Selective cellular uptake and induction of apoptosis of cancer-targeted selenium nanoparticles

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ABSTRACT

Selenium nanoparticles (SeNPs) have garnered a great deal of attention as potential cancer therapeutic payloads. However, the in vivo targeting drug delivery has been challenging. Herein, we describe the synthesis of transferrin (Tf)-conjugated SeNPs and its use as a cancer-targeted drug delivery system to achieve enhanced cellular uptake and anticancer efficacy. Tf as targeting ligand significantly enhances the cellular uptake of doxorubicin (DOX)-loaded SeNPs through clathrin-mediated and caveolae/lipid raft-mediated endocytosis in cancer cells overexpressing transferrin receptor, and increases their selectivity between cancer and normal cells. DOX-loaded and Tf-conjugated SeNPs (Tf-SeNPs) exhibit unprecedented enhanced cytotoxicity toward cancer cells through induction of apoptosis with the involvement of intrinsic and extrinsic pathways. Internalized Tf-SeNPs triggers intracellular ROS overproduction, thus activates p53 and MAPKs pathways to promote cell apoptosis. In the nude mice xenograft experiment, Tf-SeNPs significantly inhibits the tumor growth via induction of p53-mediated apoptosis. This cancer-targeted design of SeNPs opens a new path for synergistic treating of cancer with higher efficacy and decreased side effects.

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1. Introduction

Nanotechnology has raised high expectation in cancer treatment and diagnostics, and is considered as one of the most promising research orientation for oncotherapy [1,2]. Targeted nanodrug delivery systems could improve the targeted delivery, positive and stimuli-responsive drug release and mineral side effects [3–5]. Nowadays, one of the most critical obstacles for cancer chemotherapy is the limited availability of effective delivery systems for hydrophobic anticancer drugs, since low drug solubility in aqueous media prevents their intravenous administration. However, over 40% of small-molecule anticancer drugs produced by pharmaceutical companies have poor water solubility [6]. Therefore, it is of great significance to construct nanodrug delivery systems for anticancer drugs to enhance their solubility and action efficacy. A number of nanosystems with different structure and compositions, such as metals, polymers, oxides, and semiconductors, have been designed and prepared to carry anticancer drugs [7–10]. Among these nanomaterials, selenium nanoparticles (SeNPs) have garnered a great deal of attention as potential cancer therapeutic agents and drugs carriers [11–15]. As a special selenium species, SeNPs is also featured by its excellent antioxidant activity and disease prevention effects [16,17]. Abundant evidence also supports the better biocompatibility, bioefficacy and lower toxicity of SeNPs by comparing with inorganic and organic selenium compounds [13,18,19]. Recently, we reported the use of SeNPs as carriers of pharmaceutical agents such as 5-fluorouracil to enhance their anticancer outcome [11]. This strategy brings new horizon for cancer therapy and opens a new area for application of SeNPs. However, limitation still existed in this drug delivery system. The lack of targeting effects may inevitably cause drug toxicity and undesirable side effects. Besides, the in vivo anticancer activity of SeNPs-based therapeutic drugs remains unexplored. However, a cancer-targeting design could be a good strategy to overcome the drawbacks of SeNPs. Specifically, cancer-targeted ligands could be
conjugated to the surface of nanoparticles, which could provide preferential accumulation of nanoparticles in the tumor-bearing organ, enhance the selective killing abilities against cancer cells, and at the same time, reduce the toxicity toward normal cells.

Transferrin (TF), a targeting ligand, is a brilliant guide to deliver numerous therapeutic drugs into malignant sites where over-express TIRs [20–23]. TF/TIRs-mediated endocytosis has been found as a useful strategy to enhance the entrapped drug concentration in tumor sites, and keep them away from non-targeted tissues and cells that hardly express TIRs [24]. Therefore, in recent years, TF uptake pathway is widely used for targeted delivery of therapeutic agents [25–27]. By altering the surface chemistry of SeNPs, TF could be conjugated to the nanoparticles, and this drug delivery system can be utilized to target different stages of cancers, such as primary cancers and metastatic cancers. Herein, we describe the synthesis of TF-conjugated SeNPs and its use as a cancer-targeted drug delivery system for doxorubicin (DOX) to achieve enhanced cellular uptake and anticancer efficacy. The in vivo anticancer activity of TF-SeNPs and the underlying molecular mechanisms were also investigated in this study.

2. Materials and methods

2.1. Materials

Sodium selenite (Na2SeO3), transferrin, thiazolyl blue tetrazolium bromide (MTT), propidium iodide (PI), 4′, 6-Diamidino-2-phenylindole (DAPI), dihydroethidium (DHE), and bicinchoninic acid (BCA) kit were purchased from Sigma. Substrate for caspase-3 (Ac-DEVD-AMC), caspase-8 (Ac-IETD-AMC) and caspase-9 (Ac-LEHD-AMC) was purchased from Calbiochem. Terminal transferase dUTP nick end labeling (TUNEL) assay kit. Vitamin C was purchased from Guangzhou chemical reagent factory. The water used in all experiments was ultrapure by a Milli-Q water purification system from Millipore.

