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CdSe quantum dots induce apoptosis in human neuroblastoma cells via mitochondrial-dependent pathways and inhibition of survival signals

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Abstract

Quantum dots (QDs) may be useful as novel luminescent markers, but their cytotoxicity has not been fully investigated. In this report, we demonstrate that CdSe-core QDs can induce apoptotic biochemical changes, including JNK activation, loss of mitochondrial membrane potential, mitochondrial release of cytochrome *c* and activation of caspase-9 and caspase-3 in the IMR-32 human neuroblastoma cell line. Importantly, treatment of IMR-32 cells with CdSe-core QD triggered an increase in reactive oxygen species (ROS) and inhibited survival-related signaling events, such as decreased Ras and Raf-1 protein expression and decreased ERK activation. These apoptotic biochemical changes were not detected in cells treated with ZnS-coated CdSe QDs. Collectively, these results demonstrate that CdSe-core QD treatment of IMR-32 cells induced JNK activation and mitochondrial-dependent apoptotic processes while inhibiting Ras → ERK survival signaling and that a ZnS coating could effectively reduce QD cytotoxicity.

Keywords: Quantum dot; Apoptosis; JNK; ERK; ROS

1. Introduction

Quantum dots (QDs) are colloidal nanocrystalline semi-conductors that have unique light emitting properties and can be used as novel luminescent materials. Typical QDs are 1–12 nm in diameter and contain a relatively small number of atoms in a discrete cluster (Murray et al., 2000). QDs can absorb irradiated energy at any wavelength greater than that of their lowest energy transition and may then convert the irradiated energy to an extremely narrow bandwidth emission. QDs are considered good candidates for development as luminescent probes because they have the advantages of broadband excitation, narrow bandwidth emission, emission of high intensity light, resistance to quenching and good photochemical stability. These properties suggest that QDs could be useful in various biochemical assays, especial immunofluorescence staining. A previous study showed that CdSe-core QDs could induce cell death (Derfus et al., 2004), but the precise regulatory mechanisms underlying this effect have not yet been elucidated.

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Apoptosis may be triggered by a number of signals, including oxidative stress. Although the precise molecular mechanisms for apoptosis have not been clearly defined, generation of reactive oxygen species (ROS) by cadmium was found to trigger apoptosis of hepatoma G2 cells (Oh and Lim, 2006). A number of cysteine proteases called caspases are thought to play important roles in cell apoptosis (Martins et al., 1997), as are members of the Bcl-2 family of regulatory proteins (Tsujimoto and Shimizu, 2000), which regulate the release of mitochondrial cytochrome *c* by modulating the permeability of the outer mitochondrial membrane. Apoptosis in a variety of cell types has been associated with activity changes in protein kinases (Anderson, 1997) such as c-Jun N-terminal kinase (JNK) (Seimiya et al., 1997), indicating that protein phosphorylation may be involved in regulating apoptosis. Finally, the Ras-regulated extracellular signal regulated kinase (ERK) activation signal transduction pathway, which includes Raf-1 and mitogen-activated protein kinase (MAPK)/ERK kinase (MEK), appears to be involved in both proliferation and anti-apoptosis (Caraglia et al., 1999). Recent reports have shown that Ras → ERK mediated survival signaling could protect human epidermoid cancer KB cells from various apoptotic triggers (Caraglia et al., 2004, 2005).

On the basis of these study results, we herein evaluated apoptotic and anti-apoptotic signaling in CdSe-core QD-induced apoptosis of IMR-32 cells.

2. Materials and methods

2.1. Materials

The alkaline phosphatase-conjugated goat anti-rabbit and anti-mouse IgG antibodies, 2′,7′-dichlorofluorescin diacetate (DCF-DA), propidium iodide and Hoechst 33342 were acquired from Sigma (St. Louis, MO). The anti-phospho-JNK, anti-JNK1, anti-Ras, anti-Raf-1 and anti-ERK antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). U0126 was purchased from Calbiochem (Darmstadt, Germany). The monoclonal anti-cytochrome *c* antibody (6H2.B4) was obtained from Imgenex (San Diego, CA). Z-DEVD-AFC and SP600125 were obtained from Calbiochem (La Jolla, CA). CDP-Star™ (a chemiluminescent substrate for alkaline phosphatase) was purchased from Boehringer Mannheim (Mannheim, Germany).

2.2. Quantum dot preparation

Nanocrystals comprising a CdSe-core and a ZnS shell were synthesized by Professor Lu and co-workers at the Department of Chemical Engineering, National Taiwan University. Briefly, appropriate amounts of trioctylphosphine oxide (TOPO), cadmium oxide (CdO) and tetradecylphosphonic acid (TDPA) were heated to 180 °C under zargon and dried and degassed under a vacuum. The reaction temperature was then increased to 330 °C, selenium (Se) precursor solution in trioctylphosphine (TOP) was injected into the reaction flask and the mixture was allowed to cool to 240 °C. Zn and S stock solutions prepared with bis(trimethylsilyl)sulfide in TOP, along with a dimethyl zinc solution, were added dropwise with vigorous stirring until a final mole ratio of 1:4 (Cd/Se:Zn/S) was achieved in the reaction. The reaction mixture was cooled to room temperature and the nanocrystals were precipitated with anhydrous methanol, collected by centrifugation and washed three times with anhydrous methanol for removal of residual TOPO and unreacted reagents. The precipitate was dissolved in anhydrous chloroform or tetrahydrofuran (THF) for experiments. For water solubilization, the CdSe QDs were surface coupled with mercaptoacetic acid (MAA) and then suspended in PBS buffer (the modification was performed by Professor Ruaan and co-workers at the Department of Chemical and Materials Engineering, National Central University, Taiwan). A particle sizer was used to measure the CdSe QDs, which were found to be about 3.5 nm in diameter.

