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Development of a Growth-Hormone-Conjugated Nanodiamond Complex for Cancer Therapy

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It is highly desirable to develop a therapeutic, observable nanoparticle complex for specific targeting in cancer therapy. Growth hormone (GH) and its antagonists have been explored as cancer cell-targeting molecules for both imaging and therapeutic applications. In this study, a low toxicity, biocompatible, therapeutic, and observable GH–nanoparticle complex for specifically targeting growth hormone receptor (GHR) in cancer cells was synthesized by conjugating GH with green fluorescence protein and carboxylated nanodiamond. Moreover, we have shown that this complex can be triggered by laser irradiation to create a "nanoblast" and induce cell death in the A549 non-small-cell lung cancer cell line via the apoptotic pathway. This laser-mediated, cancer-targeting platform can be widely used in cancer therapy.

Introduction

Lung cancer is the leading cause of cancer deaths in men and women in America,[1] with a 5-year relative survival rate of 15.7%. Lung cancer can be classified into small cell lung cancer (SCLC) and non-small-cell lung cancer (NSCLC).[2] Traditional treatments for lung cancer include surgery, radiation, and chemotherapy, which are usually used to treat patients with late-stage lung cancer.[3] Compared with SCLC, NSCLC is quite insensitive to chemotherapy and radiation therapy.

The growth hormone receptor (GHR) is overexpressed in several cancer cell lines, including the A549 NSCLC cell line,[4] the T47D breast cancer cell line,[5] the COLO205 colon cancer cell line,[6] and the DU145 prostate cancer cell line.[7] Therefore, GHR can be used as a potential therapeutic target in cancer. Growth hormone (GH) is the ligand for GHR. In several types of cancer, expression levels of GH are associated with development and progression of the disease.[5,8] For example, an elevated GH level in various mammary carcinoma cells has been shown to increase cell proliferation, mediate anti-apoptotic effects, and affect the invasion phenotype of the cells.[9,10] Furthermore, GH is involved in the proliferation, differentiation, survival, invasion, and metastasis of cancer cells.[11–13] Accordingly, blocking the GH/GHR pathway may reduce the proliferation of cancer cells and cancer progression. Therefore, GH antagonists have been developed for therapeutic application in cancers such as breast cancer[14] and colorectal cancer.[9] While heterogeneous GH (from a different species), such as fish GH, may also be recognized by human GHR, it may have limited functional activity.[4] Therefore, antagonists of native, human GH may be preferable for specifically targeting cancer cells and reducing side effects.

Nanoparticles have been intensely studied for their potential therapeutic and imaging applications in the clinic.[15,16] A recent study has indicated that nanoparticles conjugated with chemotherapeutic drugs may improve cancer therapeutics by overcoming drug efflux and increasing apoptosis in cancer cells.[17] Nanomaterials such as carbon nanotubes,[18] nanoliposomes,[19] and nanodiamonds (NDs)[3] have been successfully conjugated with anticancer drugs. In particular, apolipoprotein B lipoparticles[20] and NDs have been applied as nanovehicles for the delivery of therapeutic agents.[17] These nanodelivery systems could enhance the therapeutic efficiency and targeting ability of drugs.

Unlike most other nanoparticles, which typically have low biocompatibility, NDs have proven to be highly biocompatible and non-cytotoxic.[21–23] Furthermore, the trace nitroso groups

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(-C-N=O) in NDs can be photolyzed by the multi-photon effect to release nitrogen monoxide (NO), creating high internal pressure and inducing a "nanoblast".[24] In addition, the surface of NDs can be modified with carboxyl groups to facilitate conjugation of proteins with high affinity.[22,25] These characteristics indicate that ND materials are ideal for biomedical applications involving protein conjugation.

We developed a protein-targeting ND complex containing a protein-indicating fluorophore by conjugating carboxylated ND (cND) with recombinant GH (rGH) and green fluorescence protein (GFP). The ND complex bound to the surface membrane of A549 NSCLC cells, and laser irradiation of the complex induced cell death via the apoptotic pathway. On the contrary, the treatment had no adverse effect on the survival of the HFL-1 lung cell line, which does not express GHR. Thus, we have developed a safe, effective, and traceable nanosurgery platform that is inducible by laser irradiation for therapeutic application in NSCLC cancer.

Results

Physical characterization of the ND complex

As particle properties such as surface modifications and size can affect the bulk properties of diamonds, the nanosize (2–10 nm) of ND powders can contribute to better abrasive pastes and suspensions for high-precision polishing than those of micro- and macroscale diamonds.[26] The cND used in this study was 5 nm in diameter. The conjugation procedure is illustrated in Scheme 1.

After conjugation with rGH and GFP, the size of the ND complex was approximately 9.3±0.16 nm in diameter, as determined by atomic force microscopy (AFM; Figure 1 a) and dynamic light scattering (DLS; Figure 1 b). As illustrated in Figure 1 c, analysis by fluorescent microscopy showed co-localization of GFP with cND particles, suggesting that GFP and cND were linked.