2.2. Preparation of TF-SeNPs

Before the experiment, the solution of 20 mg ascorbic acid (Vc) was freshly prepared by dissolving 35.2 mg Vc powder in 10 mL of Milli-Q water. 5 mg/mL of fresh selenium dioxide (Na2SeO3) solution, 5 mg/mL of transferrin (TF) solution, 0.1 mM of 1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC) and 0.8 mg/mL of chitosan (CS) solution was prepared. A 1 mL aliquot of Na2SeO3 solution was mixed with 50 mL of 5 mM DOX/DMSO solution and 100 mL of CS solution, then the mixture was dropwise added to 1 mL of Vc solution. The mixed solution was reconstituted to a final volume of 5 mL with Milli-Q water. After reaction for 12 h at 4 °C, the mixed solution was dialyzed against Milli-Q water for 6 h to remove the excess DOX and Na2SeO3. For TF conjugation, 50 mL of EDC solution was precisely stirred with 200 mL of TF for 2 h. Then CS-SeNPs-DOX solution was mixed with TF-EDC and reacted overnight at 4 °C. The excess TF, EDC and reaction by-product were removed by dialysis against Milli-Q water for 6 h. For the concentration of Se was determined by ICP-AES analysis. To quantify the in vitro cellular uptake of loaded TF-SeNPs, a fluorescein coumarin-6 at a final concentration of 5 μg/mL was mixed with Na2SeO3 solution instead of DOX to form coumarin-6-loaded TF-SeNPs. The incorporated 6-coumarin acted as a probe for TF-SeNPs and offered a sensitive method to determine its intracellular uptake and localization.

2.3. Characterization of TF-SeNPs

The as-prepared TF-SeNPs was characterized by different microscopic and spectroscopic measurements including transmission electron microscopy (TEM), scanning electron microscopy (SEM), high-resolution TEM (HR-TEM), Fourier transform infrared spectroscopy (FTIR), UV–vis spectroscopy, Zetasizer particle size analysis. TEM samples were prepared by placing two drops of nanoparticles suspension onto holey carbon films on copper grids. The images were visualized at an accelerating voltage of 80 kV under TEM microscope (Hitachi H-7650). The SEM images of nanoparticles were obtained by SEM microscope (EX-250 system, horiba). The HR-TEM images and the corresponding selected area electron diffraction (SAED) patterns of nanoparticles were obtained on JEOL 2010 high-resolution TEM at 200 kV. The size distribution and zeta potential of the nanoparticles were measured using Zetasizer Nano ZS particle analyzer (Malvern Instruments Limited). To determine the chemical composition of nanoparticles, FTIR (Equinox 55 IR spectrometer) and UV–vis (Carry 5000 spectrophotometer) studies were conducted in the range of 400–500 cm−1 and 450–700 nm, respectively. Bicinchoninic acid (BCA) kit (Pierce) for TF determination was conducted followed by manufacturer’s instructions.

2.4. Determination of loaded amount of DOX in TF-SeNPs

The loaded amount of DOX in TF-SeNPs was quantified by a DMSO-digesting method. Briefly, TF-SeNPs undergoing centrifugation and DMSO digestion for 1 h were decomposed to release the encapsulated DOX. The released DOX was analyzed by fluorescence microplate reader with the excitation and emission wavelength set as 485 nm and 590 nm respectively. A rectilinear calibration curve from 0.195 to 25 μg (r2 = 0.9925) was conducted to determine the concentration of the loaded DOX from nanoparticles, using a control DOX solution in DMSO. The Se concentration of TF-SeNPs was determined by ICP-AES analysis.

2.5. Cell lines and cell culture

All human cell lines used in this study, including MCF-7 breast adenocarcinoma cells, HepG2 hepatocellular carcinoma cells, A375 melanoma cells, HUVEC human umbilical vein endothelial cells which were purchased from American Type Culture Collection (ATCC, Manassas, Virginia). These cell lines were incubated in DMEM media supplemented with penicillin (100 units/mL), streptomycin (50 units/mL) and fetal bovine serum (10%) at 37 °C in a humidified incubator with 5% CO2 atmosphere.

2.6. In vitro cellular uptake of TF-SeNPs

Cellular uptake of TF-SeNPs was quantified by fluorescence microplate reader. Briefly, MCF-7 and HUVEC cells were seeded in 96-well plates at a density of 8000 cells/well (0.1 mL) and allowed to attach for 24 h. The medium in the well was replaced with different concentrations of coumarin-6-loaded TF-SeNPs and incubated for various periods of time at 37 °C in CO2 incubator. At the end of the incubation, the medium was removed from the wells and the cells were rinsed three times with cold PBS to remove the nanoparticles outside the cells. After that, 100 μL of 0.1% Triton X-100 in 0.1 N NaOH solution was added to lyse the cells. Fluorescence intensity from coumarin-6-loaded nanoparticles inside the cells with excitation and emission wavelengths set at 430 and 485 nm, respectively. The cellular uptake efficacy was expressed as the percentage of the fluorescence of the testing wells over that of the positive control wells.

