Articles

Integrin-Supported Fast Rate Intracellular Delivery of Plasmid DNA by Extracellular Matrix Protein Embedded Calcium Phosphate Complexes†

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ABSTRACT: Extracellular matrix (ECM) proteins, such as collagen and fibronectin, play vital roles in development and maintenance of hard tissue (bone or tooth) and are, consequently, thoroughly investigated for construction of biomimetic scaffolds in combination with calcium phosphate (CaP) material (the major component of hard tissue) for bone or dental tissue engineering. Realizing the natural affinity of ECM components toward inorganic constituents of hard tissue, we successfully constructed the nanohybrids of DNA/CaP particles with either collagen 1 or fibronectin, which finally possessed the capability of specific recognition of integrin receptor for being swiftly internalized across the plasma membrane, leading to remarkably high transgene expression in mammalian cells. This new approach with precise receptor-specific delivery as well as 10- to 50-fold enhanced efficiency level compared to the classical one, has immediate applications for basic research and large scale production of recombinant therapeutic proteins and looks promising for gene therapy.

Extracellular matrix (ECM)[1] proteins, especially collagen 1 and fibronectin which are intrinsically involved in development, homeostasis, and maintenance of bone or tooth, closely associate with deposited calcium phosphate (CaP) ceramics and thus act as scaffold for neighboring cells (*1*−*5*) in the hard tissues. ECM molecules have the inherent tendency of binding to the charged surface of CaP derivatives by electrostatic interactions and to the cells by corresponding integrin receptors. Based on this natural phenomena, a new field has been developed for creating inorganic−organic hybrid scaffolds in vitro, that could substitute hard tissue in vivo (*6*−*8*). However, on the other hand, despite the existence of a popular gene delivery device which is completely based on CaP nanoparticles and has been being used intensively for more than 30 years (*9*), no attention was paid on complexation of the particles with ECM proteins, that might dramatically accelerate the transfection efficiency. Thus, we report here for the first time how simply we could develop surface-functional nanocomposites of CaP with the help of collagen and fibronectin and succeed in enabling the DNA carrying composite particles to be internalized by specific cell surface integrin receptors. The rapid cellular endocytosis resulted in significantly high DNA delivery in cytoplasm and eventual drastic enhancement in transgene expression.

EXPERIMENTAL PROCEDURES

Cell Culture. HeLa and NIH 3T3 cell lines were cultured in 25 cm^2 flasks in Dulbecco's modified Eagle's medium (DMEM, Gibco BRL) supplemented with 10% fetal bovine serum (FBS), 50 μg of penicillin mL^{-1}, 50 μg of streptomycin mL^{-1}, and 100 μg of neomycin mL^{-1} at 37 °C in a humidified 5% CO_2-containing atmosphere.

Turbidity Measurements. After addition of 1 μg of a reporter plasmid (pGL3, Promega) carrying a luciferase gene under SV40 promoter to a total of 100 μL of a 1× Hepes-buffered solution (HBS) (25 mM Hepes, 70 mM NaCl, 0.75 mM Na_2HPO_4, pH 7.5) containing 18 mM $CaCl_2$ and 0−100 μg/mL collagen 1, fibronectin, or bovine serum albumin (BSA), the resulting solution was incubated at 37 °C for 30 min. Spectroscopic reading of the particle suspension was taken at 320 nm for turbidity measurement by SmartSpec 3000 (BIO-RAD).

Quantitative Fluorescence Measurement. All types of samples containing fluorescent compounds, ethidium bromide or fluorescence isothiocyanide (FITC) were added in 100 μL to each of the wells of a 96-well plate and estimated for total fluorescence intensity by fluorescence imaging at first and then counting by Typhoon 8600, a variable mode imager (Amersham Biosciences).

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[1] Abbreviations: ECM, extracellular matrix; CaP, calcium phosphate; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; HBS, Hepes-buffered solution; BSA, bovine serum albumin; FITC, fluorescence isothiocyanide; SEM, scanning electron microscope; PI, propidium iodide.

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Particle Size Visualization by Scanning Electron Microscope (SEM). A drop of 10× diluted protein-embedded DNA/CaP particle suspension prepared according to the protocol described above was added to a carbon-coated SEM stage and dried, followed by observation by a high-resolution SEM (S-800, Hitachi, Japan).