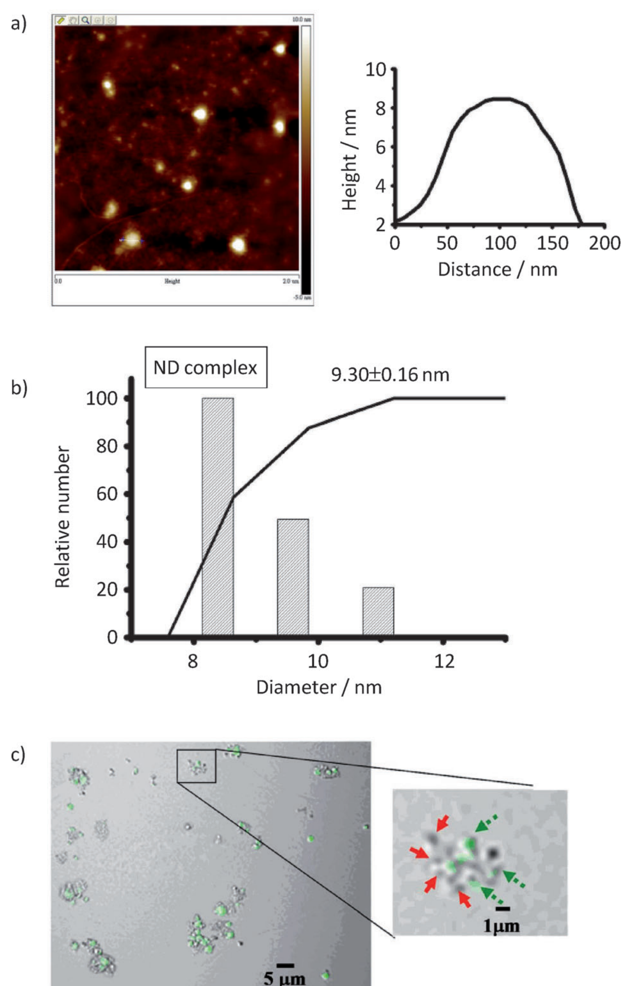
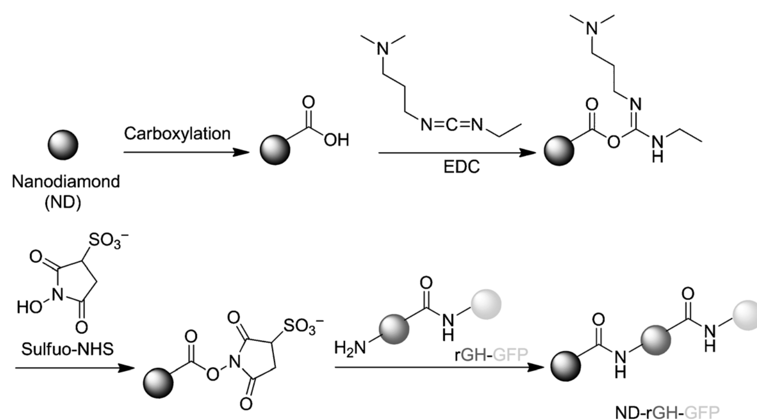


Figure 1. The shape and particle size of the nanodiamond (ND) complex were measured by a) atomic force microscopy and b) dynamic light scattering. c) Observation of the ND complex by fluorescence microscopy. Carboxylated ND particles were detected under bright field microscopy (red arrows), and green fluorescence protein (GFP) was detected by an FITC fluorescence filter (green arrows).



Scheme 1. Illustration of the process for synthesizing the nanodiamond (ND) complex via conjugation of carboxylated ND (cND), green fluorescence protein (GFP), and recombinant growth hormone (rGH) with *N*-hydroxysulfosuccinimide (sulfo-NHS). The well-established EDC/sulfo-NHS zero-length crosslinking system was applied for oriented ND production. The semi-stable, amine-reactive NHS-ester of cND was synthesized to conjugate with oriented rGH-GFP conjugated protein. An ND-complex of the ND-rGH-GFP arrangement was produced for testing. Details of the series of conjugation procedures are described in the Experimental Section.