2.3. Cell culture and quantum dot treatment

Human neuroblastoma IMR-32 cells were cultured at 37 °C in a humid 95% air/5% CO_2 atmosphere in 90% minimum essential medium (Biochrom KG, Berlin, Germany) supplemented with 10% heat-inactivated fetal bovine serum, 100 IU/ml penicillin and 100 μg/ml streptomycin. Cells (~5–6 × 10^6) were incubated in medium containing various concentrations of CdSe QDs for 24 h and cell lysates were collected for further analysis.

2.4. MTT assay

The percentage of cell survival was measured using the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) colorimetric assay. Following treatment of cells with CdSe QDs, 100 μl of 0.45 g/l MTT solution was added to each well of a 96-well cell culture plate. The plates were incubated at 37 °C for 60 min to allow color development and 100 μl of 20% SDS in DMF:H_2O (1:1) solution was added to each well. The plates were then incubated overnight at 37 °C for solubilization of the formazan products. Spectrophotometric data were measured using an ELISA reader at a wavelength of 570 nm.

2.5. Assessment of necrosis and apoptosis

Oligonucleosomal DNA fragmentation in the apoptotic cells was measured using the Cell Death Detection ELISA^{plus} kit (TUNEL apoptosis assay kit), according to the manufacturer's protocol (Roche Molecular Biochemicals, Mannheim, Germany). Cells (1 × 10^5) were treated with or without the indicated concentrations of CdSe QDs for 24 h at 37 °C prior

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to ELISA detection and spectrophotometric data were obtained using an ELISA reader at a wavelength of 405 nm. Necrosis was assayed by determining the percentage of cells with plasma membranes permeable to propidium iodide and apoptosis was additionally assayed by staining with propidium iodide (1 μg/ml) and Hoechst 33342 (2 μg/ml) at room temperature for 10 min. Apoptotic cells were identified as being impermeable to propidium iodide and showing condensed/fragmented nuclei under Hoechst 33342 staining, as observed under fluorescent microscopy. In each experiment, 7–10 independent fields (∼600–1000 nuclei in total) were counted per each condition and the percentage of apoptotic cells was calculated.

2.6. DNA fragmentation

DNA fragmentation was analyzed according to the method of Zhu and Wang (1997). Briefly, cells (∼1×10^6 cells) were detached from culture dishes by trypsin/EDTA. They were then collected, washed once with ice-cold PBS and centrifuged. The supernatants were removed, cells were dispersed in 30 μl of lysis buffer (10 mM Tris–HCl, pH 7.4, 100 mM NaCl, 25 mM EDTA and 1% sarkosyl) by gentle vortexing and lysates were incubated with 4 μl proteinase K (10 μg/μl) at 45 °C for 2 h and then with 2 μl RNase (10 μg/μl) at room temperature for 1 h. The resulting reaction mixtures (20 μl per sample) were subjected to electrophoresis on 2% agarose gels for DNA fragmentation analysis.

2.7. Measurement of ROS generation

ROS were measured in arbitrary units using 2′,7′-dichlorofluorescein diacetate (DCF-DA) and dihydrorhodamine 123 (DHR 123) dye. Cells (1.0×10^6) were incubated in 50 μl PBS containing 20 μM DCF-DA or DHR 123 dye for 1 h at 37 °C and relative ROS units were determined using a fluorescence ELISA reader (excitation 485 nm, emission 530 nm).

2.8. Caspase activity assays

Caspase-3 activity was measured using the Z-DEVD-AFC fluorogenic substrate as previously described (Chan et al., 2003; Hsieh et al., 2003). Caspase-8 and caspase-9 activities were assayed using the Colorimetric Caspase-9 Assay kit and the Fluorometric Caspase-8 Assay kit (Calbiochem, La Jolla, CA).

2.9. Immunoblotting

Immunoblotting was performed essentially as described (Chan, 2005). Briefly, proteins were resolved by SDS gel electrophoresis, blotted to PVDF membranes and detected with commercial anti-JNK, anti-phospho JNK, anti-Ras, anti-Raf-1, anti-ERK or anti-phospho ERK antibodies (0.25 μg/ml) followed by alkaline phosphatase-conjugated goat anti-rabbit or anti-mouse IgG secondary antibodies. The results were visualized using the CDP-Star™ kit according to the procedure provided by the manufacturer (Mannheim, Germany).

2.10. JNK activity assay

JNK activity, as assayed by the presence of phosphorylated c-Jun protein, was analyzed with the AP-1/c-Jun ELISA kit, according to the manufacturer's protocol (Active Motif, Carlsbad, CA). AP-1 heterodimeric complexes in cellular nuclear extracts were collected by binding to a consensus 5′-TGA(C/G)TCA-3′ oligonucleotide coated on a 96-well plate. Phospho-c-Jun was assayed using a phospho-c-Jun primary antibody and a secondary horseradish peroxidase-conjugated antibody in a colorimetric reaction.

