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A protein kinase signal-responsive gene carrier modified RGD peptide

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Abstract—We have previously reported artificial gene-regulation systems responding to cyclic AMP-dependent protein kinase (PKA) using a cationic polymer. However, this polymer alone cannot deliver any gene into living cells. In the present work, we modified the signal-responsive polymer to the RGD peptide for the introduction of a polymer/DNA complex into living cells and succeeded in regulating the gene expression responding to intracellular PKA activation.

Gene therapy is one of the most promising medical approaches.[1–3] However, with regard to gene delivery, it has not been easy to distinguish target abnormal cells from normal cells. Thus the practical use of gene therapy has been strictly limited due to the undesired serious side effects caused by the transgene expression in the undesired normal cells. To overcome this issue, many targeting strategies have been investigated using molecular markers on the target disease cell surface. However, these strategies have not satisfactorily controlled the transgene expression between the target and non-target cells. Another strategy should be coupled to these target strategies for the establishment of an ideal gene therapy. Recently, we proposed another concept that may improve the inter-cellular contrast of the transgene expression. In this concept, we aimed at the use of intracellular information rather than cellular surface information.

Living cells possess a complicated enzymatic cascade to respond to extracellular information, and certain enzymes are often hyperactivated in diseased cells.[4–6] In such enzymes, protein kinases, which catalyze the protein phosphorylations, are one of the most important aspects of the cellular signal-transduction system.[7] Thus, we reported the protein kinase-responsive gene regulation system using a polymer that possessed the target kinase-specific cationic substrate peptides as pendant groups. This polycationic polymer formed an electrostatic complex with DNA and suppressed the gene transcription. However, if the target protein kinase is activated, the pendant substrate is phosphorylated. This event introduces the disintegration of the polymer–DNA complex so that the DNA is released due to the neutralization of the polymer cationic net charges. Thus, the gene expression occurs only in the target protein kinase-activated cell. We termed this concept D-RECS, which refers to DDS responding to cellular signals.

In this study, we tried combining our D-RECS system with an ordinary targeting system. As the target protein kinase, we used cyclic AMP-dependent protein kinase (PKA),[8] because extraordinary activation of the kinase is related to various diseases such as colon cancer[4] and breast cancer.[5,6] In a previous study, we reported the design of a PKA substrate pendant polymer (PAK) and its PKA-specific gene expression in living cells.[9–12] However, this PAK alone cannot deliver any genes into living cells, so that it is difficult to evaluate the function of this polymer in living cells. Thus, an integrin receptor-binding tripeptide, RGD, unit was introduced into the PKA-responsive D-RECS system. Integrin receptors are heterodimer, transmembrane receptors that have several functions (e.g., cell survival, migration, and differentiation), and their high-level expression is well recognized

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in various cancer cells. There are over 25 known integrin receptors, and most of these recognize the tripeptide turn sequence arginine-glycine-aspartic acid (RGD)[13]. An RGD peptides modified polymer (e.g., polyethyleneimine[14]) has efficiently delivered genes into cells, in which integrin receptor was highly expressed, through receptor-mediated endocytosis.

A synthesized new polymer (NPAK-RGD) that possesses the RGD peptide (GRGDS) was composed of poly *N*-isopropyl acrylamide (NIPAM) as a main chain and a substrate peptide of PKA and RGD peptide as side chains. NIPAM has good hydrophobicity and can enhance uptake efficacy of complex. This polymer actually showed integrin receptor-dependent uptake in its DNA complex, and it also showed PKA signal-responsive gene expression.

