Communication

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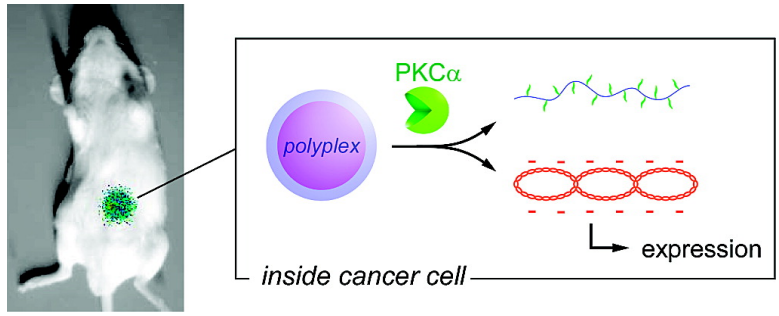

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Design of Polymeric Carriers for Cancer-Specific Gene Targeting: Utilization of Abnormal Protein Kinase Cα Activation in Cancer Cells

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Development of gene carriers for safe and effective gene therapy has been extensively studied for two decades.[1] Currently, gene targeting to a disease site is one of the most important aspects of gene therapy. This is especially true for cancer and inflammatory disease gene therapy, in which therapeutic genes with the ability to induce destruction of transfected cells are used.[2] In this regard, the following two approaches have been developed: an active targeting strategy utilizing disease cell-specific ligand molecules,[3] and plasmid DNA (pDNA) engineering in which disease cell-specific promoters were utilized.[4]

As an alternative disease cell-specific gene targeting strategy, we recently proposed the concept of a gene transcriptional regulation system that responds to malfunctions of signal transduction occurring inside disease cells. In this system, hydrophilic polymers, grafted with cationic peptides, are used for preparation of polyplex with pDNA (Figure 1).[5] Transcription of pDNA is repressed when it forms a polyplex, due to steric hindrance. The grafted cationic peptides are substrates for the target signal transduction proteins (STPs), which are activated specifically in disease cells, and which decrease the peptide cationic charge, resulting in release of pDNA from the polyplex (Figure 1 shows the case of protein kinase as an STP). We called the targeting strategy a "drug or gene delivery system responding to cellular signals (D-RECS)".[5] In our previous reports, we succeeded in activating gene expression, in response to certain STPs, such as protein kinase A[5a] and caspase-3[5b]. However, these STPs were not practical because chemical stimulation was used to raise their low activities in cells. Moreover, another obstacle was the poor transfection ability of the polyplex due to its neutral surface charge; to achieve transfection we had to encapsulate the polyplex into an inactivated virus envelope.[5]

In this study, we aimed to develop our gene delivery system to be cell-permeable without the assistance of such formulation and to be practically responsive to a disease cell-specific STP. Here we focused on protein kinase Cα (PKCα) as a practical marker of cancer cells because PKCα is known to be activated in many cancers and have negligible activity in other normal tissues.[6a] Generally, it is difficult to find a substrate peptide for a certain kinase with sufficient selectivity and reactivity. In particular, a selective PKCα substrate has not been found because the PKC family is composed of many isozymes. Recently, we built a library composed of more than 1700 candidate peptides which were designed based on a computer program (Scansite). Peptide **1** (H-FKKQGSFAKKK-NH_2) was isolated as the most selective and sensitive substrate for PKCα; we named the peptide Alphatomega.[7] First, we examined the reactivity of peptide **1** toward intracellular PKCα of typical cancer cell lines. As shown in Figure S1, a high degree of phosphorylation was observed in lysates of all cell lines examined, while the phosphorylation was mostly inhibited (<10%) in the presence of 1.0 μM of a PKCα-selective inhibitor (Ro31-7549), indicating the specificity of peptide **1** toward PKCα. Furthermore, peptide **1** was highly phosphorylated by tumor lysates, which were obtained by xenografting cancer cell lines into mice, whereas only a low level of phosphorylation (<13%) was observed in normal tissue lysates. These results indicated that peptide **1** is a selective and sensitive substrate for cancer cells and tissues.

