CdSe Quantum Dots Induce Apoptosis via Activation of JNK and PAK2 in a Human Osteoblast Cell Line

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Abstract

Quantum dots (QDs) have been proposed as novel luminescent markers for research applications, but a recent study showed that oxidation of CdSe-core QDs could release cadmium ions and trigger cell death. Here, we sought to elucidate the precise apoptotic mechanisms governing this effect. Our results revealed that CdSe-core QDs induced apoptotic biochemical changes, including activation of c-Jun N-terminal kinase (JNK), caspase-3 and p21-activated protein kinase 2 (PAK2), in a human osteoblast cell line. Treatment of osteoblasts with a JNK-specific inhibitor (SP600125) reduced CdSe-core QD-induced activation of both JNK and caspase-3, indicating that JNK activity is required for CdSe QD-induced caspase activation. Experiments using caspase-3 inhibitors and antisense oligonucleotides against PAK2 showed that caspase-3 activation is required for PAK2 activation, and both of these activations are required for CdSe QD-induced apoptosis in osteoblasts. Interestingly, ZnS surface-modified CdSe QDs were not cytotoxic to osteoblasts at any tested concentration. These findings provide important new insights into the apoptotic mechanisms triggered by CdSe QDs in a human osteoblast cell line, and suggest that surface modification might be a useful strategy for minimizing the cytotoxicity of CdSe QDs for research applications.

Keywords: Quantum dots, Apoptosis, PAK2, JNK

Introduction

Quantum dots (QDs), colloidal nanocrystalline semi-conductors, 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 [1]. 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. As luminescent probes, QDs have the benefits 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 for certain cell biological assays that are currently limited by the specific excitation frequencies and broad emission spectra of conventional organic fluorescent probes. However, there may be some issues with cytotoxicity, as indicated by a previous report that CdSe-core QDs, which are commonly used for biological imaging analysis, could induce apoptosis in rat hepatocytes [2].

Apoptosis is a unique morphological pattern of cell death characterized by chromatin condensation, internucleosomal DNA cleavage, membrane blebbing and cell fragmentation [3,4]. Although the precise molecular mechanisms governing apoptosis have not been clearly defined, a number of apoptosis-associated signaling molecules have been elucidated. For example, cysteine proteases called caspases are thought to play important roles in apoptosis [5,6]; caspase zymogens are activated by proteolysis, and may be inhibited in vitro and in vivo by small tetrapeptidic inhibitors [6,7]. Members of the Bcl-2 family also play important roles in regulating apoptosis [8]; the Bcl-2 family proteins, which can be divided into anti-apoptotic and pro-apoptotic subgroups [8,9], regulate the release of mitochondrial cytochrome C by modulating the permeability of the outer mitochondrial membrane. Finally, changes in protein kinase activity can be observed during apoptosis in a variety of cell types [10], indicating that protein phosphorylation is likely to be involved in the regulation of apoptosis.

Among the protein kinases, c-Jun N-terminal kinase (JNK) is known to regulate entry into apoptosis in several cell types [11-13]. In addition, p21-activated protein kinase (PAK) is activated during apoptosis and may be involved in apoptotic signaling events [14-18]. Three isoforms of PAK (called α-, β- and γ-PAK, or alternatively termed PAK1, -3 and -2, respectively) have been identified in mammalian tissues. All three are similar in sequence and comprise a p21 binding site-containing an N-terminal regulatory region and a C-terminal kinase domain [19]. PAK can be activated in vitro
by Cdc42/Rac-mediated autophosphorylation or by proteolytic removal of its N-terminal regulatory region [20-22], followed by autophosphorylation/autoactivation of the free C-terminal fragment at a known phosphorylation site [21,23]. Although the direct downstream substrates of the PAKs are largely unknown, recent findings have suggested that PAK may act as an upstream regulator of the JNK and p38 MAPK pathways. For example, recombinant JNK can be activated in Xenopus oocyte lysates by addition of constitutively active PAK1 [24], and overexpression of PAK1 or 2 was shown to activate JNK and p38 MAPK in COS-7 and HeLa cells [25,26]. In addition, we recently reported that activation of PAK2 is required for the photodynamic treatment (PDT)-induced apoptosis of epidermal carcinoma A431 cells [18]. However, while numerous reports have demonstrated that PAK2 plays an important role in apoptotic signaling, the direct downstream substrates and precise regulatory mechanisms governing this signaling pathway remain unknown.