Biocompatibility testing of the ND complex

To evaluate the suitability of the ND complex for biomedical applications, the cytotoxicity and effect on cell proliferation of the ND complex and its components (cND, rGH, and GFP) were quantitatively assessed by the MTT assay. Only high concentrations of cND ($10\ \mu g\,mL^{-1}$) and ND complex ($10^3\ \mu g\,mL^{-1}$) caused approximately 20% cell toxicity after 24 h of treatment (Figure 2 a,d, respectively). The rGH and GFP were highly biocompatible with A549 cells, even at high doses of treatment (Figure 2 b,c, respectively). In addition, the ND complex and its components did not stimulate cell proliferation in A549 cells. Accordingly, the ND complex (at less than $10^2\ \mu g\,mL^{-1}$) is a biocompatible material and may be used in bio-

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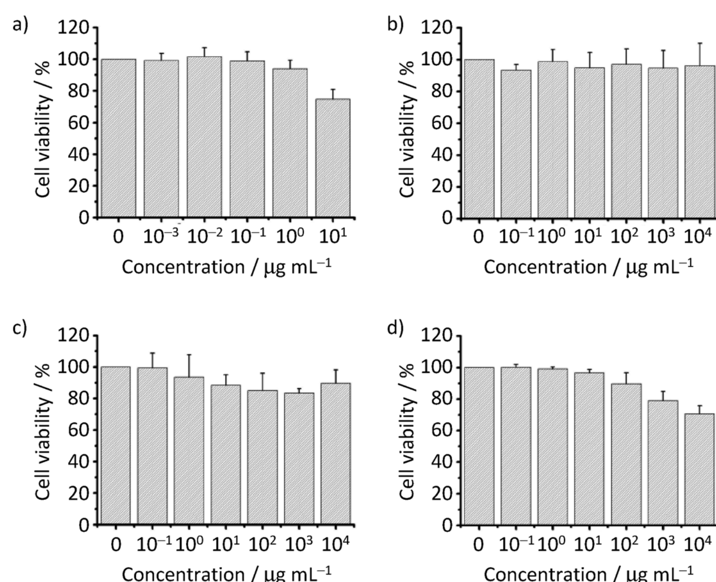

Figure 2. The cytotoxicity of a) 5 nm carboxylated nanodiamond (cND), b) recombinant growth hormone (rGH), c) green fluorescence protein (GFP), and d) the nanodiamond (ND) complex was tested by an MTT assay using the A549 non-small-cell lung cancer cell line at various concentrations (0–10 $\mu g\,mL^{-1}$ for 5 nm cND; and 0–10^4 $\mu g\,mL^{-1}$ for rGH, GFP, and the ND complex).

medical applications. Based on these results, we chose 1 $\mu g\,mL^{-1}$ as the final concentration of the ND complex in subsequent experiments.

Localization of the ND complex in cancer cells

GHR is an integral membrane protein[27] expressed in various cancer cells.[5–7] Confocal microscopy images of A549 cells treated with ND complex indicated that the ND complex could be observed on the cell surface (Figure 3 a). Moreover, three-dimensional reconstitution of the images indicated that the ND complex was localizing on the cell membrane and not entering the cells, corresponding to the location of GHR (Figure 3 b).

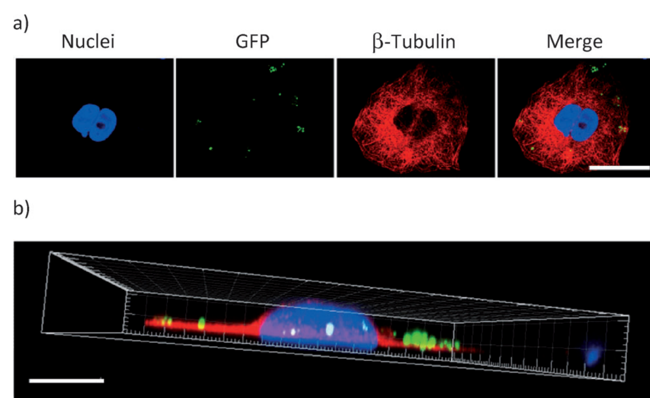

Figure 3. a) Confocal fluorescence microscopy image of A549 non-small-cell lung cancer cells treated with the nanodiamond (ND) complex. b) Three-dimensional image constructed from confocal images. Nuclei were stained with Hoechst 33342 (blue), β-tubulin was stained with a Cy3-conjugated antibody (red), and green fluorescence protein (GFP) was detected by a FITC fluorescence filter (green). The scale bar is 30 µm in a) and 15 µm in b).

Cell death mediated by ND complex and laser irradiation

It has previously been determined that irradiation with a UV-C laser at 266 nm induces the death of A549 cells death directly.[28] In contrast with the complete cell damage induced by 266 nm laser treatment (Figure 4 a), the ND complex induced selective damage after laser irradiation at 532 nm (Figure 4 b). The viability of the A549 cells was significantly reduced ($P < 0.001$) to 20% (Figure 4 c) by 532 nm laser irradiation. However, the viability was also slightly reduced for cells exposed to the same laser treatment but not to the ND complex (Figure 4 b, left panel, and Figure 4 c). Cell morphology analysis by scanning electron microscopy indicated that in the A549 cells treated with ND complex but no laser irradiation, the cell membrane was intact (Figure 4 d), whereas after 532 nm laser irradiation, the cell membrane was disrupted and collapsed (Figure 4 e). It has been proposed that ND complex-mediated cell death might be attributed to the two-photon absorption effect of ND.[24]

