Cervical Cancer HeLa Cell autocrine Apoptosis Induced by Coimmobilized IFN-γ plus TNF-α Biomaterials

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EXPERIMENTAL SECTION

1. Imaging with Confocal FRET Microscopy. In the FP-FISH method confocal images are acquired with a laser scanning spectral confocal microscope. To correct for spectral bleed-through (SBT) and for uncontrolled variations in donor acceptor concentrations, a combination of donor, FRET, and acceptor filter sets is to be used to isolate and maximize three specific signals: donor fluorescence, acceptor fluorescence resulting from FRET, and the directly excited acceptor fluorescence, respectively. Typical filter sets to be used are as follows: the green channel (donor excitation / donor emission = 543 nm / 575 nm : FITC), the red channel (acceptor excitation / acceptor emission = 633 nm / 680 nm: Rhodamine), and the FRET channel (donor excitation/acceptor emission = 543 nm / 680 nm: FRET). This SBT correction is performed with three different hybridization samples, containing just donor PNA probe, just acceptor PNA probe, and both donor and acceptor probe for their examination with each of the three filters. The later procedure is essential to perform before any of the FRET analyses.

2. X-ray Diffraction Detection. The diffraction intensity was measured using a Bruker D8FOCUS type X-ray diffractometer, and the continuous scanning mode, step size and scanning speed were selected to be 0.02^{o} and 4^{o} / min, respectively. The obtained XRD patterns was qualitatively analyzed using the MDI Jade 6.5 software kit (USA Materials Data Inc), and the qualitative phase was analyzed by XRD full spectrum method.

3. Confocal Laser Scanning. Every two hours until forty-eight hours after different treatments were mounted in distilled water and imaged with Zeiss LSM 710 confocal laser-scanning microscope (LSM710 / Con-focor2; Carl-Zeiss, Jena, Germany) for guard cell assays. FITC fluorescence was captured following excitation at 494 nm and detection at 505 to 550 nm using a band-pass filter, for dyeing TNF-α, Cy3 (555 nm excitation) for dyeing IFN-α, and DAPI (340 nm excitation) used to stain nuclei.

4. Contact Angle Detection. Prepare the measurement sample, place the sample flat on the stage, and the contact angle was measured with a contact angle meter. After the drop is dropped on the sample, the angle between the drop and the sample is measured, droplets using ultra-pure water. After the angle is taken, absorb the droplets with absorbent paper, each sample taken 12 points for testing.

5. Detection of Nano-Drug by Infrared Spectroscopy, Particle Size and Scanning Electron Microscopy. Infrared spectroscopy, particle size and scanning electron microscopy detections are reference to the laboratory preparatory work.[57]

6. Measurement of the Expression of IFN-a and TNF-α by ELISA Analysis. For ELISA procedure, the protein concentration of homogenates was calculated by the Bradford method. The different antigens were coated on 96 well multiplates overnight at 4 ℃. The plates were washed with TBS containing 0.05 % Tween 20 and blocked with 1 % BSA in TBS for 1 h at room temperature, and incubated with the different antibodies (anti-IFN-γ, IFNγ-Rα, IFNγ-Rβ) for 3 h also at room temperature. After a new wash, the biotinconjugated anti-rabbit or anti-goat immunoglobulins (Dako) were added to each well, incubated for 1 h at room temperature, and then washed and incubated with the avidin-biotin-peroxidase complex (Vector). The interactions were visualized with 0.05 % 2,2 azino di-3-etilbenziatioazolina sulphonic acid (ABTS) (Sigma) in 100 mM citrate buffer, and were measured (optical density at 405nm) in a spectrophotometer (Multiskan Bichromatic, Labsystems, Finland).

7. Western Blot analysis. After 12 h and 24 h of culturing on PSt, the total proteins were extracted from the cells by lysed in cell lysis buffer (Beyotime) to determine the product of IFN-α and TNF-α protein. Aliquots of 20-30 mg of the cell lysates per sample were separated by 10 % SDS-PAGE and then transferred to a polyvinylidene fluoride membrane (Bio-Rad). After blocking with 5 % bovine serum albumin (BSA, Gibco) for 1 h, the membranes were incubated with primary antibodies overnight. Theimmune complexes were incubated with horseradish peroxidase conjugated anti-rabbit antibody (Boshide, China). Immunodetection was performed using the Western-Light Chemiluminescent Detection System (Peiqing, China).

