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Gold nanoparticles with different charge and moiety induce differential cell response on mesenchymal stem cell osteogenesis

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ABSTRACT

Stem cells exist in an *in vivo* microenvironment that provides biological and physiochemical cues to direct cell fate decisions. How the stem cells sense and respond to these cues is still not clearly understood. Gold nanoparticles (AuNPs) have been widely used for manipulation of cell behavior due to their ease of synthesis and versatility in surface functionalization. In this study, AuNPs with amine (AuNP−NH_2), carboxyl (AuNP−COOH) and hydroxyl (AuNP−OH) functional groups possessing different surface charge were synthesized. Human bone marrow-derived mesenchymal stem cells (hMSCs) were treated with the surface functionalized AuNPs and assessed for cell viability and osteogenic differentiation ability. The surface functionalized AuNPs were well tolerated by hMSCs and showed no acute toxicity. Positively charged AuNPs showed higher cellular uptake. AuNPs did not inhibit osteogenesis but ALP activity and calcium deposition were markedly reduced in AuNP−COOH treatment. Gene profiling revealed an upregulation of TGF-β and FGF-2 expression that promoted cell proliferation over osteogenic differentiation in hMSCs. These results provide some insight on the influence of surface functionalized AuNPs on hMSCs behavior and the use of these materials for strategic tissue engineering.

1. Introduction

Human mesenchymal stem cells (hMSCs) are an important cell type and used in many tissue engineering strategies due to their great potential to differentiate into many cell types. The differentiation and fate of hMSCs are known to be governed and regulated by a variety of biological and physiochemical cues in the cellular microenvironment surrounding stem cells *in vivo* [1]. Recent studies have shown that specific chemical moieties may contribute significantly towards direction of stem cell fate [2,3]. Functional groups like amines (−NH_2), carboxyl (−COOH) and hydroxyl (−OH) are widely found on biomolecules such as proteins, nucleic acids and polysaccharides and are important factors in influencing stem cell behavior and differentiation [4].

Nanoscale materials have attracted broad attention in many tissue engineering studies because of their similarity to the nanostructured nature of microenvironment. Not only do nanomaterials possess a large surface area for interaction with biological molecules and cells (due to their high surface area to volume ratio), they are also able to traverse biological barriers and even enter the cell nuclei [5]. These factors imply a greater biological response from nanoscale materials in contrast with the bulk materials. Presenting functional chemical moieties on the surface of nanomaterials is anticipated to more closely mimic the microenvironment and to be appropriate to investigate the influence of the physiochemical cues on stem cell functions.

Gold nanoparticles (AuNPs) are ideal candidates of choice for biological studies due to their ease of synthesis, relative biocompatibility and versatility in surface modification [5]. Functionalized AuNPs are especially useful for therapeutic strategies in drug delivery [6], diagnostic imaging [7] and antibody labeling and targeting [8]. Surface charge and different surface chemical moieties can influence many cell behaviors particularly uptake of the nanoparticles [9] as well as cytoskeletal remodeling [10]. Gold nanoparticles (AuNPs) have been found to promote osteogenesis in hMSCs [11]. However it is still unknown how the AuNPs functionalized with different functional groups affect the behaviors of hMSCs. Therefore in this study, AuNPs were surface modified with three differently charged alkanethiols to produce AuNPs with amine (AuNP−NH_2), carboxyl (AuNP−COOH) and hydroxyl (AuNP−OH) functional groups and investigated for their

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interactions with hMSCs. We aimed to investigate if such functional groups on nanomaterials will have an effect on hMSCs behavior and osteogenesis.

2. Materials and methods

2.1. Gold nanoparticle synthesis and characterization

Depending on the type of surface modification, the AuNPs were synthesized under different methods. AuNP−NH_2 was synthesized using the 1-step method while the AuNP−COOH and AuNP−OH synthesis was conducted by 2-steps.

(i) One-step process:

Aminethanthiol surface modified AuNPs were synthesized according to the method as described by Niidome et al. and Techane et al. [12,13]. In brief, 56 mg of gold salt $HCl_4Au \cdot 4H_2O$ (Wako Chemicals, Japan) was dissolved in 100 mL of ultra-pure water and 2.42 mg of aminethanethiol (Wako Chemicals, Japan) was added to the gold salt solution. Next, 250 μl of 10 mM $NaBH_4$ was added dropwise and the solution was left to stir in the dark for at least 2 h after which the AuNP solution was stored at 4 °C in the dark.