GHR expression-dependent cell death induced by laser and ND complex treatment

Previous studies have indicated that GHR may be considered a specific target for therapeutic application in several cancers.[9,14] Therefore, we tested the specific binding ability of the ND complex and GHR, located on the cell membrane. As indicated in Figure 5 a, the HFL-1 normal human lung fibroblast cell line showed no GHR expression by Western blot analysis. To avoid hyperthermia damage by 532 nm laser irradiation, as shown in Figure 4 c, we reduced the laser power from 30 mW to 20 mW. Under these conditions, the viability of A549 cells was still reduced after treatment with the ND complex and laser irradiation (Figure 5 b). However, the viability of HFL-1 cells was not affected by these same conditions. These results suggest that the ND complex can specifically target cancer cells expressing GHR and trigger cell death when induced by laser irradiation.

Laser irradiation-induced cell death via the apoptotic pathway

An elevated expression level of cyclooxygenase 2 (COX-2) is one of the signs that indicate NF-κB mediated necrotic cell death. Figure 6 a shows that the COX-2 protein was expressed in A549 cells that had undergone 266 nm laser irradiation, regardless of whether they were treated with the ND complex. Moreover, the cell skeleton, actin, was also rapidly disrupted. This phenomena is similar to what has been observed in previous studies,[29–31] indicating that the cells entered the necrosis pathway. Docetaxel, an anticancer drug, was used as a control for cell apoptosis.[32,33] The expression level of COX-2 was not elevated by 532 nm laser irradiation or docetaxel treatment.

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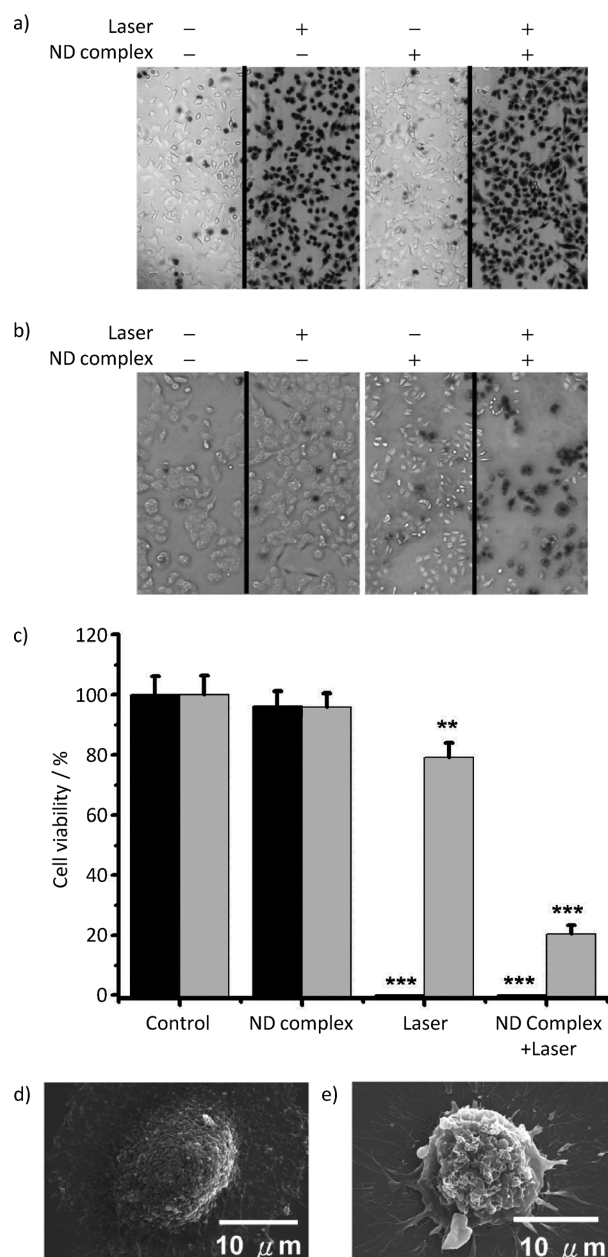

Figure 4. A549 non-small-cell lung cancer cells were irradiated with a laser at a) 266 nm or b) 532 nm. Dead cells were visualized by trypan blue staining. c) The bar diagram shows the viability of A549 cells treated with and without laser irradiation and the nanodiamond (ND) complex. Data show the mean ± standard deviation of $n \geq 5$ experiments. $^*P<0.05$, $^{**}P<0.01$, and $^{***}P<0.001$. Scanning electron micrograph of d) A549 cells treated with ND complex only and e) A549 cells treated with ND complex plus laser irradiation at 532 nm.