In an effort to gain new insight into the mechanisms involved in CdSe QD-induced apoptosis, we examined JNK- and PAK2-mediated signaling in CdSe QD-treated osteoblasts. Our results revealed that PAK2 activation, which is necessary for CdSe QD-induced apoptosis, is mediated via JNK- and caspase-dependent signaling in CdSe QD-treated osteoblasts. In addition, we demonstrated that a surface coating of ZnS effectively decreased the cytotoxicity of CdSe QDs. These findings provide important new insights into the apoptotic mechanisms triggered by oxidation of CdSe QDs in a human osteoblast cell line, and suggest that surface modification might be a useful strategy for minimizing the cytotoxicity of CdSe QDs.

**Materials and methods**

[γ-32P]ATP was purchased from Amersham (Buckinghamshire, UK). Dulbecco's modified Eagle's medium (DMEM) was obtained from Sigma (St. Louis, MO). Z-DEVD-AFC was purchased Calbiochem (La Jolla, CA). Anti-JNK1 (C17) antibody was bought from Santa Cruz Biotechnology (Santa Cruz, CA). The BCA protein assay reagent was a product of Pierce (Rockford, IL). Protein A Sepharose CL-4B was purchased from Pharmacia (Uppsala, Sweden). Myelin basic protein (MBP) was purified from porcine brain as previously described [27].

**CdSe QD preparation and surface modification**

Nanocrystals comprised of a CdSe core and a ZnS shell were synthesized by Prof. Lu and coworkers 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 dimethylzinc 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 mercaptopetacetic acid (MAMA) and then suspended in PBS buffer (the modification was performed by Prof. Ruaan and coworkers at the Department of Chemical and Materials Engineering, National Central University, ROC). A particle sizer was used to measure the CdSe QDs, which were found to be about 3.5 nm in diameter.

**Cell culture and CdSe QD treatment of cells**

Human osteoblasts were cultured at 34°C in a humid 95% air/5% CO2 atmosphere in DMEM/F12 medium supplemented with 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine and 0.3 mg/l G418. Cells (5-6 × 10^5) were plated on 60 mm culture dishes and incubated in medium containing various concentrations of CdSe QDs for 24 h. The cells were then washed twice with ice-cold PBS and lysed in 600 µl of lysis solution (20 mM Tris-HCl, pH 7.4, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 1 mM benzamidine, 1 mM phenylmethylsulfonyl fluoride, 50 mM NaF, 20 mM sodium pyrophosphate and 1 mM sodium orthovanadate) on ice for 10 min. The cell lysates were collected, sonicated on ice for 3 x 10 sec, and then centrifuged at 15,000 x g for 20 min at 4°C. The resulting supernatants were used as the cell extracts.

**MTT assay**

Cell survival was assayed using the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) colorimetric test. CdSe QD-treated cells or untreated controls were incubated at 37°C for 60 min with 100 µl/well of MTT solution (0.45 g/l), and then incubated overnight at 37°C with 100 µl/well of 20% SDS in DMF:H2O (1:1 solution). The formazan products were solubilized, and spectrophotometric data were obtained using an ELISA reader at a wavelength of 570 nm.

**TUNEL apoptosis assay**

Oligonucleosomal DNA fragmentation in apoptotic cells was measured using the Cell Death Detection ELISAplus 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, and spectrophotometric data were obtained using an ELISA reader at a wavelength of 405 nm.

**Caspase-3 activity assay**

Caspase-3 activity was measured with the fluorogenic substrate, Z-DEVD-AFC. Cell lysates were prepared from CdSe QD-treated and untreated osteoblasts, and 100 µg total cellular proteins were incubated with 0.1 mM Z-DEVD-AFC in 250 µl of caspase assay buffer [25 mM HEPES, pH 7.5, 0.1% CHAPS, 10 mM dithiothreitol (DTT) and 100 units/ml aprotinin] for 3 h at 37°C. Ice-cold caspase assay buffer (1.25
ml) was then added to the mixture and the relative caspase-3 activity was determined by fluorescence spectrophotometry (excitation 400 nm, emission 505 nm; F-2000; Hitachi, Japan).

**JNK assays**

JNK activity, as assayed by the presence of phosphorylated c-Jun protein, was analyzed with an 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. The phospho-c-Jun was assayed using a phospho-c-Jun primary antibody and a secondary horseradish peroxidase-conjugated antibody in a colorimetric reaction.

