light via light-emitting diodes (LEDs) which emit light at wavelengths of 670 to 710 nm at 2 J/cm^2.
      Note: LED light can penetrate maximum depth of approximately 5 inches. Cytotoxic effects of NIR-PIT is dependent only on given energy regardless of power density and duration of exposure[23].
5. After irradiation, transfer the spheroid into a new hanging drop plate with 50 µl of fresh culture media and incubate for 1 day in a humidified incubator at 37 °C and 5% carbon dioxide.
6. Gently transfer the spheroid to a glass-bottomed dish with 100 µl of fresh culture media (phenol red free) using a sterile 200 µl pipette tip with the tip cut off. Observe the spheroid with a fluorescence microscope 1 day after NIR-PIT using filter settings described in step 3.3.
   1. Detect dead cells by adding propidium iodide to the media at a final concentration of 2 µg/ml, or by loss of cytoplasmic GFP-fluorescence using a fluorescence microscope (**Figure 2B**)[14,18,22].
7. Repeat steps 3.1-3.6 if target cells are not completely eliminated.

Representative Results

To optically monitor the effect of NIR-PIT, the A431 cell line, which overexpresses EGFR, was genetically modified to also express GFP and luciferase (A431-luc-GFP). As a non-target of NIR-PIT, the Balb/3T3 cell line was optically modified to express RFP (3T3-RFP). The APC, panitumumab-IR700 (pan-IR700), was synthesized. Mixed spheroids, which were composed of various ratios of cells (A431-luc-GFP and 3T3-RFP) were fabricated according to this protocol (**Figure 1**). Repeated NIR-PIT was performed with the incubation of pan-IR700 (see regimen in **Figure 2A**). Target cell elimination from this mixed 3D culture was achieved and monitored with fluorescence and luciferase activities (**Figure 2B, C**). On the other hand, non-target cells continued to grow.

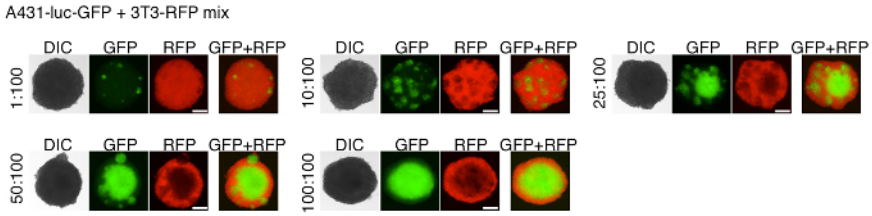

Figure 1. Representative images of various ratios of mixed spheroids. Representative images of mixed spheroids various ratios of A431-luc-GFP cells and 3T3-RFP cells at day 7 are shown. Scale bar = 200 µm. This figure has been modified from[16]. Please click here to view a larger version of this figure.

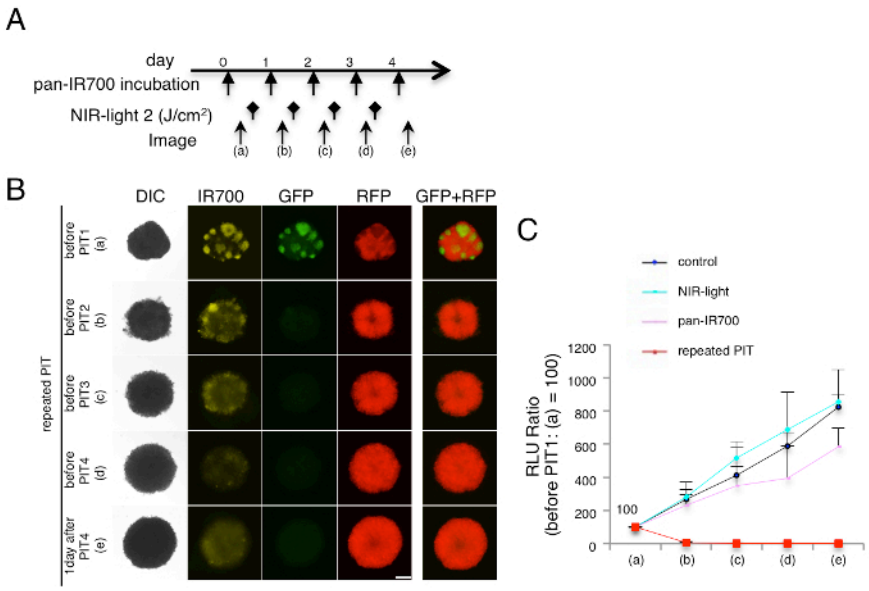

Figure 2. Target cell elimination in 3D cell spheroids (mixed spheroids of A431-luc-GFP and 3T3-RFP cells). (**A**) NIR-PIT (2 J/cm^2) regimen is shown. (**B**) Repeated NIR-PIT completely eliminated target cells (A431-luc-GFP) with no harm to non-target cells (3T3-RFP), in a mixed 3D spheroid. Bar = 200 µm. (**C**) Quantification of luciferase activities (RLU ratio) demonstrated complete elimination of target cells (n = 10 spheroids in each group). This figure has been modified from[18]. Data are expressed as means ± s.e.m. Please click here to view a larger version of this figure.

Discussion

We demonstrate a method of specific cell elimination from a mixed 3D cell culture without damage to non-target cells by using NIR-PIT. So far, there is no practical method of cell elimination once the tissue is established or after transplantation. Thus, NIR-PIT is a promising method to accomplish this. This technique could also be utilized *in vivo*[18,22], since APCs show similar pharmacokinetics as mAb itself. The target cell type can be adapted with various APC. Various antibodies or antibody fragments, including anti-EGFR, anti-PSMA, anti-mesothelin, and anti-CEA were already used as APCs[15-21]. Additionally, changing the region of NIR-irradiation can minimize the region treated. Here, with quantification of luciferase activity and fluorescence on target cells, complete elimination of specific cells was confirmed.

NIR-PIT is a light based therapy, thus a critical point is the distance between the NIR-light source and the target, since light energy and therefore cell necrosis decrease according to an inverse-square law (step 3.4). To make the 3D spheroid precisely, the plate should be treated and incubated as gently as possible. The drops containing cells easily are destroyed or fall off by a little shock before forming 3D shapes.

NIR-PIT has several unique advantages. First, NIR-PIT APCs demonstrate similar intravenous pharmacokinetics to parental non-conjugated antibodies, due to the hydrophilic characteristics of IR700, that lead to highly specific binding to target cells and minimal non-target accumulation. Thus, even after transplantation of the tissue, NIR-PIT can treat target cells via intravenous injection of the APC followed by exposure of the region of interest to NIR. Second, NIR-light can penetrate much deeper into tissue than UV or visible light, therefore, NIR-PIT could treat not only the surface but also the entire thickness of the transplanted tissue by exposure to NIR at the surface. Finally, NIR-PIT can be repeatedly used to eliminate target cells without limitation. This method is useful when target cells express sufficient copies of target molecules on the cell surface[18].

However, there are a few limitations to this technique. Firstly, the permeability of the APC is limited since it is a large molecule. To overcome this problem, repeated NIR-PIT treatment or antibody fragments can be exploited[18,19,22]. Another limitation is light penetration. Although the NIR-light can properly penetrate *in vitro* culture, translation to *in vivo* treatment would require adapted approaches such as digestive endoscopy, bronchoscopy, or direct thoracoscopy with fiberoptics to move the light source closer to the target region.

Further adaptations of NIR-PIT will allow for the use of this method in both local and specific cell elimination in many fields, such as immunomodulation or tumor microenvironments[24-26].

Disclosures

The authors have nothing to disclose.

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