PI Labeling of Plasmid DNA. A 1:1 weight ratio of DNA to PI was mixed in a total of 100 μL of Hepes-buffered solution (HBS) (pH 7.5) prior to addition of other components and incubation for 30 min at 37 °C for particle preparation.

Cell Adhesion Assay. Collagen 1 solution was added at a concentration of 10 μg/mL or 100 μg/mL to the wells of a 24-well plate, followed by incubation at 37 °C for 3 h. HeLa or NIH 3T3 cells were seeded onto the coated plate, and cell spreading was visualized under optical microscope.

Transfection of Cells. Cells from the exponential growth phase were seeded at 50 000 cells per well into 24-well plates the day before transfection. The medium was changed 1 h before transfection. For transfection experiments, 1 μg of a reporter plasmid (pGL3, Promega) carrying a luciferase gene under SV40 promoter was added to a total of 100 μL of a 1× Hepes-buffered solution (HBS) (25 mM Hepes, 70 mM NaCl, 0.75 mM Na_2HPO_4, pH 7.5) containing 18 mM $CaCl_2$ and 0−100 μg/mL collagen 1, fibronectin, or BSA. This solution was then incubated at 37 °C for 30 min. After addition of the resulting particle suspension to the cultured cells in each well in the presence of 1 mL of media (with 10% FBS), cells were incubated for 4 h at 37 °C. The medium was replaced with fresh medium, and the cells were further incubated at 37 °C for 36 h. The transfected cells were lysed using the luciferase assay system (Promega), and the luciferase activity was measured on a luminometer (TD-20/20 Luminometer). Transfection efficiency was measured as mean light units per milligram of cell protein. Lipofectamine-mediated transfection was done in the same manner following preparing DNA−lipofectamine complexes (lipoplexes) at a weight ratio of 1:5 according to the protocol provided by the manufacturer (Invitrogen).

RESULTS AND DISCUSSION

We have recently developed a highly simplified transfection methodology based on the generation of CaP/DNA particles at higher pH (7.5) and temperature (37 °C) (*10*). First we investigated the effects of ECM proteins on the growth kinetics of CaP particles generated by the new technique (Figure 1). The basic protein (isoelectric point is above pH 7), collagen, which could bind the PO_4^{3-}-rich domain of particles, slightly inhibits particle growth with increasing its concentrations and then induces the growth at higher concentration. On the contrary, the acidic proteins (isoelectric point is below pH 7), fibronectin and BSA, which could bind the Ca^{2+}-rich domain, induced the growth of particles continuously with an increase in the concentration, suggesting that acidic proteins act as nucleation sites for accelerating particle synthesis which resulted in increased number of particles (not shown here). In order to show direct evidence that an ECM protein indeed binds to the CaP particles, we admitted FITC-labeled collagen, as an example, to the reaction mixture prior to formation of the particles and observed later, following centrifugation and dissolution

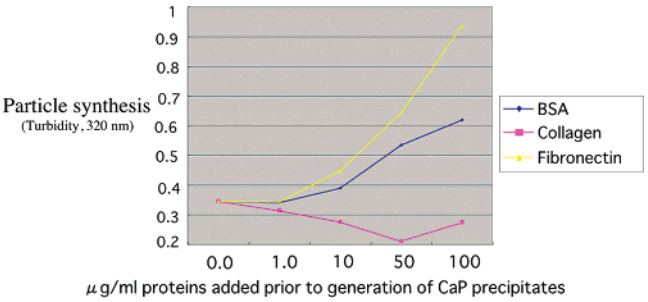

FIGURE 1: Monitoring CaP precipitate formation profile influenced by collagen 1, fibronectin, and BSA. Turbidity measurement was done at 320 nm as an indicator of precipitation which was induced by addition of 18 mM Ca^{2+} along with DNA (1 μg) and increasing concentrations of collagen 1, fibronectin, or BSA (0 to 100 μg/mL) to a 100 μL Hepes-buffered solution (pH 7.5) containing 0.75 mM inorganic phosphate, followed by incubation at 37 °C for 30 min.