2.7. Transferrin competing assay

Competitive binding of transferrin receptors (TIRs) between TF-SeNPs and excess amount of TF was performed on MCF-7 cells. 8 × 104 cells/mL of MCF-7 cells were seeded in 96-well plates and allowed to attach for 24 h. Excess concentrations of TF ranging from 0 to 5 mg/mL were added to the wells and incubated at 37 °C in CO2 incubator for 1 h. Then 80 μM of coumarin-6-loaded TF-SeNPs was added. After the incubation for 2 h, the cells were washed with 3 times of cold PBS to remove the nanoparticles outside the cells, followed by the lysis of cells and fluorescence intensity measurement of internalized TF-SeNPs.

2.8. Intracellular trafficking of TF-SeNPs

The intracellular behavior of TF-SeNPs was monitored by fluorescence microscopy (DSI, Olympus). Briefly, MCF-7 cells cultured in 2 cm cell culture dishes until 70% confluence were stained by 80 μM of lysotracker DND-99 (Sigma–Aldrich) for 2 h and 1 μg/mL of DAPI H33258 (Sigma–Aldrich) for 30 min. After rinsed by PBS for 3 times, the cells were incubated with 80 μM of coumarin-6-loaded TF-SeNPs for various periods of time and observed by fluorescence microscopy.

2.9. Mechanisms of cellular uptake of TF-SeNPs

MCF-7 cells seeded in 96-well plates at a density of 8000 cells/well were incubated in complete medium for 24 h. The cells were treated with endocytosis inhibitors for 1 h, except for nystatin (Sigma–Aldrich) was incubated for 30 min. Treated cells were then incubated with 80 μM of coumarin-6-loaded TF-SeNPs for another 3 h. The control samples were received 80 μM of coumarin-6-loaded TF-SeNPs without the addition of inhibitors. Final concentration of specific endocytosis inhibitors were listed as follows: sodium azide (NaN3) 100 mM, 2-deoxy-D-glucose (DOG, Sigma–Aldrich) 50 mM, sucrose 0.45 μM, dynasore (Sigma–Aldrich) 80 μM and nystatin 10 μg/mL. For investigation of energy-dependent pathways, the cells were treated in complete medium at 4 °C for 4 h, followed by incubation of coumarin-6-loaded TF-SeNPs for another 3 h.

2.10. In vitro drug release of TF-SeNPs

Two copies of 10 mg of TF-SeNPs were respectively suspended in 10 mL PBS solution at pH 5.3 and pH 7.4 with constantly shaking in dark tubes at 37 °C. At specific intervals, a certain volume of buffer was taken out from tubes and same volume of fresh buffer was replaced. The collected buffer was centrifuged and the DOX concentration was determined by fluorescence microplate reader. (With excitation and emission wavelength set as 485 nm and 590 nm respectively).
2.1.1. MIT assay

Cell viability was determined by measuring the ability of cells to transform thiazolyl blue tetrazolium bromide (MTT) to a purple formazan dye as previously described [28]. Briefly, cells were seeded in 96-well plates at a density of 2.5 × 10³ cells/well for 24 h. The cells were exposed at different concentrations of TF-SeNPs for various periods of time. After treatment, 20 µL of MTT solution (5 mg/mL in PBS) was added to each well and incubated for 5 h. The medium was replaced with 200 µL/well of DMSO to dissolve purple formazan. The color intensity of the formazan solution, which is positively correlated to the cell viability, was measured by microplate spectrophotometer (VSENSOR Max) at 570 nm.

2.1.2. Synergy analysis

Isobologram method was conducted to analyze the synergistic effect between SeNPs and DOX. Briefly, line segment between the IC50 value of SeNPs and DOX on the x- and y-axes respectively represented the additive line. The data point near or on the additive line represented an additive treatment effect, while the data point below or above the additive line remarked the synergism or antagonism respectively. In addition, the extent of synergism or antagonism was evaluated by combination index (CI). CI value of 1 meant an additive effect between two drugs, while CI value < 1 represents synergism, CI value > 1 indicates antagonism. The extent of CI value below or above 1 is positively related to the extent of synergism and antagonism respectively.

2.1.3. Flow cytometric analysis

The cell cycle distribution was analyzed by flow cytometry [29]. After treatment with TF-SeNPs, the cells were harvested and washed with PBS. The cells were fixed with pre-cooled 70% ethanol overnight at -20°C overnight. The fixed cells were washed with PBS and stained with propidium iodide (PI) for 1 h at 4°C. The stained cells were determined by flow cytometer (Epics-XL, Beckman Coulter) to explore cell cycle distribution, followed by data analysis using MultiCycle software. The cell population in G0/G1, S, and G2/M phases was expressed as DNA histogram. Apoptotic cells with hypodiploid DNA content were detected by quantifying the sub-G1 peak in cell cycle pattern. For each experiment, 10,000 events per sample were recorded.

2.1.4. TUNEL-DAPI containing assay

TF-SeNPs induced DNA fragmentation in apoptotic cells were detected using TUNEL assay following the manufacturers’ protocol. Briefly, MCF-7 cells with 24 h treatment of TF-SeNPs were fixed with 3.7% formaldehyde for 10 min and lysed with 0.15 Triton X-100 in PBS. The cells were cultured with TUNEL reaction mixture for 1 h. For nuclear staining, cells were incubated with 1 µg/mL of DAPI for 15 min at 37°C. At the end of incubation, the cells were rinsed with PBS and observed under a fluorescence microscope (Nikon Eclipse 80i).