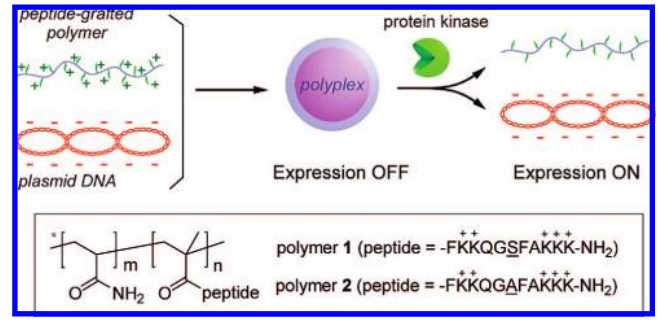

Figure 1. Polyplex formation from plasmid DNA and hydrophilic copolymer grafted with cationic substrate peptide, and dissociation of the polyplex induced by phosphorylation of the grafted peptide by protein kinase that reduces the cationic charge of the substrate peptide. Chemical structures of PKCα-responsive polymer **1** and its negative control polymer **2** are shown below.

We then synthesized polymers **1** and **2** which carry peptide **1** and its negative control peptide (the phosphorylation site Ser was substituted with Ala), respectively, by radical copolymerization between acrylamide and peptide macromonomer[5] (Table S1; M_w and macromonomer contents of the polymers). The macromonomer contents were raised (>5 mol%) compared with our previous reports; this will provide a highly cationic character to the polyplexes, resulting in an increase in cell membrane permeability. Polyplexes were prepared from the polymers and pDNA with various cation/anion (C/A) charge ratios (0.5, 1.0, and 2.0). The average diameters of the both poplyplexes formed from polymers **1** and **2** were ca. 200 nm irrespective of the C/A ratio (Table S2). The ζ-potential values of the polyplexes increased with the increase of C/A ratio and became highly positive (>20 mV) at 2.0 (Table

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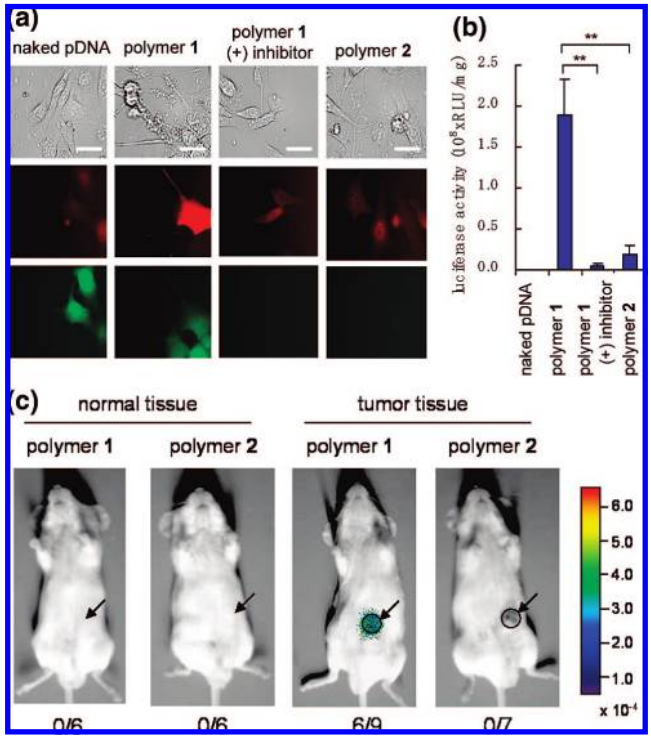

Figure 2. (a) GFP expression in B16 melanoma cells 24 h after microinjection of polyplexes (C/A = 2.0). Red and green colors result from Texas Red and GFP, respectively. Scale bars, 10 μm. (b) Luciferase expression in B16 melanoma cells 24 h after transfection of polyplexes (C/A = 2.0). Error bars represent standard deviation of three experiments. **, $P < 0.01$. (c) Images of luciferase activity in normal subcutaneous and xenografted B16 melanoma tumor 24 h after direct injection of polyplexes of polymer **1** or **2** (C/A = 2.0). Numbers of luciferase expressing mice per total mice are described below. Arrows and circles indicate the injection site of polyplexes and xenografted-tumor tissue, respectively.