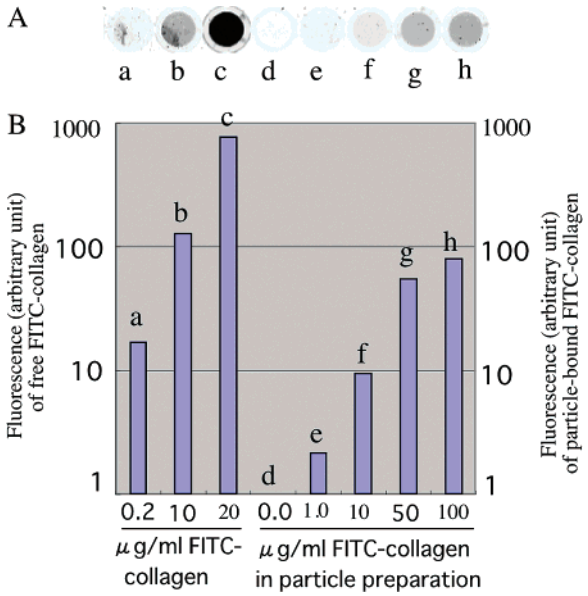

FIGURE 2: Measurement of binding affinity of collagen to CaP precipitates. Particles prepared by incubation at 37 °C for 30 min of a 100 μL Hepes-buffered solution (pH 7.5) containing 0.75 mM inorganic phosphate and 18 mM Ca^{2+} along with DNA (1 μg) and increasing concentrations of FITC-collagen 1 were centrifuged at 15 000 rpm for 5 min, followed by dissolution of the particle pellet by 50 mM EDTA in PBS for subsequent fluorescent imaging (A) and then quantitation for collagen binding (B). a, b, and c represent fluorescence intensity of 0.2, 10, and 20 μg/mL free FITC-collagen, and d, e, f, and g represent fluorescence intensity of FITC-collagen bound to the particles following particle preparation in the presence of 0, 1, 10, 50, and 100 μg/mL FITC-collagen.

of formed particles by EDTA, that a significant portion of total collagen could be associated with the particles. As shown in Figure 2, approximately 0.2 to 10 μg of collagen could be bound to the particles generated in the presence of 10 to 100 μg of total collagen (Figure 2).

Next, we evaluated the effect of protein association on the quantity of plasmid DNA embedded in the particles (Figure 3). Particle-bound DNA was released by EDTA and estimated fluorometrically using propidium iodide (PI). As shown in Figure 3c, increasing the concentrations of collagen 1, which finally resulted in increased incorporation of collagen 1 into the particles (Figure 2), gradually hampered to some extent the adsorption of DNA to the particles, indicating that DNA and collagen 1 competitively bind to

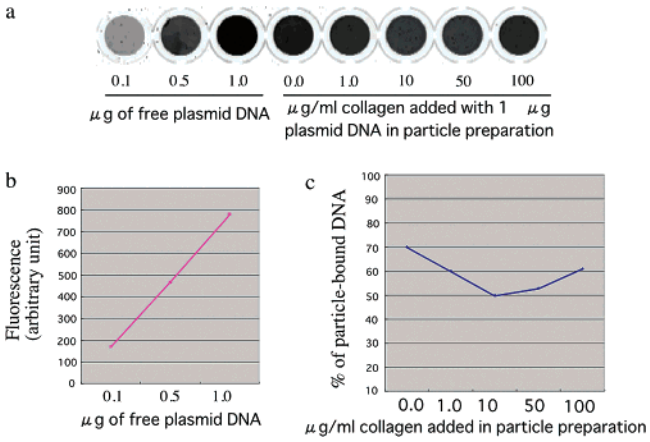

FIGURE 3: Determination of binding affinity of plasmid DNA to CaP precipitates in the presence of increasing concentrations of collagen 1. Particles prepared in the presence of a fixed amount of DNA (1 μg) and variable concentrations of collagen 1 (1 to 100 μg/mL) (as described in the legend to Figure 1) were centrifuged at 15 000 rpm for 5 min, and the resulting pellet was dissolved by 50 mM EDTA in PBS for subsequent fluorescent imaging (a) and then quantitation for calibration (b) and estimation of bound DNA.