(ii) Two-step process:

AuNPs were synthesized by the citrate reduction method [14] from gold salt $HCl_4Au \cdot 4H_2O$ (Wako Chemicals, Japan). The resultant citrate coated AuNPs were centrifuged and resuspended in a solution of equal parts of water and ethanol adjusted to pH11. Next, 2 mM of 3-mercaptoethanoic acid (Dojindo, Japan) for carboxyl group functionalization or 2-mercaptoethanol (Wako Chemicals, Japan) for hydroxyl group functionalization were added into the solution and the mixtures were stirred for at least 24 h to ensure optimal ligand exchange was carried out.

Size characterization of the AuNPs was measured by dynamic light scattering (DLS) (JASCO, Japan) and transmission electron miscroscopy (TEM) (Jeol, Japan). From the TEM images, diameter size of at least 100 individual nanoparticles was measured per sample type and recorded with the Image-J software (developed by Wayne Rasband, National Institutes of Health, USA). The surface modified AuNPs were resuspended in ultra-pure water and measured by a zeta-potential analyzer (Otsuka Electronics, Japan) to verify the surface charge of the synthesized AuNPs.

2.2. Cell culture and treatment with surface modified AuNPs

Human bone marrow-derived mesenchymal stem cells (hMSCs) were obtained from LONZA (Walkersville MD, USA). The cells were cultured in 75 cm^2 tissue culture flasks (BD Falcon, USA) with normal cell culture medium at 37 °C in humidified air containing 5% CO_2. The cell culture growth medium was Dulbecco's modified Eagle's medium (DMEM, Sigma, USA) supplemented with 10% fetal bovine serum, 4500 mg/L glucose, 4 mM glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, 0.1 mM nonessential amino acids, 0.4 mM proline, 1 mM sodium pyruvate and 50 μg/mL ascorbic acid.

The hMSCs were seeded into 24-well plates at a density of 5000 cells/cm^2 with growth medium. After 1 day, the media in the wells were changed to fresh growth media with 0.1, 0.5 and 1.0 nM of AuNP−NH_2, AuNP−COOH and AuNP−OH. Media was changed every 2–3 days until the experimental time point. Cells were also treated with AuNP−citrate as a control.

2.3. Osteogenic induction of hMSCs

The hMSCs were seeded onto 24-well plates as stated in 2.2 and then treated with 0.5 nM surface modified AuNPs in osteogenic induction medium. The osteogenic induction medium consisted of DMEM supplemented with 1000 mg/L glucose, 584 mg/L glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, 0.1 mM nonessential amino acids, 50 mg/L ascorbic acid, 10% FBS, 10 nM dexamethasone and 10 mM β-glycerophosphate. The media were changed every 2–3 days for 21 days.

2.4. Cell viability studies of AuNP treated hMSC

(i) Live/dead fluorescence staining

Cells were cultured on 48-well plates and treated with AuNPs as stated in 2.2 and 2.3. The experiment was performed using the Cellstain Live−Dead Double Staining kit (Dojindo, Japan) as per manufacturer's instructions. After 3 days of culture with AuNPs, the cells were washed with 1 × PBS thrice and incubated in 2 mM calcein-AM and 4 mM propidium iodide (PI) in PBS for 15 min. The live/dead images were observed with an inverted fluorescence microscope (Olympus, Japan).

(ii) Trypan blue exclusion assay

Cells were cultured on 24-well plates and treated with AuNPs in osteogenic induction medium as stated in 2.3. After 21 days of culture, the cells were washed with 1 × PBS and harvested by trypsinization. All media, cell suspension and wash solution were collected and centrifuged. The cells were resuspended in a known volume of medium and mixed in 0.4% trypan blue solution (Wako Chemicals, Japan) before counting with a hemocytometer (SLGC, Japan). Triplicate samples for each treatment group were used (n = 3). The cell number data were collected and analyzed by calculating the total viable cells in a volume of resuspension medium. Cell viability was calculated by dividing the total viable cell count of AuNP treated samples with total viable cell count of the control and the values represented in percentage.