**Production of antibodies**

The anti-PAK2 (C15) antibody was produced in rabbits using the antigen peptide, TPLIMAAKEAMKSNR, which corresponds to C-terminal residues 510-524 of the human and rabbit PAK2 sequences [22,28]. The peptide was synthesized by Genosys Biotechnologies. A cysteine residue was added to the N-terminus to facilitate coupling of the peptide to keyhole limpet hemocyanin, as previously described [29]. Glutaraldehyde was used as the cross-linker. The anti-peptide antibody was produced and affinity purified as previously described [30].

**Immunoprecipitation and PAK2 activity assay**

Cell extracts were first diluted to equal protein concentrations with cell lysis solution. For immunoprecipitation of the C-terminal catalytic fragment of PAK2, 0.5 ml of cell extract (1.0 mg/ml) was incubated with 10 µl of anti-PAK2 (C15) antibody (200 µg/ml) at 4°C for 1.5 h, and then with 40 µl of Protein A-Sepharose CL-4B (30% v/v; Pharmacia, USA) for another 1.5 h with shaking. The immunoprecipitates were collected by centrifugation, washed three times with 1 ml of solution A (20 mM Tris/HCl, pH 7.0, and 0.5 mM DTT) containing 0.5 M NaCl, and resuspended in 40 µl of solution A. For measurement of immunoprecipitated PAK2 activity, the immunoprecipitates were incubated in a 50 µl mixture containing 20 mM Tris/HCl, pH 7.0, 0.5 mM DTT, 0.2 mM [γ-32P]ATP, 20 mM MgCl2, and 0.1 mg/ml myelin basic protein (MBP) at room temperature for 10 min with shaking. To determine the incorporation of 32P into MBP proteins, 20 µl of the reaction mixture was spotted onto Whatman P81 paper (1 X 2 cm; Whatman), the paper was exposed to 75 mM phosphoric acid, and samples were processed as previously described [31].

**Inhibition of PAK2 by antisense oligonucleotides**

PAK2 sense (5'-ATC ATG TCT GAT AAC GGA GAA) and antisense (5'-TTC TC C G TT A TC AGA CAT GA T) oligonucleotides were obtained from Life Technologies (Grand Island, NY). These sequences represent codons -1 to +7 of human PAK2. The oligonucleotides were synthesized under phosphorothioate-modified conditions, purified by HPLC, dissolved in 30 mM HEPES buffer (pH 7.0) and transfected into cells using Lipofectamine 4 (Life Technologies). Briefly, cells grown in 60 mm culture dishes were incubated at 37°C in 1 ml of Opti-MEM I medium (modified Eagle's minimum essential medium buffered with HEPES and sodium bicarbonate; Life Technologies) containing Lipofectamine 4 (12 µg) and oligonucleotides (70 µM) for 72 h. Cells were then exposed to CdSe QDs for 24 h and cell extracts were analyzed by the immunoprecipitation kinase assay and the TUNEL apoptotic assay, as described above.

**Statistics**

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

**Results**

**JNK, caspase-3 and PAK2 are activated during CdSe QD-induced apoptosis in human osteoblasts**

MTT assays revealed that the viability of CdSe QD-treated osteoblasts was approximately 50-60% lower than that in untreated controls (Fig. 1A), while TUNEL assays revealed that CdSe QD treatment induced a 2.5-fold increase in nucleosomal fragmentation (an apoptosis-associated parameter) as compared to untreated cells (Fig. 1B). These findings confirm that CdSe QDs can induce apoptosis in osteoblasts, and indicate that our system may be used to assess apoptotic signaling during CdSe QD-induced apoptosis.
Figure 2. Activation of JNK, caspase-3 and PAK2 in CdSe QD-treated osteoblasts. Osteoblasts were incubated with various concentrations of CdSe QDs (CdSe) or ZnS-coated CdSe QDs (ZnS) for 24 h. (a) JNK/AP-1 activity was evaluated by ELISA detection of phosphorylated c-Jun. The results are expressed in relation to control values, which were arbitrarily set to 1.00. (b) Cell extracts (60 µg) were analyzed for caspase-3 activity using Z-DEVD-AFC as the substrate. (c) The C-terminal catalytic fragment of PAK2 was immunoprecipitated and kinase activities were assayed using myelin basic protein (MBP) as the substrate. Values are presented as means ± SD of three to five determinations. * ** P < 0.001 versus the untreated control group.