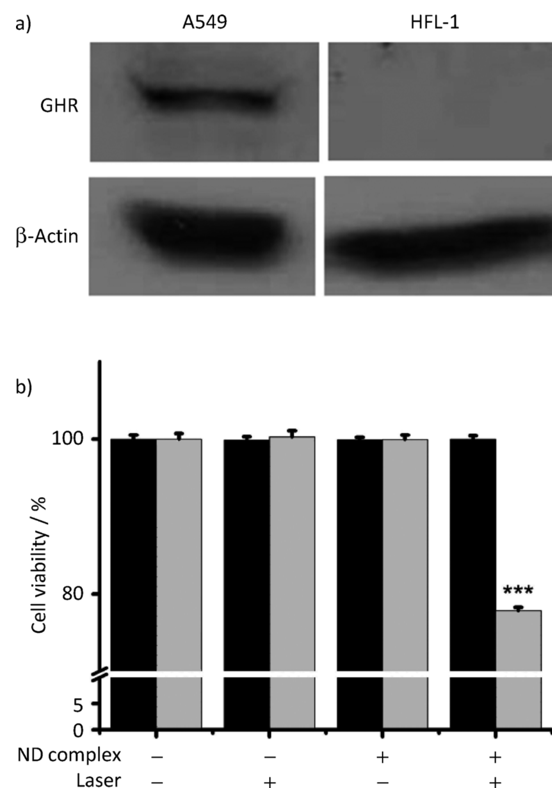

Figure 5. a) Western blot analysis of growth hormone receptor (GHR) protein in A549 non-small-cell lung cancer and HFL-1 human lung fibroblast cell lines. β-actin was used as an internal control. b) The bar diagram shows the cell viability of A549 (black squares) and HFL-1 (striped squares) cells treated with and without laser irradiation and the nanodiamond (ND) complex. The data show the mean ± standard deviation of $n \geq 5$ experiments. $^*P<0.05$, $^{**}P<0.01$, and $^{***}P<0.001$.

These results indicate that the necrosis pathway was involved in the cell death induced by 266 nm laser irradiation but not in the cell death induced by 532 nm laser irradiation. Moreover, the apoptosis markers caspase-3 (Figure 6 b) and apoptosis-inducing factor (AIF) (Figure 6 c), were translocated into the nucleus after the combined treatment with ND complex and 532 nm laser irradiation. These results were similar to those for docetaxel-treated A549 cells. In contrast, the expression level and location of caspase-3 and AIF were not changed by 266 nm laser irradiation.

ND complex and laser irradiation treatment in other GHR-expressing cancer cells

As shown in Figure 7, GHR expression levels were analyzed in different cancer cell lines, including liver hepatocellular cells (HepG2), cervical carcinoma cells (HeLa), and breast adenocarcinoma cells (MCF-7). The results indicate that cancer cell apoptosis triggered by treatment with ND complex and 532 nm laser irradiation may not be limited to lung adenocarcinoma epithelial cells (A549) but could also be applied to other cancer cells expressing GHR.

Discussion

NDs have been proved to exhibit high biocompatibility and very low cytotoxicity,[21,23] and ND-conjugated chemotherapeutic drugs can improve cancer therapeutics by overcoming drug efflux.[17] Hence, ND-conjugated drugs could be potential tools for therapeutic application in cancer. Herein we report the ND-rGH conjugated complex which, because of its size and shape, can be transformed by laser irradiation, as well as induce targeted cancer cell death through apoptosis. Our data show that

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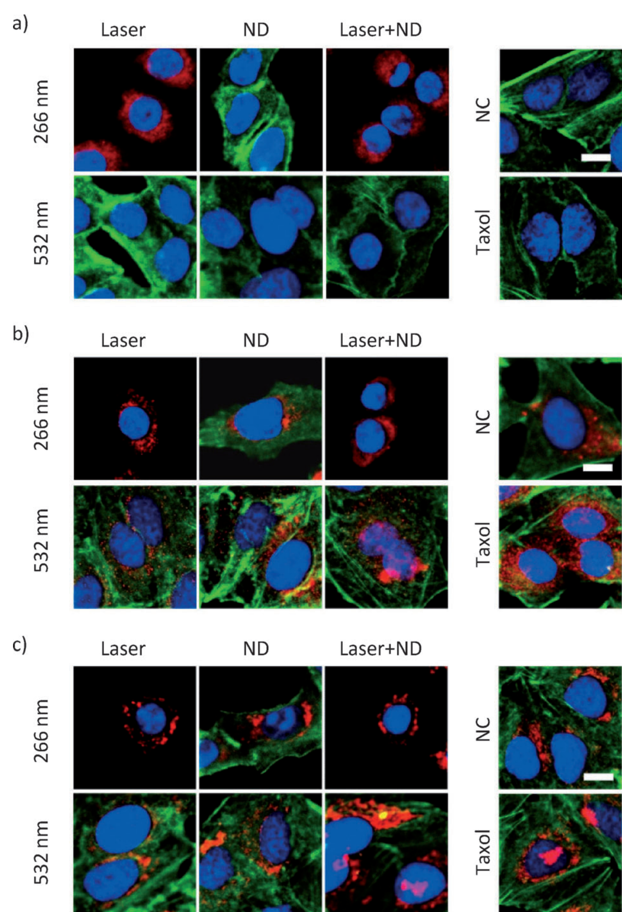