2.1.5. Caspase activity

Harvested cell pellets were suspended in cell lysis buffer (Beyotime) and incubated on ice for 1 h. After centrifugation at 11,000 g for 30 min, supernatants were collected and immediately measured for protein concentration using BCA assay. Thereafter, caspase activity was determined by a fluorometric method. In short, the cell lysates and specific caspase substrates (Ac-DEVD-AMC for caspase-3, Ac-LEHD-AMC for caspase-9) were added into 96-well plates at 37°C for 2 h. Caspase activity was measured by fluorescence intensity with the excitation and emission wavelength set as 380 nm and 460 nm respectively.

2.2. Measurement of intracellular reactive oxygen species (ROS) generation

Changes of ROS production induced by TF-SeNPs treated MCF-7 cells were evaluated by staining cells with dihydroethidium (DHE, Beyotime). Briefly, the cells harvested by centrifugation, washed with PBS twice and resuspended in PBS were incubated with DHE at a final concentration of 10 µM at 37°C for 30 min. Then the cells were incubated with different concentrations of TF-SeNPs at 37°C for different periods of time. The intracellular ROS level was examined by detecting fluorescence intensity of DHE conducted by fluorescence microplate reader (excitation and emission wavelength set as 500 nm and 520 nm respectively).

2.2.1. Western blot analysis

MCF-7 cells with TF-SeNPs treatment for 72 h were incubated with lysis buffer (Beyotime) to obtain total cellular proteins. The protein concentration was examined by BCA assay. Equal amount of proteins were electrophoresed in 12% tricine gels and then transferred to nitrocellulose membrane and blocked with 5% non-fat milk in Tris-Buffered Saline Tween-20 (TBST) buffer for 1 h. Then the membranes were incubated with primary antibodies at 1:1000 dilution in 5% non-fat milk overnight at 4°C with continuous agitation. Then the membranes were incubated with secondary antibodies conjugated with horseradish peroxidase at 1:2000 dilution for 1 h at room temperature, followed by 3 times washing with TBST. Protein bands were visualized on X-ray film using enhanced chemiluminescence detection regents (Pierce). β-Actin was used to confirm the comparable amount of proteins in each lane.

2.2.2. Statistical and synergy analysis

All experiments were carried out at least in triplicate and results were expressed as mean ± S.D. Statistical analysis was performed using SPSS statistical program version 13 (SPSS Inc., Chicago, IL). Difference between two groups was analyzed by two-tailed Student’s t-test. Difference with P < 0.05(*) or P < 0.01(**) was considered statistically significant. The difference between three or more groups was analyzed by one-way ANOVA multiple comparisons.

3. Results and discussion

3.1. Preparation and characterization of TF-SeNPs

TF-SeNPs were prepared as illustrated in Fig. 1A. To fabricate the stable and versatile nanoparticles, a four-layer structure was formed by loading DOX and conjugating chitosan (CS) and TF to SeNPs. The abundant hydroxyl groups of CS could react with SeO₂ groups to form stable chain-shaped intermediates [14], which were reduced to become Se atoms by adding ascorbic acid. The accumulative Se atoms led to a formation of SeNPs, thus CS assembled to be absorbed to the surface of SeNPs, leaving positive NH₃⁻ groups outward. Meanwhile, DOX was incorporated into SeNPs by adding the drugs in colloidal dispersion to form three-layer structure. Thereafter, the outside NH₃⁻ groups of CS were covalently bound to TF to form the final four-layer structure, TF-CS-SeNPs-DOX (TF-SeNPs). TF-SeNPs was characterized by various microscopic and spectroscopic means. TEM (Fig. 1B) and SEM images (Fig. S1A) showed that TF-SeNPs was near-spherical in shape and were monodisperse particles with high uniformity. Further structure information of TF-SeNPs was investigated by high resolution-TEM (HR-TEM) detection. As shown in Fig. 1B, the atomic lattices fringe (0.7 nm) of TF-
SeNPs reveals its single crystalline structure. Moreover, the corresponding selective area electron diffraction (SAED) pattern demonstrates the orderliness and symmetry of Tf-SeNPs (Fig. S1B).

The stability of Tf-SeNPs under aqueous and physiological conditions is of fundamentally importance for evaluating their medical applications. Therefore, the size distribution of Tf-SeNPs in aqueous and PBS (pH 7.4) solutions was monitored for over one month. As shown in Fig. 1F, the size of Tf-SeNPs in aqueous solution remained stable with an average size of 130 nm within 21 days, followed by a gradual increase in the size afterward. Tf-SeNPs dispersed in PBS solution kept stable within 21 days. The bigger size and wider distribution of Tf-SeNPs in PBS after 3-week store should be due to the interference of buffer salts that led to nanoparticle aggregation. Overall, the favorable stability of Tf-SeNPs supports their potential application in medical area.