2.11. Detection of changes in mitochondrial membrane potential

IMR-32 cells were plated and grown in 96-well plates for 24 h and then incubated with various concentration of CdSe QDs for an additional 24 h. The fluorescent dyes, DiOC6(3) (20 nM) and TMRE (0.1 μM), were added to each well and plates were incubated for 15 min. Fluorescence was measured with a plate spectrofluorometer [excitation: 485 nm (DiOC6(3)) and 535 nm (TMRE); emission: 535 nm (DiOC6(3)) and 590 nm (TMRE)].

2.12. Cytochrome c release assay

IMR-32 cells (1×10^7) were treated with CdSe QDs for 24 h and then harvested by centrifugation at $800 \times g$ at 4 °C for 15 min. After three washes with ice-cold PBS, the cell pellets were re-suspended in Hepes-buffer (20 mM Hepes, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 0.1 mM PMSF, pH 7.5) containing 250 mM sucrose, homogenized with a homogenizer and centrifuged at $800 \times g$ at 4 °C for 15 min. The resulting supernatants were centrifuged at $10{,}000 \times g$ for 15 min at 4 °C and the supernatant (cytosolic fraction) and pellet (mitochondrial pellet) were separately dissolved in SDS sample buffer, subjected to 15% SDS-PAGE and analyzed by immunoblotting with a monoclonal antibody against cytochrome *c*.

2.13. Statistics

Data were analyzed using one-way ANOVA and the differences were evaluated using a Student's *t*-test and analysis of variance. A P-value < 0.05 was considered significant.

3. Results

3.1. The effects of CdSe-core QDs on IMR-32 cells

To test the potential cytotoxicity of QDs, we used MTT assays to examine the viability of IMR-32 cells

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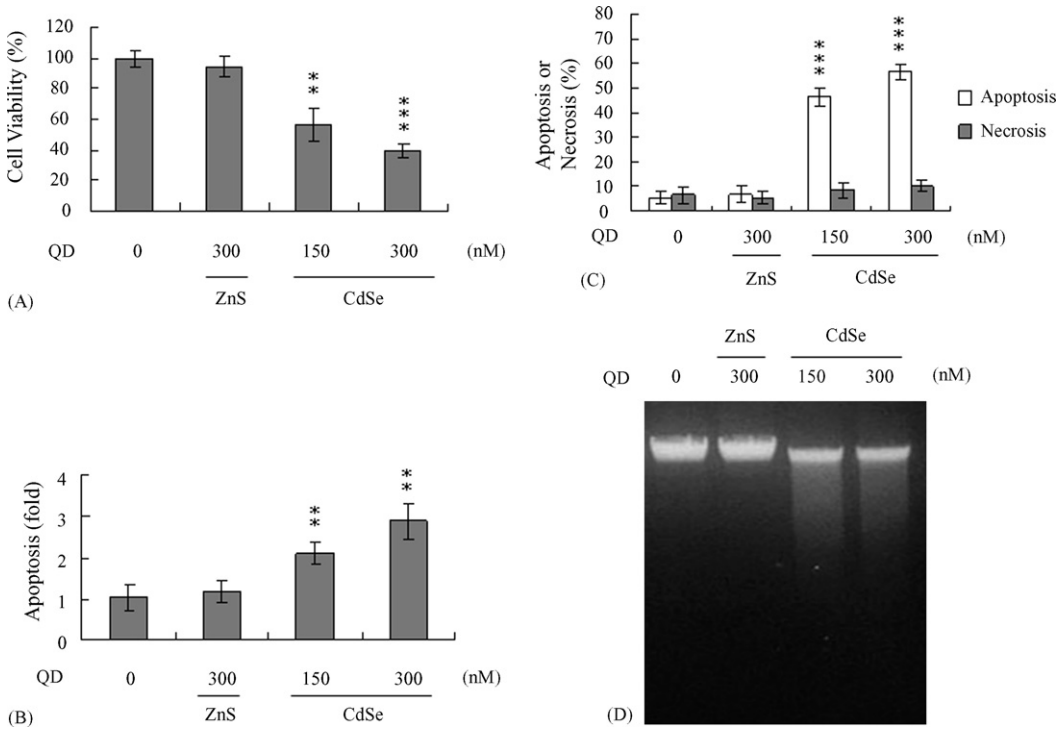

Fig. 1. Effects of QDs on IMR-32 cells. IMR-32 cells were incubated with various concentrations of CdSe-core QDs (CdSe) or ZnS-coated CdSe QDs (ZnS) for 24 h. Cell viability was determined using MTT assays (A) and apoptosis was detected with the Cell Death Detection ELISA kit (TUNEL assay kit) (B). (C) Percentages of apoptosis and necrosis were determined by staining the cells with propidium iodide and Hoechst 33342. (D) Genomic DNA was prepared from the cells and subjected to electrophoresis on a 2% agarose gel for DNA fragmentation analysis. Values are presented as means ± S.D. of five determinations. $^{**}P < 0.01$ and $^{***}P < 0.001$ vs. the untreated control group.

treated with various doses of CdSe-core QDs or ZnS-coated CdSe QDs. Our results revealed that while ZnS-coated CdSe QDs had no effect on cell viability, the viability of CdSe-core QD-treated IMR-32 cells was decreased by approximately 50–60% (Fig. 1A). To investigate the mode of CdSe-core QD-induced cell death, we used a TUNEL ELISA kit to determine cell apoptosis. We found that CdSe-core QD treatment induced a 2.8-fold increase in TUNEL positivity, as compared to untreated cells (Fig. 1B). The percentage of apoptotic and necrotic cells was further analyzed by staining with propidium iodide and Hoechst 33342. As shown in Fig. 1C, the percentage of apoptotic cells was significantly increased following treatment with 150–300 nM CdSe-core QDs. However, the necrotic cell population remained at a relatively low level (Fig. 1C). In addition, CdSe-core QD treatment of IMR-32 cells triggered chromosomal DNA fragmentation, the most prominent biochemical event in the early stages of apoptosis (Fig. 1D). These findings suggest that CdSe-core QDs induce apoptosis but not necrosis in IMR-32 cells, but that a ZnS-based coating appears to reduce this cytotoxicity.