Figure 6. Confocal images of A549 non-small-cell lung cancer cells irradiated with a laser at 266 nm or 532 nm after treatment with the nanodiamond (ND) complex. a) Cyclooxygenase 2 (COX-2), b) caspase-3, and c) apoptosis-inducing factor (AIF) were detected by their corresponding Cy3-conjugated secondary antibodies (red). NC denotes untreated cells; Taxol denotes docetaxel-treated cells. Nuclei were stained with Hoechst 33342 (blue), and β-actin was stained with a phalloidin-conjugated antibody (green). The scale bar is 10 μm.

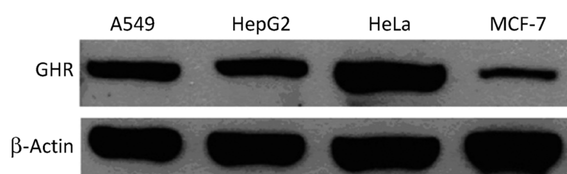

Figure 7. Western blot analysis of the expression of growth hormone receptor (GHR) protein in A549 non-small-cell lung cancer, HepG2 liver hepatocellular, HeLa cervical carcinoma, and MCF-7 breast adenocarcinoma cell lines. β-actin was used as an internal control.

the ND complex exhibits low toxicity, high biocompatibility, and the ability to specifically target cancer cells and induce their destruction.

Under dry conditions, the particle size of the ND complex is increased to micrometer scale, as observed by fluorescence microscopy (Figure 3c). Under these conditions, the ND complex may aggregate and increase in size but can be reversibly dissolved. Therefore, the particle size of the ND complex, as measured by DLS (Figure 1b), is well distributed around 9.3 nm in an aqueous environment, an ideal particle size for application in nanomedicine.

Conformational transition of NDs can be induced by laser irradiation at 532 nm.[24] After expanding in volume, the ND complex, which interacted with GHR on the cell membrane, was able to damage the cell membrane (Figure 4e) and cause cell death (Figure 4b and c). As shown in Figure 4c, the viability of cells irradiated with a 532 nm laser was slightly reduced (to approximately 80%); however, combination of laser irradiation with the ND complex treatment resulted in markedly reduced cell viability (to approximately 20%). In contrast, laser irradiation at 266 nm induced cell death directly, unrelated to the ND complex (Figure 4c). Hence, the ND complex plays an important mediator role in cell death induced by 532 nm laser irradiation, but not in cell death induced by 266 nm laser irradiation.

GHR is overexpressed in various cancer cell lines and is a potential cancer chemotherapy target.[5,7] In our previous study, we demonstrated that rGH can bind to human GHR on the A549 cell membrane.[4] In the current study, we showed that this interaction does not influence cell proliferation (Figure 2b). Hence, rGH is an ideal GH antagonist for targeting GHR overexpressed in cancer cells. As shown in Figure 3, the ND complex bound specifically to the surface membrane of A549 cells. Moreover, compared with HLF-1 cells not expressing GHR, A549 cells expressing GHR responded to the treatment with ND complex in combination with 532 nm laser irradiation (Figure 5b). In other words, cell death caused by the combination treatment is specific to GHR-expressing cancer cells. Accordingly, rGH conjugated with the ND complex has a targeting role, specifically binding to GHR on cancer cell membranes, while 532 nm laser irradiation has a cell death-triggering role, damaging the ND complex specifically bound to the cancer cells.

Unprogrammed cell death, necrosis, is typically a result of an inflammatory response.[34,35] In contrast with necrotic cell death, apoptosis is programmed cell death and occurs normally during aging and development of cells in living tissues and organisms.[36,37] As shown in Figure 6a, COX-2 was expressed when 266 nm laser irradiation was used to induce cell death, but not when the 532 nm laser was used. On the contrary, caspase-3 and AIF were expressed and translocated into the nucleus when the combined treatment of ND complex and 532 nm laser irradiation was used. In the apoptotic pathway, caspase-3 plays a central role in the caspase cascade, being cleaved and translocated into nucleus.[38,39] In addition, AIF is a recently discovered apoptotic effector protein, which can induce chromatin condensation and DNA fragmentation when it translocates from mitochondria to the nucleus.[40,41] Thus, our results indicate that the cell death induced by combined treatment of ND complex and 532 nm laser irradiation occurred via the apoptotic pathway, whereas cell death induced by 266 nm laser irradiation occurred via the necrosis pathway.