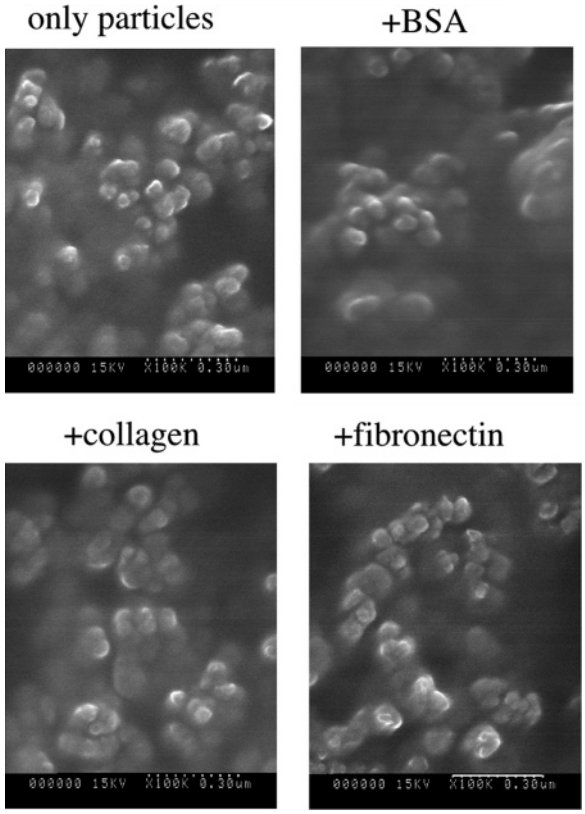

FIGURE 4: Morphology and sizes of composite particles. Scale bar, 300 nm.

the CaP particles. However, at sufficiently high collagen concentration (100 μg/mL), the increment in DNA association could be interpreted by the induction of particle growth (Figure 1) causing an increase in particle number and facilitating more DNA to be bound to the additionally synthesized particles. However, 50% binding of DNA to the collagen embedded particle which was at the minimum level was still sufficient for efficient transgene delivery. Since a significant portion of DNA (70%) could be bound to the particle only (Figure 3) in the presence of PI, DNA charge neutralizing effect of PI on the binding of DNA to the particles might be insignificant. Scanning electron microscopy (Figure 4) showed that protein adsorption apparently did not bring in the changes of particle morphology. A nonaggregated particle lay below 100 nm, a value of diameter which increased upon aggregation to 300 nm, especially when too many particles are generated in the presence of high concentrations of BSA or fibronectin (not shown here).

To analyze that ECM protein adsorption created surface functional DNA/CaP particles with the capacity of recognizing specific integrin, leading to prompt DNA delivery across the cell membrane, we labeled DNA with PI (*11*) before forming the composite particles and allowing them for cellular uptake (Figures 5 and 6). As shown in HeLa, an epithelial cancer cell line (Figure 5) which expresses integrin for both collagen 1 (*12*, *13*) and fibronectin (*14*, *15*) DNA/CaP particles with adsorbed ECM molecules surprisingly increased total internalized plasmid DNA at certain concentrations, indicating that receptor-recognizable domains for both proteins might be functional on the particle surface for quick endocytosis (*16*−*21*). However, non-ECM protein BSA and collagen at high concentration (100 μg/mL) could not enhance the process of DNA delivery. One reason behind that inefficient DNA uptake was lack of receptor for BSA and saturation of receptors at high unbound, free collagen l. The other one might be due to hydrophilic layer surrounding the particles under these two conditions. Interestingly, when we performed the same experiment in NIH 3T3, a fibroblastic cell type, with collagen 1 embedded DNA/CaP particles, no additional increment in DNA uptake was observed (Figure 6), thus suggesting that these cells have either no receptor or very few receptors for collagen 1. To prove the notion, we checked the integrin receptor levels by observing cell spreading on collagen 1 coated plates since integrin-mediated attachment almost coincides with the event of cell-spreading and thus the phenomenon is considered as strong evidence for cell-surface integrin expression (*22*). As shown in Figure 7, while HeLa cells completely spread within a few hours following addition to collagen 1 coated plate, NIH 3T3 cells showed still round-shape morphology like on hard plate (no coating with collagen 1), thus providing evidence that ECM-carrying DNA/CaP particles are fully dependent on their specific receptors for being internalized by the cells.