Fig. 2. ROS generation, activation of caspase-9 and caspase-3 in CdSe-core QD-treated IMR-32 cells. (A) IMR-32 cells were preloaded with 10 μM DCF-DA or DHR 123 for 1 h. Cells were then treated with or without the indicated concentrations of CdSe-core QDs (CdSe) or ZnS-coated CdSe QDs (ZnS) for 24 h. Generation of ROS is expressed as absorbance/mg of protein. (B and C) IMR-32 cells were incubated with *N*-acetyl cysteine (NAC; 2 mM) or α-tocopherol (Toc; 300 μM) at 37 °C for 1 h and then treated with CdSe-core QDs (CdSe; 300 nM) for another 24 h. ROS generation was assessed using DCF-DA and DHR 123 (B). Apoptosis was evaluated using the Cell Death Detection ELISA kit (C). (D-G) IMR-32 cells were incubated with various concentrations of CdSe-core QDs or ZnS-coated CdSe QDs for 24 h. Caspase-9 activities were assayed using the Colorimetric Caspase-9 Assay kit (Calbiochem) (D). Cell extracts (60 μg) were analyzed for caspase-3 activity using Z-DEVD-AFC as the substrate (E). Caspase-8 activities were assayed using the Colorimetric Caspase-8 Assay kit (F). Caspase activation was further examined by immunoblotting with antibodies specific to cleaved caspase-3, -9 and -8 (G). Values are presented as means ± S.D. of five determinations. $^{**}P < 0.01$ and $^{***}P < 0.001$ vs. the value of the untreated control group. $^{\#}P < 0.001$ vs. the "CdSe-core QD-treated only" group.

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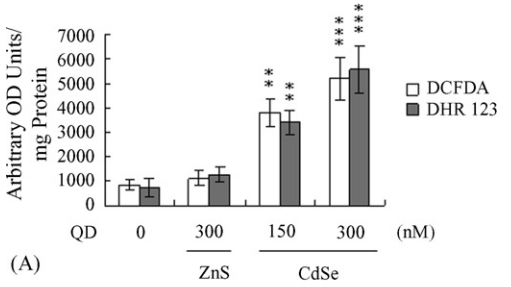

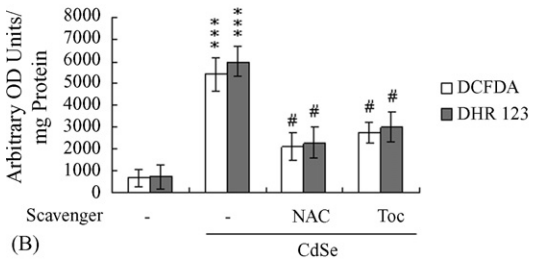

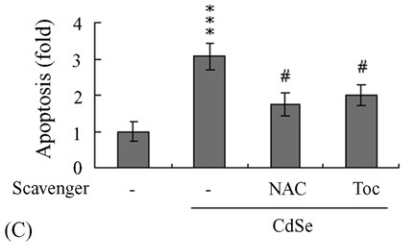

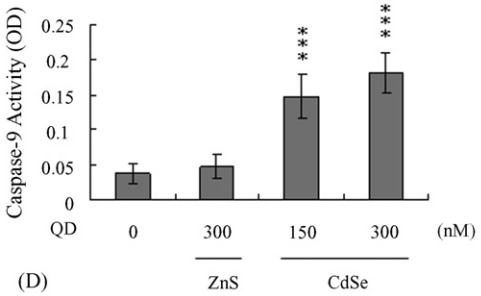

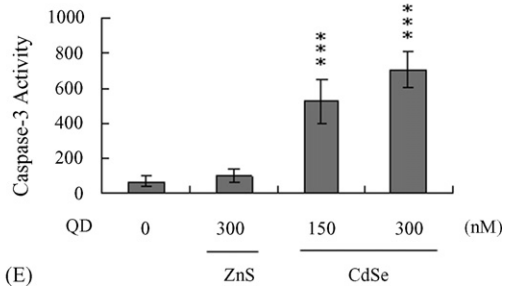

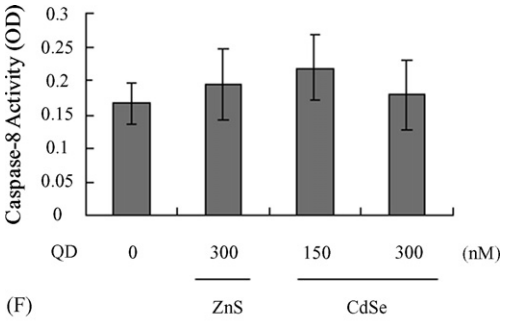

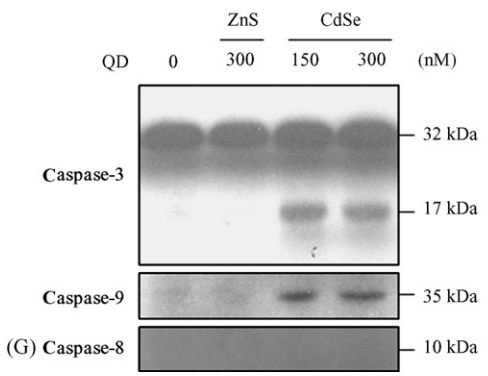

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3.2. ROS generation and apoptotic biochemical changes in CdSe-core QD-treated IMR-32 cells