2.5. Inductively coupled plasma optical emission spectroscopy (ICP-OES)

hMSCs were cultured with surface modified AuNPs as described in 2.3 for 21 days in osteogenic induction medium. The cells were washed thrice in 1 × PBS and then trypsinized and centrifuged to remove the supernatant. Then 3 mL aqua regia was added to each sample for digestion of biological material and dissolution of the AuNPs. Next, the samples were diluted in MilliQ water before measurement with the SII Nanotechnology SPS3520UV-DD ICP-OES system (Hitachi, Japan). Data were calculated to represent the amount of Au per sample normalized against the total viable cell number measured as described in 2.4. The experiment was repeated in triplicate for each treatment group (n = 3).

2.6. Histological staining for osteogenic differentiation markers

After 21 days of culture with AuNPs as described in 2.3, histological staining for alkaline phosphatase (ALP) was carried out. The cells were washed with PBS twice and subsequently fixed with 4% paraformaldehyde for 10 min at room temperature. The fixed cells were washed thrice with PBS and incubated with 0.1% naphthol AS-MX phosphate (Sigma, USA) and 0.1% fast blue RR salt (Sigma, USA) in 56 mM 2-amino-2-methyl-1,3-propanediol (pH 9.9, Sigma, USA) working solution at room temperature for 10 min, washed with PBS twice and observed using an optical microscope (Olympus, Japan).

For calcium phosphate Alizarin Red S (ARS) staining, the cells were washed twice with PBS, followed by fixation with 4% paraformaldehyde for 20 min at room temperature. After that, the cells were incubated with 0.1% Alizarin Red S (Sigma, USA) solution at room temperature for 30 min, washed twice with PBS and observed using an optical microscope. After which, the samples were air dried and the ARS staining was eluted with 5% perchloric acid. The solution from each well was then transferred to a 96-well plate and the absorbance was read with a spectrophotometer (Biorad, USA) at an absorbance wavelength of 405 nm. The experiment was repeated in triplicate for each treatment group (n = 3).

2.7. Alkaline phosphatase (ALP) activity assay

Alkaline phosphatase activity was assayed with the Sensolyte® *p*NPP Alkaline phosphatase assay kit (Anaspec, USA). Cells were seeded in 24-well plates and treated with AuNP as stated in 2.3. After 21 days, cells were harvested according to the manufacturer's protocol and the color change was measured with a spectrophotometer (Biorad, USA) at absorbance wavelength of 405 nm. Cells from each treatment group were also counted with a hemocytometer to determine total cell number per well. The amount of ALP was calculated per well and normalized to the total cell number per well. The experiment was repeated in triplicate for each treatment group (n = 3).

2.8. RNA extraction and RT^2 gene profiler PCR array

hMSCs were cultured in 6-well plates and treated with AuNPs according to the same procedure in 2.3. After 21 days of culture with AuNPs, the cells were washed once in PBS and harvested for RNA extraction with the RNAeasy Minikit (Qiagen, Netherlands) according to the manufacturer's protocol. Total RNA was then converted to cDNA by the proprietary first strand cDNA synthesis kit included in the PCR array system (Qiagen, Netherlands). The osteogenic pathway PCR array system (Cat #: PAHS-026Z, Qiagen, Netherlands) was selected for this study. The cDNA and SYBR green Master Mix were added to each well of the array plate according to the manufacturer's instruction. Real time PCR was performed on the 7500 Real-Time PCR system (Applied Biosystems, USA). All target gene expression results were normalized to GAPDH. Statistical analysis and fold change calculations were performed with the provided software at the Qiagen PCR Array Data Analysis web portal (www.SABiosciences.com/pcrarraydataanalysis.php). Gene expression changes of target genes were compared with control group using the student *t*-test and values of $P < 0.05$ were considered to be statistically significant. The experiment was repeated in triplicate for each treatment group (n = 3).

2.9. Statistical analysis

Statistical analysis was performed with the GraphPad Prism software (GraphPad Software, USA). All data were shown as mean ± standard error of the mean (SEM) with experiments repeated in triplicate (n = 3). A one-way analysis of variance (ANOVA) with Tukey's post hoc test for multiple comparisons was used for statistical analysis. A value of $P < 0.05$ was considered to be a statistically significant difference.

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