S2). Moreover, no cytotoxicity of the developed polymers toward cells was found (Figure S2).

We confirmed whether such high positively charged polyplexes (C/A = 2.0) can still result in phosphorylation of grafted peptide **1** and then release pDNA. The dissociation of the polyplex triggered by the enzymatic reaction of PKCα was monitored by the change of laser light scattering intensity of the polyplex dispersion (Figure S3a). Simultaneously, the phosphorylation of grafted peptide **1** in the polyplex was monitored using a coupled-enzyme assay[9] (Figure S3b). The abrupt decrease of the scattering intensity of the polyplex prepared from polymer **1** was synchronized with the decrease of absorbance at 340 nm (A_{340}), resulting from the consumption of ATP by phosphorylation. By contrast, changes in scattering intensity and in A_{340} were negligible in the polyplex from control polymer **2**. This result clearly indicated that the phosphorylation of the grafted peptide **1** readily took place, which weakened the polyplex due to the net cationic charge decrease (+5 to +3).

We then examined whether the polyplexes can release pDNA in response to the intracellular concentration of PKCα and whether gene expression occurs from the released pDNA. The polyplexes, assembled from the two polymers and GFP-encoding pDNA, were directly microinjected into B16 melanoma cells, which show hyperactivation of PKCα (Figure S1). Texas Red modified dextran was mixed with the polyplex dispersion and coinjected to mark the polyplex-injected cells. As shown in Figures 2a and S4, in the case of control polymer **2**, expression of GFP was completely suppressed at a C/A ratio of 2.0. By contrast, the polyplexes of polymer **1** afforded expression of GFP. The expression from the polyplex composed of polymer **1** was completely suppressed in the presence of a selective inhibitor of PKCα, indicating that efficient expression from the polyplexes of polymer **1** was triggered by enzymatic reaction of intracellular PKCα. A similar stark contrast between polyplexes of polymers **1** and **2** was also observed in HuH-7 cells (Figure S5a and b). The transfection ability of the polyplexes was then tested using luciferase-encoding pDNA (Figures 2b, S5c and S6). The results showed the same tendency as the microinjection experiments; the polyplex of polymer **1** (C/A = 2.0) showed much higher levels of luciferase expression compared with that of polymer **2**. Therefore, due to the highly positive ζ-potential values of the polyplexes, they indeed became cell membrane permeable.

Finally, we conducted an *in vivo* evaluation of our tumor-specific gene expression system. Model mice xenografted with B16 melanoma cells into the subcutaneous tissue were used. Polyplexes between the polymer and luciferase-encoding pDNA (C/A = 2.0) were directly injected into xenografted tumors or into normal subcutaneous tissue, and luciferase expression was evaluated using an *in vivo* imager (Figure 2c). When the polyplexes of polymer **1** were injected into the normal subcutaneous tissue, none of the six mice examined showed luciferase activity. On the other hand, when the polyplexes were injected into the xenografted tumor, more than half of the mice (6/9) showed luciferase expression. In the case of polymer **2** polyplex, the expression was completely suppressed (Figure S7). Moreover, the level of activated PKCα was higher in the B16 tumor than in normal tissue (Figure S8). These results clearly demonstrated that our strategy enables tumor-specific expression by utilizing the activation of PKCα in cancer cells.

In summary, we successfully developed our system to be transfectable, as well as to be applicable to cancer-specific gene targeting, both *in vitro* and *in vivo*. To our knowledge, this is the first report that utilizes the activation of protein kinase for cancer cell specific gene expression. For gene therapy that aims to kill cells, gene expression in normal cells must be avoided. Because our system can be combined with conventional disease-site targeting approaches, such as receptor-directed delivery and the promoter design of pDNA, more specific and safer gene therapy will be realized.

Supporting Information Available: Details of experimental procedures. This material is available free of charge via the Internet at http://pubs.acs.org.

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