As Cd treatment is known to provoke oxidative stress in cells (Oh and Lim, 2006), we used the DCF-DA and DHR 123 detection reagent to examine ROS generation in QD-treated IMR-32 cells. Our results revealed that the intracellular ROS content was significantly increased in IMR-32 cells treated with CdSe-core QDs, but not in cells treated with ZnS-coated QDs (Fig. 2A). In addition, we assessed the effect of two frequently used ROS scavengers, *N*-acetyl cysteine (NAC) and α-tocopherol, on CdSe QD-treated IMR-32 cells. Pretreatment of cells with NAC (2 mM) or α-tocopherol (300 μM) attenuated CdSe-core QD-induced intracellular ROS level increases and induction of apoptosis (Fig. 2B and C), suggesting that CdSe-core QDs may trigger cell apoptosis via ROS generation. To further investigate apoptotic signaling during CdSe-core QD-induced apoptosis, we evaluated the activation levels of caspase-9, caspase-8 and caspase-3, which are known to be activated during cell apoptosis (Chan and Wu, 2004; Erhardt and Cooper, 1996). Our results demonstrated that treatment of IMR-32 cells with CdSe-core QDs stimulated the activation of caspase-9 (Fig. 2D) and caspase-3 (Fig. 2E), but not caspase-8 (Fig. 2F). These results were confirmed by immunoblotting analysis with antibodies specific to cleaved caspase-3, -9 and -8, which showed cleavage of caspase-3 and -9 but not caspase-8 (Fig. 2G). In contrast, no significant caspase activation was seen in ZnS-coated CdSe QD-treated IMR-32 cells (Fig. 2D, E and G). We then used immunoblotting and ELISA to examine JNK activity during CdSe-core QD-induced apoptosis. Our results revealed that JNK was activated dose-dependently in IMR-32 cells treated with CdSe-core QDs but not in cells treated with ZnS-coated QDs (Fig. 3A and B). Previous studies have shown that the protein expression ratio of Bax versus Bcl-2 is relevant to apoptosis; a high Bax/Bcl-2 ratio is associated with a lower threshold of apoptosis and a low ratio denotes a higher apoptotic threshold. Here, we investigated whether CdSe-core QDs induced apoptosis by modulating the Bax/Bcl-2 ratio. Immunoblotting revealed that treatment of IMR-32 cells with 150 nM and 300 nM CdSe-core QDs caused increases in Bax protein levels and decreases in Bcl-2 protein levels (Fig. 3C). Densitometric analysis revealed that this led to a higher Bax/Bcl-2 ratio in CdSe-core QD-treated IMR-32 cells, thus favoring apoptosis (Fig. 3D). Collectively, these results indicate that various apoptotic biochemical changes were induced in IMR-32 cells treated with CdSe-core QDs but not in those treated with ZnS-coated QDs.