The monoclonal antibodies against IFN-α and TNF-α protein were purchased from Boster.

8. Subcutaneous Tumor Growth in Nude Mouse. Animals (nude mice) were purchased from the Experimental Animal Center of Sun Yat-sen University and cared for under the supervision of the Experimental Animal Center of Sun Yat-sen University. Xenograph flank tumors were induced in 4 - 5 week old BALB / c nude mice by subcutaneous (s.c.) injection of 0.2 mL, 1×10^7 HeLa cells/nude mouse, tumor growth and weight measured twice-weekly. After 3 weeks, when tumors had reached about 100 mm^3, mice were divided into three groups of six mice, minimizing weight and tumor size differences. Tumor-bearing nude mice were treated by intravenous injection of the three samples: normal saline (CK-group), PSt + AzPhIFN-γ plus AzPhTNF-α (F-group), and NPs-coimmobilized IFN-γ plus TNF-α(C-group) every other day. After the dosing, the mice were monitored for implanted tumor size daily for 4 weeks. The length and width of the tumors were measured by digital calipers. Tumor volume was calculated by the following formula: ((width × $length^2$) / 2). And quantification of IFN-A and TNF-A in tumor by Western blot analysis. For animals put to death by dislocation of infra-cervical spine, the tumor size at the time of death was used for the purpose of mean tumor size calculation. Animals were sacrificed when tumors reached maximum allowed size or when signs of ulceration were evident.

9. Immunohistochemistry Analysis. HeLa cells or tumor samples were collected into an Eppendorf tube of 1.5 mL and put in 10 % neutral buffered formalin for 12 h. Then they were stained with eosin for 1 min. Three micrometer-thick sections were cut onto positive-charged slides and used for immunohistochemical detection of TNF-α and IFN-α. Briefly, after the deparaffinization in xylene and rehydration through graded alcohols, these sections were microwaved in 0.01 M citrate buffer (pH 6.0) for 15 min. Endogenous peroxidase activity was blocked by immersion in 0.03 % H_2O_2 with methyl alcohol for 15 min, where 10 % normal rabbit (for mouse primary antibodies) or goat serum (for rabbit primary antibodies) was utilized to avoid non-specific reaction. These sections were then incubated using the primary antibodies for 2-3 h. After washing with PBS, biotinylated anti-muse or rabbit IgG was applied for 20 min at room temperature. The

peroxidase-conjugated-streptavidin solution was subsequently applied for 30 min and visualized using 0.05 % 3'-3' diaminobenziine (DAB). The counterstaining was performed using hematoxylin. The breast cancer cell known to be positive for TNF-α and IFN-α over-expression was used as a positive control. For a negative control, the primary antibody was replaced by a nonspecific negative control antibody.

10. Serological Detection. We used the sterilized scissors to take blood samples from the tails of tumor bearing nude mice treated by intravenous injection of three groups every other day. Before this procedure, a hair dryer blowing was used for tumor-bearing nude mice to get the blood circulation faster. For each animal, 1.5 mL blood was obtained and preserved in EP tube adding anticoagulants. When taking blood was completed, all the samples were sent to Guangdong laboratory animals monitoring institute, (Guangzhou, China) to carry out the serum detection including the white blood cells (WBC), blood platelets (PLT), and red blood cells (RBC).

11. Prussian Blue Staining. Paraffin embedded tissue was placed in water. Hydrochloric acid and potassium ferrocyanide solution in 151 volume mixing ratio was freshly prepared and used to be working solution. Sections were immersed in working solution for standing 10-30 min. After the reaction, sections were immersed in distilled water for 3 min and 3 times, and then rinsed thoroughly. Nuclear fast red solution was dropped in, covering the specimen for 5-10 min. After the reaction, sections were immersed in distilled water for 3 min and 3 times, and then sections were immersed in 95 % alcohol and xylene to dehydrate for 2 min. The mounting medium was dropped onto the sections and covered with coverslip.