For the NPAK-RGD, two kinds of polymers, NPAK-RGD2.4 and 1.6, which contained different amounts of RGD unit (2.4 and 1.6 mol%), were prepared. Both polymers were synthesized in a manner similar to that described previously.[9] Thus, 11.9 mg of *N*-isopropyl acrylamide, 1.78 mg of methacryloyl-ALRRASLGW-NH_2 (substrate of PKA), and 0.45 mg (NPAK-RGD1.6) or 0.92 mg (NPAK-RGD2.4) of methacryloyl-Lnk-Lnk-GRGDS-NH_2 (Lnk = –NH–$(CH_2CH_2O)_2$–CH_2CO–) were dissolved in degassed water and allowed to stand at room temperature for 90 min after the addition of 1.05 mg of ammonium persulfate and 1.40 μl of *N*,*N*,*N*′,*N*′-tetramethylethylenediamine as the redox initiator couple. The product was then purified by overnight dialysis against water using a semipermeable membrane bag (with a molecular-weight cutoff of 25,000), followed by lyophilization to obtain a white powder. The resulting NPAK-RGD contained the substrate of PKA and RGD peptide at a concentration of 0.8 mol%, 2.4 mol% (NPAK-RGD2.4), and 1.0 mol%, 1.6 mol% (NPAK-RGD1.6). Lnk was used as the linker to prevent affinity between RGD peptide and integrin receptor from lessening. NPAK which has no RGD peptide was also synthesized in a similar manner. The average molecular weight and M_w/M_n of NPAK were evaluated using GPC (150,000 and 1.93, respectively) (Fig. 1).

We first investigated whether NPAK-RGD formed complexes with DNA using 1% agarose gel electrophoresis. When the NPAK-RGD (charge ratio 0.5, 1.0, 3.0) was added to the pDNA (pEGFP-C1 0.25 μg) solution in PBS (−) including 0.3 mM ATP and 10 mM $MgCl_2$, the migration of the DNA was totally suppressed. This result means that the NPAK-RGD actually formed a complex through the electrostatic interaction. On the other hand, when the activated PKA (25 U) was added to each NPAK-RGD/DNA solution, the band of original DNA was completely recovered (Fig. 2). This result indicated that the PKA signal can disintegrate the complex and release the DNA similar to the previous polymer (PAK).[9]

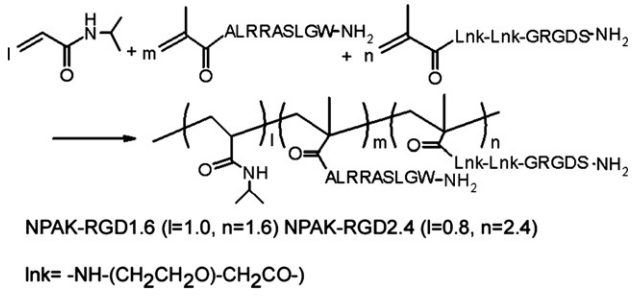

Figure 1. Synthetic scheme and chemical structures of the NPAK-RGDs. Lnk means –NH–$(CH_2CH_2O)_2$–CH_2CO–.

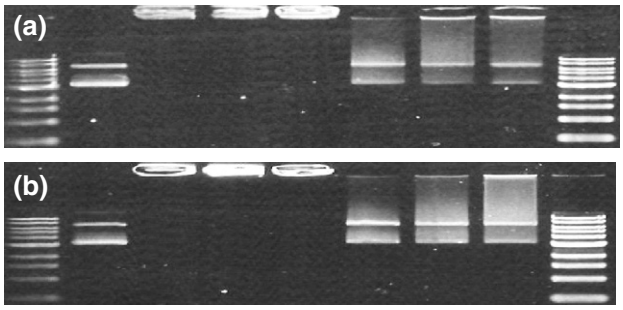

Figure 2. Formation of the NPAK-RGD/DNA complex and its disintegration with PKA signaling. (a) Lane 1, 1 kb DNA ladder. Lane 2, DNA alone. Lanes 3–5 and lanes 6–8 show the electrophoresis of the NPAK-RGD1.6 complex in the absence and presence of activated PKA. (b) Those of NPAK-RGD2.4. The charge ratio was 0.5 in lanes 3 and 6, 1.0 in lanes 4 and 7, and 3.0 in lanes 5 and 8.