Conclusions

GHR is overexpressed not only in cancer cells but also in normal organs such as the adrenal gland.[42] The wide distribu-

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tion of GHR may reduce the therapeutic efficacy and induce side effects of anticancer drugs targeting GHR. In this study, an ND complex was shown to bind specifically to GHR and induce cell death via local laser irradiation. Thus, side effects induced by the overexpression of GHR in normal cells might be reduced. Moreover, our approach induced cell death via the apoptotic pathway but did not lead to damage of the surrounding tissue caused by the inflammatory response. This study may provide a new strategy and platform for therapeutic application in cancers where GHR is overexpressed.

Experimental Section

Materials: All chemicals were purchased from Merck (Rahway, NJ) and Sigma (St. Louis, MO), cell culture plates were purchased from Corning Incorporated (Corning, NY), primers were synthesized by Biokit (MiaoLi, Taiwan, R.O.C.), and recombinant fish GH protein was obtained as previously described.[43]

GFP protein expression and purification: cDNA of GFP was subcloned from the pcDNA 3.1/CT-GFP-TOPO vector (Invitrogen, Carlsbad, CA) into the pET30a-Eam vector (a thymine vector that is a modified form of the pET30a vector (Novagen, Darmstadt, Germany)) using PCR with GFP forward (5′-AGAATGGCTAGCAAAGGAGAAG-3′) and BGH reverse (5′-TAGAAGGCACAGTCGAGGC-3′) primers. The plasmid was then transformed into BL21 (DE3) cells. The *Escherichia coli*-carrying GFP gene was expressed using a standard process, and soluble GFP molecules were further purified from the supernatant of the total cell lysate by a His GraviTrap column (GE Healthcare Bio-Sciences Corp., Piscataway, NJ). rGH protein production was carried out following our previous study.[43] Briefly, *E. coli*-carrying recombinant GH gene was grown in LB medium and induced with 0.1 mM IPTG after the culture reached an OD_{600} value of 0.3–0.6 at 37 °C for 16 h. rGH was harvested from inclusion bodies and then refolded.[44]

Conjugation of ND complex: Firstly, carboxyl groups of cND were transformed to primary amines. The carboxylated cND (final concentration 2 mg mL^{-1}) was dissolved in reaction buffer (0.02 M 2-(*N*-morpholino) ethanesulfonic acid (MES), 0.5 M NaCl, and 2 mM 1-ethyl-3-[3- dimethylaminopropyl]carbodiimide hydrochloride (EDC)). EDC was used to convert a carboxy to form the amine-reactive *O*-acylisourea intermediate. Secondly, the unstable reactive *O*-acylisourea ester was stabilized. Briefly, 5 mM sulfo-NHS and 20 mM 2-mercaptoethanol (β-Me) were added to ammonized cND, and the mixture was reacted for 10 min at room temperature. The semi-stable, amine-reactive NHS-ester of cND was then conjugated with the correctly oriented and conjugated rGh-GFP, which was produced by adding rGH to EDC-activated GFP. This produced an ND-rGH-GFP complex, with the reaction mixture further dialyzed against distilled water (ddH_2O) for 8 h. The procedure used to synthesize the ND complex is illustrated in Scheme 1.

Analysis of ND complex particle size by dynamic light scattering (DLS): DLS was measured using a goniometer, obtained from Brookhaven Instruments Corp. (BIC, Holtsville, NY) and equipped with a diode-pumped laser (Coherent, Santa Clara, CA) with a wavelength λ of 532.15 nm and at a power of 10 mW. Scattered light was collected at an angle of 90°. The chamber temperature was controlled at 20 °C by a water circulator. The autocorrelation function was computed using a digital correlator (BI 9000, BIC, Holtsville, NY), and analyzed by the negatively constructed least squares (NNLS) method.[45]

Molecular image analysis of ND complex by AFM: The ND complex (100 μL at 1 mg mL^{-1}) was dropped in mica for 20 min and washed with ddH_2O to remove remaining chemical compounds in the reaction buffer. Water molecules bound to the sample were removed by nitrogen purge. The ND complex was imaged with a diInnova SPM (Bruker AXS, Karlsruhe, Germany) in tapping mode. The AFM probe used was SSS-SEIHR (Nanosensors, Neuchatel, Switzerland), with a force constant of 15 N m^{-1} and a frequency of 130 kHz.

Cell culture: The A549 and HFL-1 cell lines were cultured in RPMI-1640 medium (Invitrogen, Carlsbad, CA) supplemented with bovine fetal calf serum (10 %), penicillin (100 unit mL^{-1}), and streptomycin (100 μg mL^{-1}).