To demonstrate the final outcome of efficiently delivered plasmid DNA by virtue of integrin-mediated endocytosis, we assessed the transgene expression level by luciferase assay system (Figure 8). Luciferase expression remarkably increased far beyond 50- and 10-fold at a collagen concentration of 10 μg/mL and a fibronectin concentration of 50 μg/mL, respectively, and then declined with increasing the concentrations of both ECM molecules, suggesting the previous argument of receptor saturation by high amount of free proteins and creation of hydrophilic environment on DNA/CaP particles, so preventing nonspecific adsorptive endocytosis (*23*). On the other hand, no improvement in transfection efficiency could be observed for BSA, but decreased, instead, due to hydrophilic particle surface (*23*). Transfection efficiency was compared with lipofectamine, a widely used transfecting agent, which showed that collagen associated nanocrystals were at least 3 times more effective than lipofectamine under the same experimental conditions

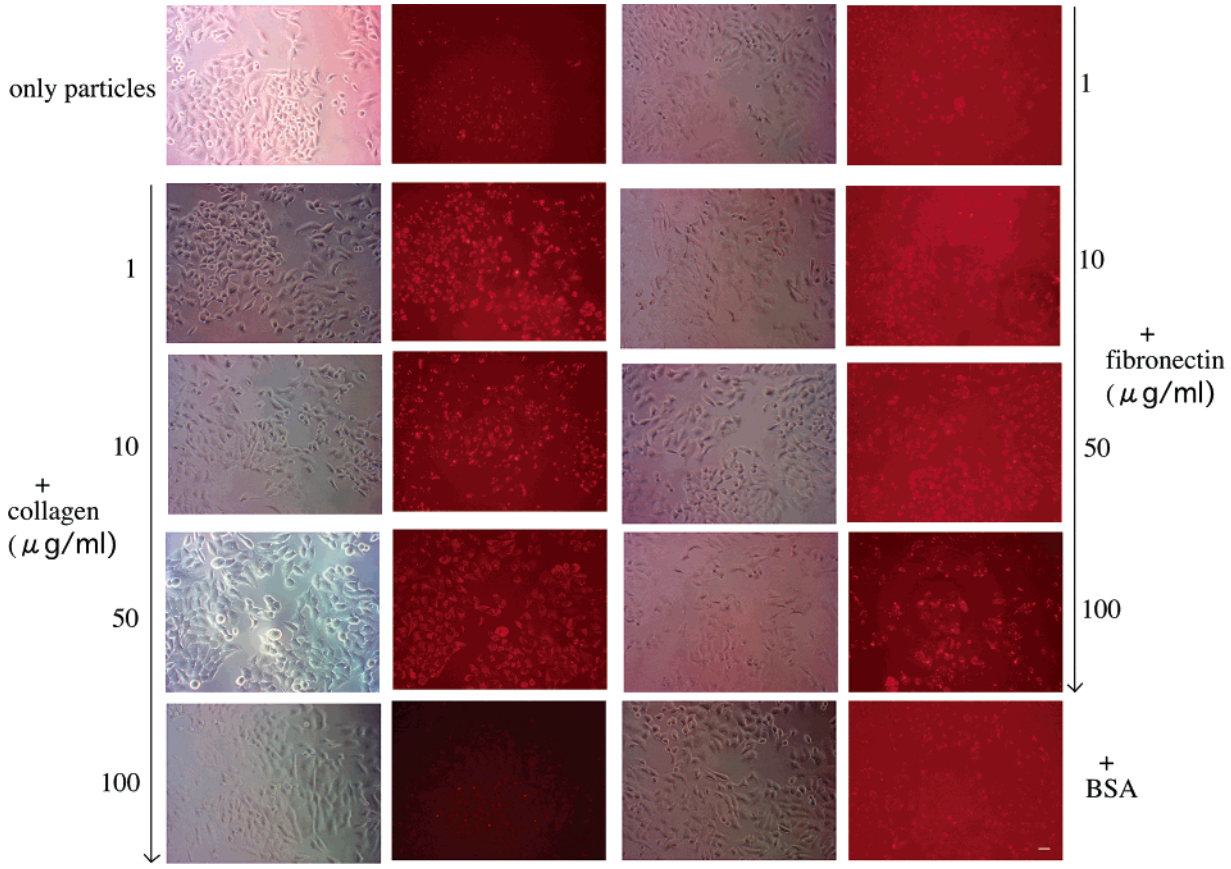

FIGURE 5: Enhanced uptake of DNA in HeLa cells by ECM protein-coated nanoparticles. DNA/CaP particles were prepared (as described in the legend to Figure 1) in the presence of PI before being added onto the cells in the presence of 10% serum-supplemented medium and kept for 4 h in the incubator for cellular internalization. Extracellular particles were removed by EDTA prior to observation of the cells by a fluorescence microscope. Pictures in the first and third columns and those in the second and fourth columns were taken under visible and fluorescent light, respectively. Scale bar, 50 μm.

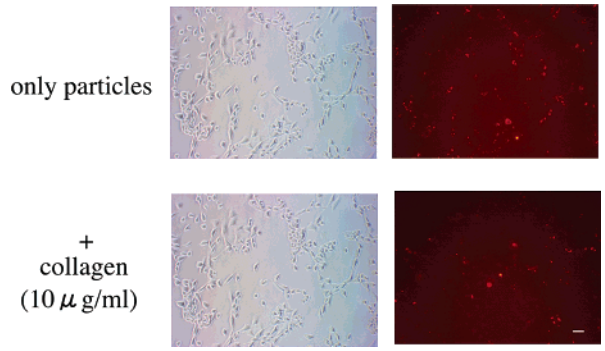