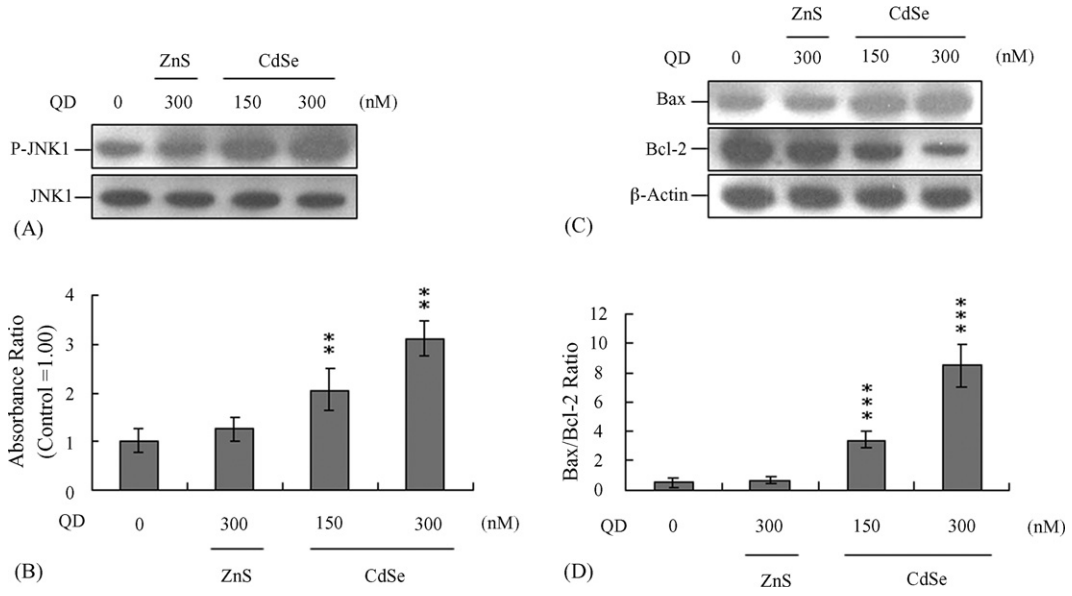

Fig. 3. Changes in JNK activation and Bcl family protein expression in CdSe-core QD-treated IMR-32 cells. IMR-32 cells were incubated with various concentrations of CdSe-core QDs (CdSe) or ZnS-coated QDs (ZnS) for 24 h. (A) Cell extracts (60 μg) were immunoblotted with anti-p-JNK and anti-JNK1 antibodies. (B) JNK/AP-1 activity was evaluated by ELISA detection of phosphorylated c-Jun. The results were expressed in relation to the values of untreated controls, which were arbitrarily set to 1.00. (C and D) Bax and Bcl-2 protein levels were analyzed by immunoblotting (C) and analyzed by densitometry (D). Values are presented as means ± S.D. of five determinations. $^{**}P < 0.01$ and $^{***}P < 0.001$ vs. the untreated control group.

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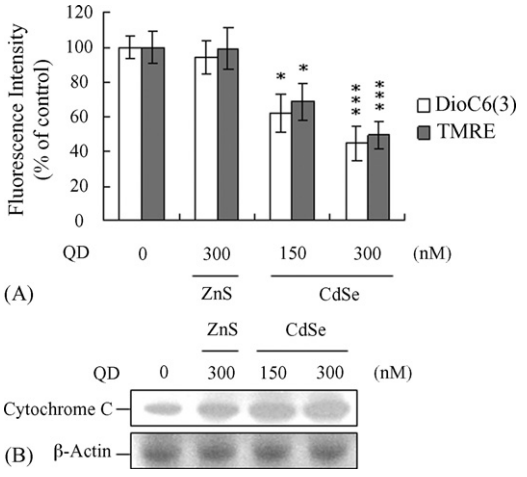

Fig. 4. Analysis of mitochondrial membrane potential and cytochrome *c* release in CdSe-core QD-treated IMR-32 cells. IMR-32 cells were incubated with various concentrations of CdSe-core QDs (CdSe) or ZnS-coated QDs (ZnS) for 24 h. (A) To examine mitochondrial membrane potential changes, cells were then incubated with 40 nM DiOC6(3) or 1 μM TMRE at 37 °C for 1 h and analyzed by spectrofluorometry. Values are presented as means ± S.D. of five determinations. $^{*}P<0.05$ and $^{***}P<0.001$ vs. the untreated control group. (B) To examine cytochrome *c* release from the mitochondria to the cytosol, cytosolic and mitochondrial fractions were separated and the cytosolic fractions were immunoblotted with an anti-cytochrome *c* antibody. The presented data are representative of five independent experiments.

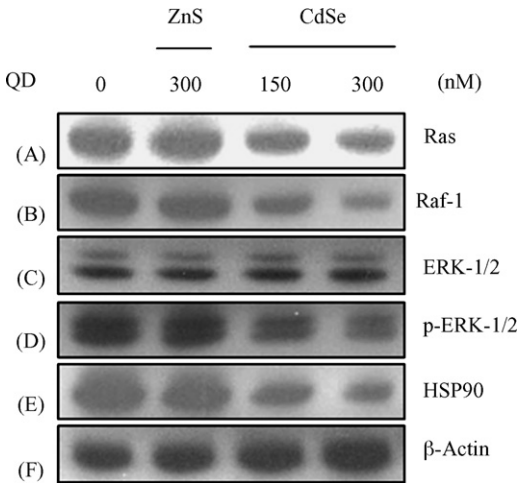

Fig. 5. Effects of CdSe-core QDs on survival signaling molecules and heat shock protein 90. IMR-32 cells were incubated with various concentrations of CdSe-core QDs (CdSe) or ZnS-coated QDs (ZnS) for 24 h and the expression levels of survival signaling proteins and heat shock protein 90 (HSP90) were determined. (A–C) Immunoblot assay for protein expression of Ras (A), Raf-1 (B) and ERK-1 and -2 (C). (D) The phosphorylation levels of ERK-1 and -2 were evaluated with immunoblotting using an anti-pMAPK antibody. (E) Immunoblot assay for the expression of HSP90. (F) Levels of β-actin (loading control).

We then investigated the effect of CdSe-core QDs on mitochondrial membrane potential change and cytochrome *c* release. Our results revealed that treatment of IMR-32 cells with CdSe-core QDs led to significant losses of mitochondrial membrane potential, whereas treatment with ZnS-coated QDs did not (Fig. 4A). Immunoblotting analyses showed that significant amounts of cytochrome *c* were released into the cytosol of IMR-32 cells treated with CdSe-core QDs but not from cells treated with ZnS-coated QDs (Fig. 4B). These findings indicate that CdSe-core QDs triggered apoptosis via decreased mitochondrial membrane potential and increased cytochrome *c* release, whereas ZnS-coated QDs did not.