Cytotoxicity of cND, rGH, GFP, and ND complex: A549 cells were cultured in 96-well culture plates (2×10^4 cells per well) for 24 h to 70 % confluence, then washed twice with phosphate-buffered saline (PBS) and incubated in serum-free medium for 16 h. cND (final concentrations 0–10 μg mL^{-1}), rGH (final concentrations 0–10^4 μg mL^{-1}), GFP (final concentrations 0–10^4 μg mL^{-1}), and ND complex (final concentrations 0–10^4 μg mL^{-1}) were added to the A549 cells and incubated for 24 h. Cell viability was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) dye reduction assay. For the MTT assay, serum-free RPMI-1640 medium containing MTT (0.5 mg mL^{-1}) was added to each well. Plates were incubated for 4 h in the absence of light, the medium was removed, and precipitates were dissolved in DMSO (100 μL). Absorbance at 565 nm was measured using a plate reader. Experiments were performed in triplicate.

Confocal fluorescence microscopy for ND complex localization: A549 cells were cultured in six-well culture plates (2×10^5 cells per well) for 18 h, washed twice with PBS, incubated in serum-free medium, and treated with ND complex (final concentration 1 μg mL^{-1}) for 30 min. The cells were then washed twice with PBS to remove free ND complex and incubated in RPMI-1640 medium for 24 h. The cells were washed twice with PBS and fixed by paraformaldehyde (4 %). Permeabilization of the cell membrane was induced by Triton X-100 (0.2 %). Nuclei and β-tubulin were stained with Hoechst 33342 (Invitrogen, Carlsbad, CA) and Cy3-conjugated β-tubulin antibody (Sigma, St. Louis, MO), respectively. The fluorescence of GFP, β-tubulin, and nuclei were observed by confocal fluorescence microscopy (FV300, Olympus Corporation, Shinjuku-ku, Tokyo, Japan).

Single- and multi-photon effect of A549 cells: A549 cells were cultured in six-well culture plates (2×10^5 cells per well) for 18 h, washed twice with PBS, incubated in serum-free medium, and treated with ND complex (final concentration 1 μg mL^{-1}) for 30 min. The cells were washed twice with PBS to remove free ND complex and incubated with RPMI-1640 medium for 24 h. The cells were irradiated with an Nd:YAG (yttrium aluminum garnet) pulse laser (LS2137U/2, Lotis TII Ltd., Minsk, Belarus) at a wavelength of 266 nm (0.1 s/step, 5 mW average power, 10 Hz repetition rate, 2 mm beam size) or 532 nm (10 s/step, 30 mW average power, 10 Hz repetition rate, 2 mm beam size) and cultured with RPMI-1640 medium for 24 h. Trypan blue staining was used to detect cell death.

Next. the laser-irradiated cells were fixed by paraformaldehyde (4 %) and cell membrane permeabilization was induced by Triton X-100 (0.2 %). The nuclei, actin, COX-2, caspase-3, and AIF were stained with Hoechst 33342, phalloidin-conjugated anti-actin antibody (Molecular probe, Life Technologies, NY), anti-COX-2 antibody (Santa Cruz Biotechnology, Inc. Santa Cruz, CA), anti-caspase-3 antibody (Abcam, Cambridge, UK), and anti-AIF antibody (Santa Cruz

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Biotechnology, Inc. Santa Cruz, CA), respectively. The fluorescence of nuclei, actin, COX-2, caspase-3, and AIF were observed by confocal fluorescence microscopy.

Cell viability analysis of cells with and without GHR expression: A549 and HFL-1 cells were cultured in 96-well culture plates (2×10^4 cells per well) for 24 h to 70% confluence, then washed twice with PBS, incubated in serum-free medium, and treated with ND complex (final concentration 1 $\mu g\,mL^{-1}$) for 30 min. The cells were washed twice with PBS to remove free ND complex and incubated with RPMI-1640 medium for 24 h. The cells were irradiated with an Nd:YAG pulse laser at a wavelength of 532 nm (10 s/step, 20 mW average power, 10 Hz repetition rate, 2 mm beam size) and cultured for 24 h. Cell viability was determined by the MTT dye reduction assay.

Western blot analysis to detect GHR expression: Cell lysates were denatured and resolved on a 12% SDS-PAGE gel, then electrotransferred to a polyvinylidene difluoride (PVDF) membrane using semi-dry transfer blots. The membrane was incubated in blocking solution (10 mM Tris, 100 mM NaCl, 0.1% Tween-20, and 5% nonfat milk, pH 7.5) for 30 min at room temperature. GHR was detected by a goat anti-GHR antibody (Santa Cruz Biotechnology, Santa Cruz, CA), which was recognized by a donkey anti-goat antibody (Santa Cruz Biotechnology, Santa Cruz, CA). Chemiluminescent HRP substrates (Millipore Corporation, Billerica, MA) were used to enhance the chemiluminescence signal.

Statistical analysis: All quantitative assays were carried out at least five times, and the data were expressed as mean $\pm$SEM values. A *P* value less than 0.05 was considered to indicate statistical significance.

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Keywords: A549 lung cancer cells · apoptosis · lasers · nanodiamond complexes · recombinant growth hormones

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