Since activation of the JNK pathway is essential for induction of apoptosis in some cell types [11,12], and we previously demonstrated that JNK activity mediates apoptosis induced by environmental stress and various chemical stimuli [32-34], we used immunoblotting and ELISA to examine JNK activation during CdSe QD-induced apoptosis. Our results revealed that JNK was dose-dependently activated in CdSe QD-treated osteoblasts (Fig. 2A). To further investigate apoptotic signaling during CdSe QD-induced apoptosis, we used an in vitro ELISA assay to monitor the activation levels of caspase-3, which is activated during apoptosis of multiple cell types triggered by a variety of apoptotic stimuli [35,36]. Our results demonstrated that treatment of osteoblasts with CdSe QDs stimulated the activation of caspase-3 (Fig. 2B). Finally, since previous studies have shown that the activation of PAK2 is relevant to apoptosis [18], we investigated whether PAK2 was involved in CdSe QD-induced apoptosis. Immunoprecipitation assays revealed that treatment of osteoblasts with 250 and 500 nM CdSe QDs activated PAK2 by ~21-37-fold versus untreated controls (Fig. 2C). Collectively, these results indicate that JNK, caspase-3 and PAK2 are activated in CdSe QD-treated osteoblasts.

**JNK activation is required for caspase-3 activation and apoptosis in CdSe QD-treated osteoblasts**
To determine the relationship between JNK activity and that of caspase-3 during CdSe QD-induced apoptosis, we examined the effect of the specific JNK inhibitor, SP600125, in this system [37]. As shown in Figure 3A, pretreatment with SP600125 dose-dependently reduced CdSe QD-stimulated JNK activity, with 20 µM SP600125 inhibiting CdSe QD-stimulated JNK activity by ~50%. We further found that inhibition of JNK activity by SP600125 significantly reduced caspase-3 activation and apoptosis, with 20 µM SP600125 inhibiting these effects by ~57% (Fig. 3B) and ~77% (Fig. 3C), respectively. These findings indicate that caspase-3 activation and apoptosis are downstream of the critical JNK activation step during CdSe QD-induced apoptosis of osteoblasts.

**Caspase-3 activation is required for PAK2 activation in CdSe QD-treated osteoblasts**

To elucidate the relationship between caspase-3 activation and that of PAK2 during CdSe QD-induced apoptosis, we examined the effects of the specific tetrapeptidic caspase inhibitors, Ac-DEVD-cho or Ac-YVAD-cmk [16,18], on CdSe QD-treated osteoblasts. Our results revealed that CdSe QD-induced activation of caspase-3 was markedly inhibited by pretreatment with either of the tested inhibitors (Fig. 4A). Furthermore, pretreatment with the caspase inhibitors blocked CdSe QD-induced activation of PAK2 (~30-36% versus cells treated with QDs alone) (Fig. 4B), indicating that caspase-3 functions upstream of PAK2 activation during CdSe QD-induced apoptosis.

**PAK2 activation is required for CdSe QD-induced apoptosis in osteoblasts**

We then investigated the role of PAK2 activation in CdSe QD-induced apoptosis by pre-incubating the cells with antisense oligonucleotides targeting PAK2. This led to a significant decrease (~60%) in CdSe QD-induced PAK2 activation (Fig. 5A). No such change was observed in caspase-3 activation (Fig. 5B), confirming that caspase-3 is upstream of PAK2 in this signaling pathway. Notably, PAK2 knockdown was associated with a significant decrease in CdSe QD-induced apoptosis (~24% of that seen in cells treated with QDs alone) (Fig. 5C), demonstrating that PAK2 activation is directly involved in CdSe QD-induced apoptosis in osteoblasts.

**ZnS coating decreases the cytotoxic effects of CdSe QDs on osteoblasts**

Lastly, we tested whether ZnS coating of the CdSe QDs might ameliorate some or all of their cytotoxic effects on osteoblasts. MTT assays revealed that ZnS-coated CdSe QDs had no significant effect on the viability of human osteoblasts (Fig. 1A), while TUNEL assays failed to reveal any significant increase in nucleosomal fragmentation in ZnS-coated CdSe QD-treated cells versus untreated controls (Fig. 1B). Furthermore, we did not observe any significant activation of JNK (Fig. 2A), caspase-3 (Fig. 2B) or PAK2 (Fig. 2C) in ZnS-coated QD-treated cells. Collectively, these findings indicate that the addition of a ZnS coating to CdSe QDs seems to decrease their cytotoxicity in human osteoblasts.

**Discussion**

In the present study, we showed that CdSe QDs could induce apoptosis in a human osteoblast cell line via activation of JNK and caspase-3, and report for the first time that PAK2 is involved in this effect. Furthermore, we suggest a possible apoptotic signaling pathway, and provide additional evidence that a ZnS coating can effectively reduce CdSe QD-induced cytotoxicity.