Besides, the change in zeta potential of the nanoparticles was also recorded to monitor the different surface decoration. As shown in Fig. 1C, zeta potential of bare SeNPs and SeNPs-DOX were $-17.9$ mV and $-24.8$ mV, while attachment of CS elevated the zeta potential to $44.5$ mV, probably due to the positive-charged NH$_3^+$ groups from CS. With the conjugation of TF onto the nanoparticles, the zeta potential decreased to $20.6$ mV. These varied data of zeta potential demonstrated the existence of TF, CS and DOX in the nanosystem. The chemical structure of Tf-SeNPs was further confirmed by FTIR. As shown in Fig. 1D, the spectrum of Tf-SeNPs (line c) exhibited characteristic peak $1389$ cm$^{-1}$ from SeNPs (line a). The peak at $1450$ cm$^{-1}$ was assigned to the benzene ring from DOX (Fig. S2A). The peaks at $1156$ cm$^{-1}$ and $1081$ cm$^{-1}$ corresponded to the stretching vibration of C–O, which is the characteristic peak of CS (Fig. S2A). The peaks appeared at $1649$ cm$^{-1}$ and $1539$ cm$^{-1}$ were assigned to the first and secondary $–$CO$–$NH$–$ groups of TF (Fig. S2A). Specifically, new amide groups from TF were found in Tf-SeNPs, indicating linking of TF to the nanoparticles via amide groups in CS. The presence of TF was further verified by using a protein staining BCA assay. As shown in Fig. S2B, the UV–vis spectrum shows an increase in absorbance at 560 nm, due to the presence of protein in the nanoparticles. This spectroscopic difference is apparent in the particle color, as the nanoparticles with TF are stained purple while the bare SeNPs maintain the standard green BCA color (in web version) (Fig. 1E). These results support the successful conjugation of TF to SeNPs.

**3.2. Selective cellular uptake, intracellular localization and release of Tf-SeNPs**

Selective cellular uptake of therapeutic drugs remains a formidable obstacle for cancer therapy. Specific ligand-mediated targeting may be a feasible strategy to solve this problem. To examine the selectivity between cancer and normal cells, we compared the internalization of coumarin-6 loaded Tf-SeNPs in MCF-7 cells overexpressing TIR and HUVEC cells that hardly expressed TIR (Fig. 2). The cells seeded in 96-well plates were incubated with 20, 40 and 80 $\mu$M of Tf-SeNPs for 0.5, 1.0 and 2.0 h, and the cellular uptake was quantified by measuring the fluorescence intensity of coumarin-6. As shown in Fig. 2B and C, cellular uptake of Tf-SeNPs in MCF-7 cells at 20 $\mu$M was much higher than that in HUVEC cells at much higher concentration (80 $\mu$M). For instance, after 0.5, 1.0 and 2.0 h of incubation with 20 $\mu$M of Tf-SeNPs in MCF-7 cells, the cellular concentrations Tf-SeNPs reached $22.2$, $66.5$ and $102.4$ $\mu$g/$10^6$ cells respectively, which were $1–6$ folds higher than those of HUVEC cells at 80 $\mu$M.

To examine the contribution of TIR to the cellular uptake of Tf-SeNPs, firstly, we examined the expression level of the receptor on the cell membrane. As shown in Fig. 2D, the expression levels of TIR in human cancer cells (A375, HepG2 and MCF-7) were
significantly higher than HUVEC cells. These results suggest the feasibility of Tf-guided selectivity between cancer and normal cells. Tf protein competing assay was used to further confirm this hypothesis. The cells were pretreated with excess amount of Tf and then incubated with Tf-SeNPs for various periods of time. As shown in Fig. 2E, Tf protein significantly inhibited the uptake of Tf-SeNPs in a dose-dependent manner, even to the level similar to that in HUVEC human normal cells. These results are consistent with the appealing suggestion, namely that the selective uptake of Tf-SeNPs in human cancer cells, but not in normal cells, can be traced to TfR-mediated endocytosis.

Fluorescence imaging technique was also employed to gain more insights into the intracellular trafficking of Tf-SeNPs. Two special fluorescent tracers, lyso-tracker (red, in web version) and DAPI (blue, in web version) were used to labeled lysosomes and nucleus. The results reveal that Tf-SeNPs moved across the cell membrane in 1 h, accumulated in lysosomes afterward, and finally dispersed in the whole cytoplasm, with bright, large fluorescence observed after 12 h (Fig. 2F). Meanwhile, no green fluorescence was observed in the cell nucleus during the whole process, suggesting that lysosome, but not nucleus, is the main cellular target of Tf-SeNPs.