FIGURE 6: Uptake of composite particle-associated DNA by NIH 3T3 cells. DNA/CaP particles were prepared (as described in the legend to Figure 1) with PI before being added onto the cells in the presence of 10% serum-supplemented medium and kept for 4 h in the incubator for cellular uptake. Extracellular particles were removed by EDTA prior to observation of the cells by a fluorescence microscope. Scale bar, 50 μm.

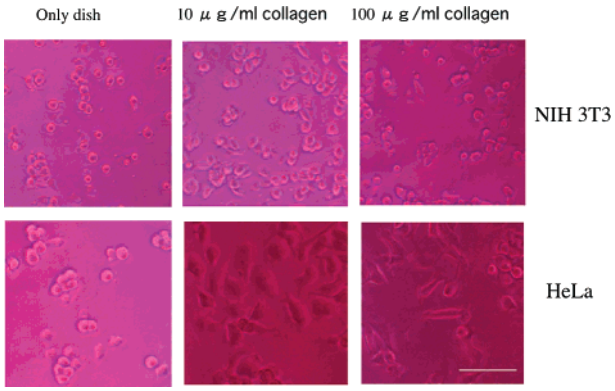

FIGURE 7: Cell adhesion test on collagen 1. NIH 3T3 or HeLa cells were seeded onto a collagen 1 coated plate and allowed for attachment within 2 h before observation of adhesion-dependent spreading on the plate by an optical microscope. Scale bar, 50 μm.

(Figure 9). In NIH 3T3 cells having few or no collagen 1 receptor (Figure 7), almost no enhancement could be seen at 10 μg/mL collagen 1 and noticeably, with increasing the concentrations, a more drastic decrease in transfection activity was found (Figure 10), compared to that observed in collagen 1 receptor bearing HeLa cells (Figure 8). This difference was expected because, even at a high concentration (50 μg/mL) of collagen 1, cellular uptake of DNA was prominent due to integrin-mediated endocytosis (Figure 5) while uptake was inhibited in NIH 3T3 cells owing to the absence of an integrin-mediated binding and the hydrophilic particle surface-assisted diminishment of nonspecific interactions between the cell surface and the particles. All of the evidence, thus, suggested that internalization of the ECM-anchored nanocomposite particles was fully mediated by the corresponding integrin receptor for rapid delivery of genetic material across the plasma membrane for subsequent high level protein expression.