3.3. Effects of CdSe-core QDs on survival signaling molecules and heat shock protein 90

As the Ras → ERK-mediated survival signaling pathway is known to protect cells from apoptotic triggers (Caraglia et al., 2003, 2005), we examined the effects of CdSe-core QDs on components of this pathway. Immunoblotting revealed a dose-dependent decreased in Ras and Raf-1 protein expression in IMR-32 cells treated with CdSe-core QDs but not in cells treated with ZnS-coated QDs (Fig. 5A and B). In addition, immunoblotting of the phosphorylated (active) forms of ERK-1 and ERK-2 proteins revealed an approximately four-fold decrease in the activations of these kinases in CdSe-core QD-treated IMR-32 cells (Fig. 5C and D). These results suggest that the CdSe-core QD-induced decreases in ERK-1/2 activity were mediated by decreased expression of the upstream enzymes, Ras and Raf-1. As previous reports have indicated that heat shock protein 90 (HSP90) prevents proteasome-mediated degradation of several signaling proteins, including Raf-1 (Blagosklonny, 2002; Pratt and Toft, 2003), we further analyzed the effect of CdSe-core QD treatment on HSP90 protein expression in IMR-32 cells. Our results revealed that HSP90 was down-regulated in IMR-32 cells treated with CdSe-core QDs but not in cells treated with ZnS-coated QDs (Fig. 5E). These data suggest that CdSe-core QD-induced apoptosis could be mediated by suppression of HSP90 expression, which triggers down-regulation of Ras and Raf-1 and subsequent decreases in ERK 1 and ERK 2 activity.

3.4. Effect of inhibition of JNK and ERK activation on CdSe-core QD-induced apoptosis

To further determine the relationship between JNK and mitochondrial membrane potential changes dur-

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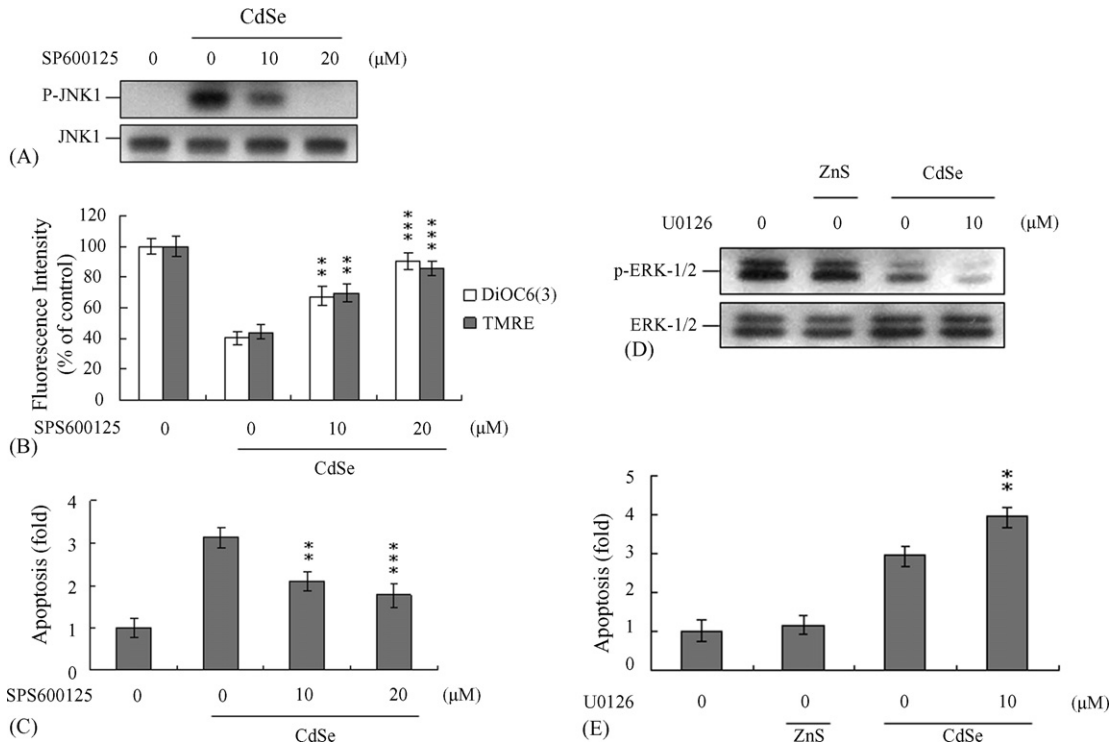

Fig. 6. Effect of inhibition of JNK and ERK activation on CdSe-core QD-induced apoptosis. (A–C) IMR-32 cells were preincubated with various concentrations of SP600125 at 37 °C for 1 h and then treated with 300 nM CdSe-core QDs (CdSe) for another 24 h. (A) Cell extracts (60 μg) were prepared and immunoblotted with anti-p-JNK1 and anti-JNK1 antibodies. (B) Mitochondrial membrane potential changes were measured with DiOC6(3) (40 nM) or TMRE (1 μM), followed by spectrofluorometry. (C) Apoptosis was detected with the Cell Death Detection ELISA kit. (D and E) IMR-32 cells were preincubated with or without 10 μM U0126 for 1 h and then treated with 300 nM ZnS-coated QDs (ZnS) or CdSe-core QDs (CdSe) for another 24 h. The phosphorylation levels of ERK-1 and -2 were evaluated with immunoblotting using an anti-pMAPK antibody (D) and apoptosis was detected with the Cell Death Detection ELISA kit (E). Values are presented as means ± S.D. of five determinations. $^{**}P < 0.01$ and $^{***}P < 0.001$ vs. the value of the "CdSe-core QD-treated only" group.