To further dissect the Tf-SeNPs internalization pathway, cells were pretreated with different endocytosis inhibitors before the addition of Tf-SeNPs (Fig. 2G). Treatments of sodium azide (NaNO3) in combination with 2-deoxy-D-glucose (DOG), or low temperature (4 °C), strongly inhibited the Tf-SeNPs internalization to 41.1% and 63.5% of control, which suggest that Tf-SeNPs is transported into the cells by means of energy-dependent endocytosis. Phagocytosis/
macropinocytosis, caveolae/lipid raft-mediated and clathrin-mediated endocytosis are three main mechanisms of endocytosis [30]. Since MCF-7 cells are known to be not phagocytic, the other two pathways were studied. TIR is known to bind to Tf with bound ferric ions through clathrin-coated vesicles [31,32]. Therefore, we investigated the cellular uptake of Tf-SeNPs with continuous treatment of sucrase, a specific inhibitor of clathrin-mediated endocytosis. As we expected, sucrase markedly decreased the internalization of TF-SeNPs to 35.3% of control, indicating that the clathrin-mediated endocytosis was the main pathway. In parallel, nystatin, an inhibitor of lipid raft-dependent endocytosis, caused a reduction to 57.4% of TF-SeNPs uptake, demonstrated that lipid raft-mediated endocytosis was also involved in the endocytosis of TF-SeNPs. Dynamin, a GTP-binding protein, is essential for receptor-mediated endocytosis [33,34]. Dynasore, a specific inhibitor essential for dynamin-mediated lipid raft endocytosis, halted the internalization of TF-SeNPs to 51.9% of control, suggesting that dynamin-mediated pathway was the main pattern of lipid raft-dependent endocytosis of TF-SeNPs in MCF-7 cells. Taken together, both dynamin-dependent lipid raft-mediated and clathrin-mediated endocytic pathway were involved in the cellular uptake of TF-SeNPs in MCF-7 cells.

The drug release behavior from TF-SeNPs was investigated in PBS solution at pH 7.4 and pH 5.3 to simulate the blood and lysosome environments in vivo. As shown in Fig. 2H, the cumulative release amount of DOX from the nanoparticles at pH 5.3 was 49.0% within 1 h and 90.0% for 48 h, whereas the release rate at pH 7.4 was 12.7% in 1 h and finally reached 39.5% for 48 h. These results demonstrated that DOX from nanoparticles should be regulated by the existing amino groups in CS layer. At pH 7.4, CS was orderly aggregated due to the deprotonation of its amino groups, which hindered the release of DOX from nanoparticles [35,36]. At pH 5.3, the protonized behavior of amino groups led to CS dissolution, which facilitated the release of DOX from TF-SeNPs. These features of CS endow TF-SeNPs system the ability of drug controlled release.

3.3. Induction of cancer cell apoptosis by TF-SeNPs

The anticancer efficacy of TF-SeNPs was evaluated against various human cancer and normal cell lines by MTT assay. TF-SeNPs exhibited broad-spectrum growth inhibition on MCF-7, HepG2, and A375 cancer cell lines, with IC50 values ranging from 7.1 to 11.1 μM (Fig. 3A). A time-course analysis revealed that TF-SeNPs inhibited MCF-7 cell growth in a time- and dose-dependent manner (Fig. 3B). Moreover, the IC50 value suggested lower than those on cancer cells (Fig. 3A). These results indicate that, TF-SeNPs is efficient in reducing the toxicity in normal cells without sacrifice of its anticancer activity. In agreement with these results, we found that, MCF-7 cells expressed higher level of TIR than HepG2 and A375 cells, while HUVEC cells hardly expressed TIR. Consequently, TF/TIRs-mediated cell-specific delivery contributes to the cellular selectivity of TF-SeNPs.

To understand the synergistic interaction between SeNPs and the loaded drug (DOX), the growth inhibition of TF-SeNPs were analyzed by isobologram examination. According to the results of MTT assay, the IC50 value for TF-SeNPs, SeNPs and DOX was 7.1, 234.0 and 0.3 μM, respectively. Isobologram analysis shows that the viability inhibition of combined SeNPs and DOX treatment was significantly synergistic, as evidenced by the location of data point in the isobologram far below the line defining additive effect (Fig. 3B). Besides, the combination index (CI) of the TF-SeNPs was calculated as 0.1, which indicates the strong synergistic effects between SeNPs and DOX.

Apoptosis is the main mechanism accounting for the anticancer action of Se. Therefore, cell cycle distribution was performed by flow cytometric analysis to determine whether apoptosis was involved in the TF-SeNPs-induced cell death. Fig. 3C shows that exposure of MCF-7 cells to TF-SeNPs for 72 h resulted in a significant dose-dependent increase in sub-G1 cell population from 11.8% to 79.6%. Apoptotic cell death was further confirmed by DNA fragmentation and nucleolus condensation by TUNEL and DAPI co-staining assay. Fig. 3D shows that TF-SeNPs caused a dose-dependent increase in DNA fragmentation (green fluorescence) and nucleolus condensation (blue fluorescence) in MCF-7 cells. These results demonstrate that cell death induced by TF-SeNPs is mainly caused by apoptosis.

Caspases, a family of cysteine proteases, are vital components in response to apoptosis. To identify the signaling pathways involved in TF-SeNPs-induced apoptosis, fluorometric assay was conducted to analyze the activation of caspase-8, caspase-9 and caspase-3. As shown in Fig. 3E, two initiator caspases, caspase-8 (Fas/CD95-mediated) and caspase-9 (mitochondrial-mediated), as well as an effector caspase (caspase-3), were triggered by TF-SeNPs in a dose-dependent manner in MCF-7 cells. These results indicate that both intrinsic and extrinsic pathways contribute to TF-SeNPs-induced apoptosis.