Finally, we have unveiled a new and superior transfection methodologyy based on anchoring in DNA/CaP nanocrystals

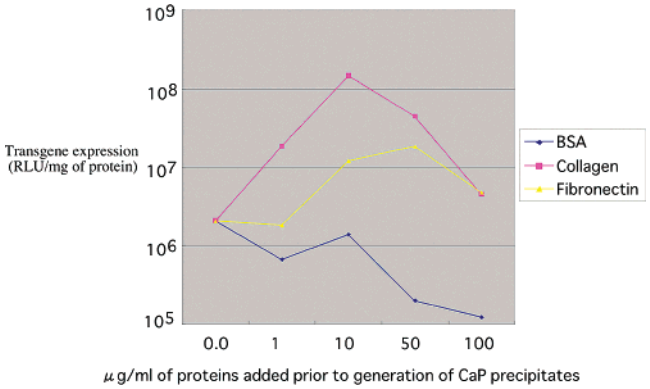

FIGURE 8: Luciferase expression in HeLa cells for ECM protein-associated DNA/CaP nanoprecipitates. Precipitates were generated in accordance with the procedure described in the legend to Figure 1 and added onto the cells being cultured in a well of a 24-well plate in the presence of 10% FBS-supplemented 1 mL DMEM. After incubation for 4 h, cells were rinsed with fresh medium and recultured for 1 day and luciferase expression was quantitated by a luminometer using a luciferase detection kit.

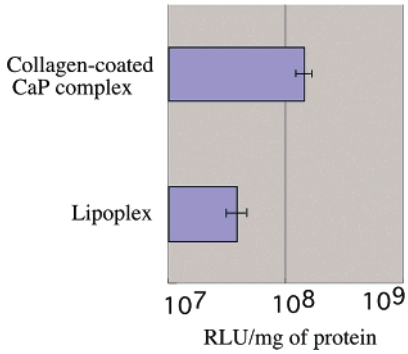

FIGURE 9: Comparison of transfection efficiency between collagen-associated CaP precipitates and lipofectamine. DNA embedded, collagen anchored CaP particles were generated in accordance with the procedure described in the legend to Figure 1, and lipoplexes were formed at a weight ratio (DNA to lipofectamine) of 1:5, followed by addition of each of the transfection complexes in 10% FBS-supplemented DMEM onto HeLa cells being cultured in a well of a 24-well plate. After incubation for 4 h, cells were rinsed with fresh medium and recultured for 1 day and luciferase expression was quantitated by a luminometer using a luciferase detection kit.

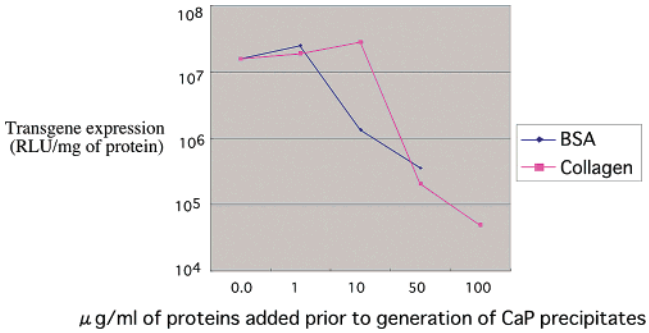

FIGURE 10: Luciferase expression in NIH 3T3 cells for ECM protein-associated DNA/CaP nanoprecipitates. Precipitates were generated according to the protocol mentioned in the legend to Figure 1 and added onto the cells being cultured in a well of a 24-well plate in the presence of 10% FBS-supplemented 1 mL DMEM. After incubation for 4 h, cells were rinsed with fresh medium and recultured for 1 day and luciferase expression was quantified by a luminometer using a luciferase detection kit.

the ECM macromolecules with their exposed integrin-binding domains that could effectively interact with corresponding receptors, ultimately allowing DNA in the hybrid particles to be rapidly internalized and strongly expressed to a desirable protein. Considering the simplicity, level of efficiency, and minimal cost, the technique would emerge as a valuable tool in molecular biology laboratories for routine uses and industries for production of increasingly demanding therapeutic proteins and seems pivotal for gene therapy. Moreover, the new, biomimetic concept for development of biorecognizable inorganic nanoparticles would pave the way for delivery of other therapeutics, such as protein, siRNA, or drug in order to treat and control human diseases.

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