ing CdSe-core QD-induced apoptosis, we examined the effect of the specific JNK inhibitor, SP600125, on CdSe-core QD-treated IMR-32 cells. As shown in Fig. 6A, pretreatment with SP600125 reduced CdSe-core QD-stimulated JNK activity in a dose-dependent manner, but had no effect on JNK protein levels. We also found that inhibition of JNK activity by SP600125 significantly prevented mitochondrial membrane potential losses (Fig. 6B) and apoptosis (Fig. 6C) in CdSe-core QD-treated IMR-32 cells. These findings indicate that JNK activity is required for loss of mitochondrial membrane potential and subsequent apoptotic biochemical changes during CdSe-core QD-induced apoptosis. Moreover, pretreatment of cells with U0126, a specific inhibitor of MEK1 (an upstream activator of ERK-1/2), enhanced CdSe-core QD-induced ERK-1/2 inactivation (reflected as decreased phospho-ERK-1/2) (Fig. 6D). Importantly, increased apoptosis was correlated with decreased ERK-1/2 activity in treated cells (Fig. 6D and E). Taken together, these findings indicate that CdSe-core QDs induce apoptosis in IMR-32 cells through JNK activation, mitochondrial-dependent apoptotic processes and inhibition of survival signaling components such as ERK.

4. Discussion

A recent report showed that Cd could induce ROS generation (i.e. oxidative stress) and trigger apoptosis via a caspase-dependent pathway (Oh and Lim, 2006). However, the mechanisms of Cd-induced apoptosis, cell cytotoxicity and carcinogenesis remain unclear. Recent studies have showed that CdSe-core QDs could induce cell death under certain conditions and that the cytotoxicity of CdSe-core QDs was correlated with the release of free Cd^{2+} from the CdSe lattice. However, these effects could be significantly reduced by the addition of a ZnS coating (Derfus et al., 2004). Photoluminescent semiconductor QDs have been used as novel nanometer-size probes for bioimaging of immunostained cells (Goldman

et al., 2002) and fluorescent QD probes were useful as bioimaging tools for tracing target cells over the course of a week in a mouse model (Hoshino et al., 2004). In the future, fluorescent QD probes might be developed as biotracers for application in human disease diagnosis. Here, we showed that CdSe-core QDs could induce apoptosis and ROS generation in IMR-32 cells, but that a ZnS coating could effectively reduce this cytotoxicity (Figs. 1 and 2). Thus, it is noteworthy that our results suggest that QDs are likely to have latent cytotoxicity if their coatings are destroyed in vivo.

Previous studies have shown that mitochondria act as important signaling conduits during programmed cell death and that loss of mitochondrial integrity can be promoted or inhibited by many key regulators of apoptosis (Green and Reed, 1998; Kroemer et al., 1997). To further elucidate the mechanisms underlying CdSe-core QD-induced apoptosis, we examined mitochondrial membrane potentials and mitochondrial release of cytochrome *c*. We found that CdSe-core QDs concentration-dependently induced loss of mitochondrial membrane potential and mitochondrial release of cytochrome *c* (Fig. 4A and B).

JNK plays roles in many cell responses, including entry into apoptosis. We previously showed that JNK activation is an important trigger for cytochrome *c* release and subsequent activation of caspases in UV irradiation-, photodynamic treatment- and methylglyoxal-induced apoptosis (Chan et al., 2003; Chan and Wu, 2004; Hsuuw et al., 2005). Previous studies demonstrated that ROS is a upstream regulator for JNK activation, which is an important event for downstream apoptotic processes, such as mitochondrial membrane potential changes, cytochrome *c* release and caspase activation (Chan et al., 2003; Chan and Wu, 2004; Hsuuw et al., 2005). In the present study, we used ROS scavengers and a specific JNK inhibitor to show that ROS play important roles in CdSe QD-induced apoptosis and further showed that JNK is an upstream regulator for this mitochondria-dependent apoptotic pathway (Figs. 2 and 6). Based on previous reports and the results of this study, we conclude that CdSe-core QD treatment-induced apoptosis appears to occur through ROS generation, JNK activation and mitochondrial-dependent processes.

Heat shock proteins (HSP) can protect proteins from proteasome- and ubiquitin-dependent degradation (French et al., 2001; Leszczynski et al., 2002). HSP90, the major and most abundant molecular chaperone protein in the intracellular system, is involved in maintaining the correct conformation of intracellular proteins and kinases, such as Raf-1 (Mayer and Bukau, 1999), which is involved in regulating cell proliferation and survival. Here, we found that CdSe-core QD-induced apoptosis was associated with reduced protein expression of HSP90 and its downstream targets, Ras, Raf-1, ERK-1 and ERK-2 (Fig. 5A–F). These findings may indicate that a CdSe-core QD-induced decreases in HSP90 expression lead to increased proteasome-dependent degradation of Ras and Raf-1, with decreased Raf-1 levels resulting in subsequent downregulation of ERK-1 and ERK-2.

In sum, we herein showed that CdSe-core QDs induced apoptosis through ROS-, JNK-, caspase-9- and caspase-3-mediated apoptotic pathways in IMR-32 cells, with attendant downregulation of survival signaling molecules such as HSP90, Ras, Raf-1 and ERK-1/2. In contrast, these apoptotic biochemical events were not detected in cells treated with ZnS-coated CdSe QDs. This is the first report that QD treatment can induce apoptotic signaling cascades and affect survival signaling components, providing important new insights into potential safety risks for the use of QD labeling in vivo.

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