3.4. Activation intracellular apoptotic signaling pathways by TF-SeNPs

Reactive oxygen species (ROS) has been postulated to be closely related to cell apoptosis activated by anticancer drugs. High level of ROS can cause damage to macromolecules and further induce cell apoptosis [37,38]. In order to investigate whether TF-SeNPs triggers ROS-mediated apoptosis, we examined the intracellular ROS level by measuring dihydroethidium (DHE) fluorescence intensity. As shown in Fig. 4B, TF-SeNPs, SeNPs and DOX elevated ROS generation in MCF-7 cells after 5 min of treatment, followed by a gradually downfall to 120 min. Importantly, TF-SeNPs triggered higher level of ROS than SeNPs and DOX, indicating that the combination of SeNPs and DOX synergistically elevated intracellular ROS level, which resulted in dramatical enhancement in anticancer efficacy.

Mitochondrial respiratory chain has been regarded as the major source of ROS in cancer cells, especially superoxide and hydrogen peroxide. The observation of ROS overproduction and activation of mitochondria-mediated apoptosis confirms the induction of mitochondrial dysfunction by TF-SeNPs. Bcl-2 family proteins could regulate outer mitochondrial membrane permeability and control the on/off of intrinsic apoptotic pathway [39]. In this study, we show that, TF-SeNPs suppressed the expression of Bcl-1, a pro-survival member of Bcl-2 family protein, and increased the expression of pro-apoptosis member Bad (Fig. 4C), while didn’t affect the expression of Bax and Bcl-2. Moreover, the truncation of Bid enhanced the crosstalk between extrinsic and intrinsic pathways to promote caspase activation and PARP cleavage (Fig. 4D).

A number of evidence has supported that ROS-mediated DNA damage can cause cell death by induction of apoptosis via various signaling pathways, such as p53, ATM/ATR, AKT and MAPKs pathways [37]. p53 tumor suppressor protein acts as transcription factor that regulate the expression of a variety of genes correlated to cell cycle arrest and apoptosis [40]. Phosphorylation of p53 at Ser 15 can lead to cell apoptosis caused by chemotherapeutic drugs and chemopreventive agents, especially selenocompounds [41]. To investigate whether cell apoptosis induced by TF-SeNPs was regulated by p53 pathway, we examined the expression of p53 and its phosphorylation status in MCF-7 cells. Western blot analysis shows that TF-SeNPs treatment did not affect the expression level of total p53 and its phosphorylation status in MCF-7 cells. However, significantly increased the phosphorylation of p53 at ser 15 site (Fig. 4E). To examine how TF-SeNPs activates p53 in cancer cells, we examined the effects of the nanoparticles on the status of DNA, since DNA damage is one kind of stimuli which enables to activate P53 expression [42,43]. Importantly, the phosphorylated action of histone at Ser 139 is
Fig. 3. Induction of cancer cell apoptosis by Tf-SeNPs. (A) Growth inhibition of Tf-SeNPs in MCF-7, HepG2 and A375 cancer cells for 72 h. (B) Isobologram examination of growth inhibition of SeNPs and DOX on MCF-7 cells. (C) Flow cytometric analysis of Tf-SeNPs-treated MCF-7 cells for 72 h. (D) Representative images of DNA fragmentation and nuclear condensation caused by 24 h-treatment of Tf-SeNPs, as detected by TUNEL assay and DAPI staining. (E) Analysis of caspase activity in Tf-SeNPs-induced apoptosis in MCF-7 cells. MCF-7 cells were treated with Tf-SeNPs for 72 h. Caspase activities were determined by synthetic fluorogenic substrate. Values represented were means ± SD of triplicates. *P < 0.05 vs. control. **P < 0.01 vs. control.
Fig. 4. Activation intracellular apoptotic signaling pathways by Tf-SeNPs. (A) The main signaling pathway of Tf-SeNPs-induced apoptosis. (B) Changes of intracellular ROS generation induced by SeNPs, DOX and Tf-SeNPs in MCF-7 cells. Cells incubated with 10 μM DHE in PBS for 30 min were exposed to 200 μM Tf-SeNPs, 200 μM SeNPs and 0.7 μM DOX, respectively. The intracellular ROS level was determined by measuring the fluorescent intensity of DHE. Values represented were means ± SD of triplicates. Western blot analysis for the expression of (C) Bax, Bcl-xl, Bad, Bid; (D) PARP; (E) p53, p-p53, p-His, and (F) ERK, p-ERK, P38, p-P38, JNK, AKT. β-Actin was used as loading control.
Fig. 5. In vivo anticancer activity of Tf-SeNPs. (A) Records of the changes of tumor volume in Tf-SeNPs-treated xenograft MCF-7 cancer nude mice for 16 days. Treated mice were given through intravenous injection with different dosages of Tf-SeNPs for 16 days. Control mice received an equal volume of PBS only. The tumor size of mice was measured every 2 days using calipers. (B) Effect of Tf-SeNPs on Tumor weight. (C) Body weight of xenograft MCF-7 cancer nude mice treated by Tf-SeNPs. The data was obtained at the last day (16th day) of experiment. (D) Analysis of caspase activity in Tf-SeNPs-treated xenograft MCF-7 cancer nude mice. Xenograft MCF-7 cancer nude mice treated with Tf-SeNPs for 16 days were sacrificed and the tumors were isolated. The total protein extracted and caspase activities were determined by synthetic fluorogenic substrate. (E) Western blot analysis of the expression of p-ERK, p-histone, p-p53. β-Actin was used as loading control. (F) Immunohistochemical analysis of Tf-SeNPs-treated tumor sections by TUNEL, H&E, Ki67 and phosphorylated p53 staining. Values represented were means ± SD of triplicates. *P < 0.05 vs. control. **P < 0.01 vs. control.
closely associated with DNA double strand break, and has been identified as a biochemical marker of DNA damage [44]. In this study, we show that, Tf-SeNPs significantly triggered the phosphorylation of histone at Ser 139 site, indicating that Tf-SeNPs triggers cancer cell apoptosis through DNA damage-mediated p53 activation.