In our examination of CdSe QD-induced apoptotic signaling, we began by assessing the involvement of JNK, which is known to play roles in many cell responses, including entry into apoptosis [32-34]. Co-treatment of osteoblasts with CdSe QDs and SP600125, a JNK-specific inhibitor, demonstrated that JNK activity mediates CdSe QD-induced caspase-3 activation and apoptosis in osteoblasts (Fig. 3). We also investigated the involvement of another kinase, PAK2.

PAK2 has been demonstrated to involve in several cell regulations, including apoptosis and cell cycle control. For example, microinjection of active PAK into early frog embryos causes cleavage arrest [43,44], and endogenous Xenopus PAK2 has been identified as a key enzyme in the negative regulation of M phase promoting factor (MPF) during G2/M.
antisense techniques to show for the first time that PAK2 activation is a critical mediator for CdSe QD-induced apoptosis (Fig. 5). Our finding may explain the previous observation that apoptosis is delayed by transfection of dominant-negative PAK2 (either full-length or an N-terminally truncated form) into CHO cells stably expressing a CD4-Fas chimera [15], since the dominant-negative PAK2 could interfere with activation of the JNK/SAPK pathway required for caspase activation in these cells.

Various studies have indicated that PAK2 lies upstream of JNK in some signaling pathways. For example, transfection of cultured cells with vectors encoding constitutively activated Rac and Cdc42 (both activators of PAK2) triggered potent activation of JNK [48], while another study showed that transfection of cultured cells with N-terminal truncated PAK2 or constitutively active PAK1 led to JNK activation [15]. Cells transfected with this N-terminal truncated PAK2 expressed a constitutively active version of PAK2 (c-terminal kinase fragmentation; 36 kD), due to autophosphorylation/autoactivation [15]. In contrast to these previous findings, our present results show that PAK2 functions downstream of JNK in CdSe QD-induced apoptosis (Fig. 3). Future work will be required to fully define the relationship(s) between PAK2 and JNK during apoptosis and other biological processes.

Previous reports have indicated that inhibition of ROS generation and JNK activation could prevent apoptotic biochemical changes induced by UV irradiation or photodynamic treatment. These studies further showed that UV irradiation- or photodynamic treatment-induced ROS generation and JNK activation were both important triggers for activating caspase-3 and subsequent apoptotic biochemical changes (32, 33). In the present study, we show that JNK activation is an important trigger for caspase-3 activation and subsequent activation of PAK2, and that all three of these activations are essential for CdSe QD-induced apoptosis in a human osteoblast cell line. We propose that our present results may be combined with the previous findings to suggest a model for CdSe QD-triggered apoptotic signaling as followed: CdSe QDs → oxidation → Cd$^{2+}$ release → ROS generation → JNK activation → caspase-3 activation → PAK2 cleavage/activation → cell apoptosis.

Lastly, we examined the ability of a ZnS coating to potentially reduce Cd-induced cytotoxicity, which has previously been associated with inflammation, fibrosis, organ dysfunction [38,39] and the development of various cancers [40] in vivo, as well as caspase-dependent apoptosis in Hep G2 cells in vitro [41]. The cytotoxicity of CdSe QD treatment has been correlated with the release of free Cd$^{2+}$ from the CdSe lattice, which appears to be associated with surface oxidation [2]. However, a previous report showed that these effects could be significantly reduced by the addition of a ZnS coating [2]. Consistent with this finding, the results of our present study also demonstrated that a ZnS coating could effectively reduce CdSe QD-induced cytotoxicity. We propose that the ZnS coating may prevent CdSe QD-induced cell death and cytotoxicity by blocking surface oxidation and the release of Cd$^{2+}$ ions. However, future studies will be required to assess...
this possibility in detail.

In sum, we herein show CdSe QDs could induce cell apoptosis through ROS generation and activation of JNK, caspase-3, and PAK2. These new insights into CdSe QD-induced apoptotic signaling, along with our confirmation that ZnS surface modification decreases the cytotoxicity of CdSe QDs in our experimental system, provide new insight into CdSe QD-mediated cytotoxicity and may facilitate new improvements in CdSe QDs and their application as a novel biomarker.

Acknowledgments
We thank Dr. Chuan-Hsin Lu (Department of Chemical Engineering, National Taiwan University, ROC) and Dr. Ruoh-Chyu Ruan (Department of Chemical and Materials Engineering, National Central University, ROC) for providing the modified CdSe quantum dots. This work was supported by a grant (NSC 94-2120-M-033-001) from the National Science Council of Taiwan, ROC.

References
[31] E. M. Reimann, D. A. Walsh, and E. G. Krebs, “Purification and