Next, we investigated whether the stability change in the NPAK-RGD/luciferase-encoded DNA complex is directly related to the regulation of gene expression using a cell-free expression system (T7 S30 Extract System for Circular DNA; Promega). The luciferase expression was suppressed up to 30–40% in NPAK-RGD/DNA complexes (0.25 μg DNA, charge ratio 1.0 and 3.0). On the other hand, the expression level was significantly recovered to 60–80% compared with that of free DNA 1 h after the addition of activated PKA (25 U) (Fig. 3). The tendency of gene regulation was nearly the same between NPAK-RGD2.4 and NPAK-RGD1.6. These results indicate that NPAK-RGD can regulate gene expression responding to PKA activation.

All the results described above were similar to those obtained in our previous research using PAK.[9] However, PAK itself cannot deliver any genes into living cells. Thus, we then evaluated the effect of the RGD unit in NPAK-RGD on the DNA delivery into living cells, and we also investigated its PKA-responsive regulation ability of transgene expression.

On the other hand, since several studies reported the toxicity of polycationic polymer on cells,[15,16] the NPAK-RGDs, polycationic polymers, may have toxicity on cells. On account of these reasons, we have examined whether the polymers have toxicity on cells. Our results have confirmed that NPAK-RGDs do not significantly affect cell viability (viability >90%) in the concentration range employed in this study, using the WST assay (Fig. 4).

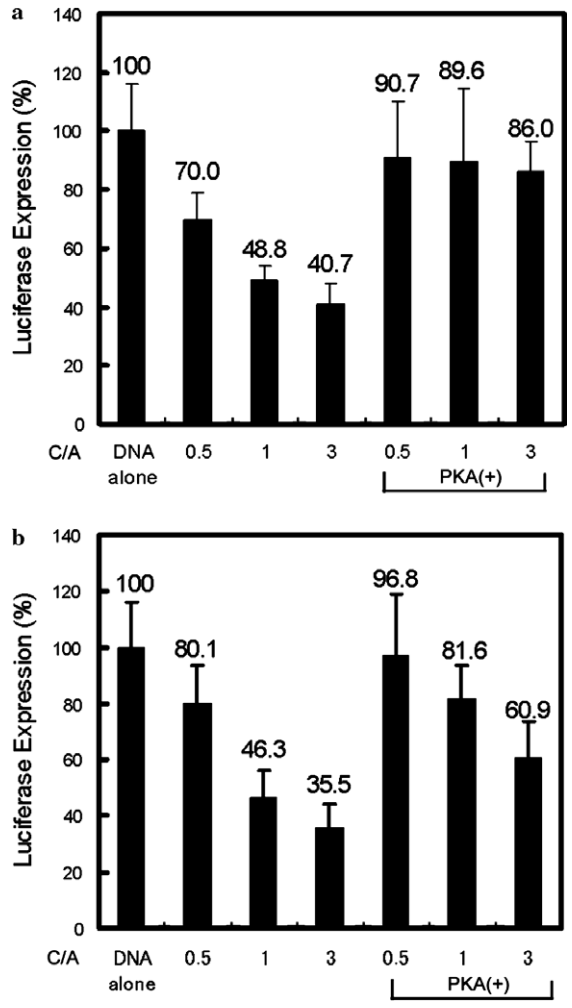

Figure 3. Suppression of luciferase expression with the NPAK-RGDs and their cancellation with PKA signaling in a cell-free system. Luciferase expression in the presence of (a) NPAK-RGD1.6 and (b) NPAK-RGD2.4 with or without activated PKA. Data represent the average ±SEM ($n = 3$).