To determine the possible role of MAPKs in Tf-SeNPs-induced apoptosis, we checked the status of MAPKs in treated cells. As shown in Fig. 4F, TF-SeNPs exhibited differential effects on the phosphorylation of p38, JNK, ERK, and AKT. The phosphorylation of pro-apoptotic kinases p38 displayed a trend of up-regulation in a dose-dependent manner. In contrast, the phosphorylation of anti-apoptotic kinases ERK was effectively suppressed by TF-SeNPs, while the phosphorylation of JNK and AKT was not affected by TF-SeNPs. Taken together, these results suggest that regulation of p38 and ERK pathway contributes to apoptosis induced by TF-SeNPs.

3.5. In vivo anticancer activity of TF-SeNPs

In vivo therapeutic efficacy of TF-SeNPs is a crucial index for its future medical potential. Therefore, we treated MCF–7 xenografts nude mice with different dosages of TF-SeNPs to examine its in vivo anticancer efficacy. At the end of the experiments, the mice were sacrificed and the tumor weight and tumor volume were measured. The action mechanisms accounting for the in vivo growth inhibitory effects were also elucidated. The results show that TF-SeNPs significantly inhibited the proliferation of MCF-7 cells in a dose-dependent manner, as represented by the decrease in tumor volume and tumor weight (Fig. 5A and B, Fig. S4). Besides, no distinct reduction was observed in the body weight of nude mice, indicating the mineral side effect of SeNPs after TF surface decoration (Fig. 5C). These results demonstrate the effective in vivo tumor-suppressed capacity of TF-SeNPs.

Our previous data have demonstrated that TF-SeNPs caused caspase-mediated apoptosis in MCF-7 cells. To examine whether TF-SeNPs was able to activate the caspase-mediated apoptosis in vivo, the total protein of tumor was extracted and analyzed by a fluorometric caspase activity assay. As shown in Fig. 5D, after treatment with TF-SeNPs, the activities of caspase-3, caspase-8 and caspase-9 were significantly evoked, which demonstrate that both intrinsic and extrinsic apoptotic pathways are both activated by TF-SeNPs in nude mice.

To investigate the upstream events of TF-SeNPs-mediated apoptosis, the expression of essential apoptosis-related protein was detected in the tumor tissues. As shown in Fig. 5E, TF-SeNPs effective inhibited the phosphorylation of ERK, whereas dramatically up-regulated p-histone and p-p53. In consistent with the results obtained in cell model, TF-SeNPs could inhibit MCF-7 tumor growth by suppression of ERK pathway and activation of p53 pathway. Protein expression in section was also examined by immunohistochemistry using specific antibodies. As shown in Fig. 5F, from the results of Ki67 staining and TUNEL assay, dose-dependent decrease in cell proliferation and increase in cell apoptosis were observed in TF-SeNPs groups. The results of HE and CD34 staining shows that, heteromorphism in nucleus and tube formation in vivo were evident in the control group, which were significantly inhibited by TF-SeNPs treatment. Moreover, TF-SeNPs activated the phosphorylation of p53 at Ser 15 site in a dose-dependent manner. Taken together, these findings all indicated that TF-SeNPs showed potential therapeutic effect in vivo through induction of apoptosis.

4. Conclusions

In summary, we describe the synthesis of TF-conjugated SeNPs and its use as a cancer-targeted drug delivery system to achieve enhanced cellular uptake and anticancer efficacy. TF as targeting ligand significantly enhances the cellular uptake of drug-loaded SeNPs through clathrin-mediated and dynamin-dependent lipid raft-mediated endocytosis in cancer cells overexpressing TfRs, and increases their selectivity between cancer and normal cells. TF-SeNPs exhibits unprecedented enhanced cytotoxicity toward cancer cells through induction of apoptosis with the involvement of intrinsic and extrinsic pathways. Internalized TF-SeNPs triggers intracellular ROS overproduction, thus activates p53 and MAPKs pathways to promote cell apoptosis. We report that, TF-SeNPs significantly inhibits in vivo tumor growth in nude mice model through induction of p53-mediated apoptosis. This cancer-targeted design of SeNPs opens a new path for synergistic treating of cancer with higher efficacy and decreased side effects.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.biomaterials.2013.04.067.

References