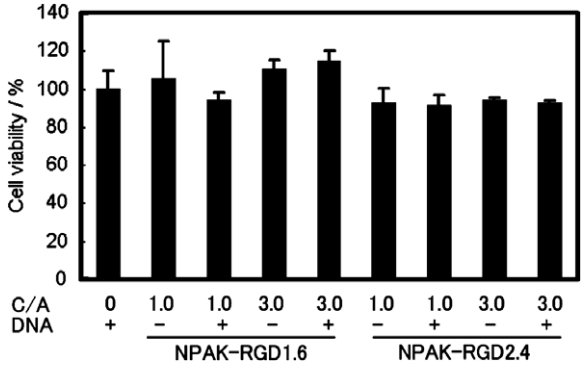

Figure 4. Cytotoxic activities on NPAK-RGDs or complexes of NPAK-RGDs with plasmid DNA at C/N ratios of 1.0 and 3.0. Cell viabilities were evaluated by WST assay. Data represent the average ±SEM ($n = 3$).

NPAK-RGD/DNA complexes that involved 1.25 μg of the luciferase-encoded DNA (charge ratio 1.0 and 3.0) were added to each well, in which HeLa cells were preincubated. To stimulate the cells to activate PKA continuously, the cell sample was treated with Folskolin (Fsk, 10 μM) and isobutylmethylxanthine (IBMX, 100 μM). Fsk activates adenylate cyclase which produces cAMP, and IBMX inhibits phosphodiesterase which degrades cAMP. When the NPAK-RGD2.4/DNA complexes (charge ratio 1.0 and 3.0) were transfected into HeLa cells, in which PKA was activated, the gene expression was approximately three times higher than the case of an unstimulated sample (Fig. 5). Moreover, we confirmed the intracellular activation of PKA using pCRE-Luc and pRL-CMV (CREB assay system) by measuring the luminescence intensity ratios from *Firefly* and *Renilla* luciferase (data not shown). This reporter gene assay using the gene with the CRE promoter has been widely used to assess the intracellular PKA activity. These results indicated that NPAK-RGD/DNA complex was uptaken to cells and regulated gene expression responding to PKA activity. On the other hand, when the NPAK-RGD1.6/DNA complexes were transfected, gene expression was not recovered significantly. The RGD modification ratio appeared to influence the uptake efficiency of the polymer/DNA complexes.

In conclusion, we exploited the novel signal-responsive polymer modified RGD peptide (NPAK-RGD) and characterized it. The polymers, which possessed the RGD peptide in different concentrations (NPAK-RGD 1.6 and 2.4), can form a complex with DNA through the electrostatic interaction and suppress gene expression. However in the presence of activated PKA, the complex was disintegrated and gene expression was recovered. Moreover, we indicated that NPAK-RGD2.4 could deliver a gene into a living cell and regulate the gene expression, responding to intracellular PKA activation. Since this modification strategy can possibly be applied to other peptides (e.g., cationic NLS peptide and anionic endosome-disruptive peptide[17]) or other small molecules for cellular targeting (e.g., sugar), it may be useful for realizing a more ideal delivery system.

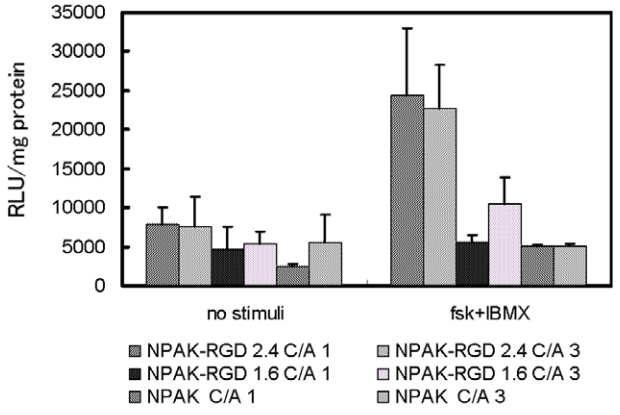

Figure 5. Delivery of the luciferase-encoding DNA/NPAK-RGD complexes to PKA stimulated or non-stimulated cells. Data represent the average ±SEM ($n = 3$).

Acknowledgment

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