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Rapid communication

Liver-specific expression of an exogenous gene controlled by human apolipoprotein A-I promoter

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ABSTRACT

Liver-specific gene therapy is advantageous to minimize the possible adverse effects caused by non-target gene expression. The CMV promoter of the enhanced green fluorescent protein (EGFP) expressing plasmid pCMV-EGFP was replaced with the liver-specific promoter apolipoprotein A-I (ApoAI) generating pApoAI-EGFP plasmid. *In vitro* expression experiments performed in various cell lines including HepG2, SMMC-7721, MCF7, ACC-2 and Lo2 indicated that pCMV-EGFP treatment caused gene expression in all the cell lines, whereas pApoAI-EGFP treatment only induced EGFP expression in cells of liver origin including the liver cancer cells HepG2 and SMMC-7721 and the normal liver cells Lo2. Either pCMV-EGFP or pApoAI-EGFP was formulated as pegylated immuno-lipopolyplexes (PILP), a novel and efficient gene delivery system. Following intravenous administration of the PILP in H22 tumor-bearing mice, there was significant EGFP expression in liver, tumor, spleen, brain and lung in the pCMV-EGFP treated mice, whereas in the pApoAI-EGFP treated mice there was only gene expression in liver and tumor and the non-liver organ gene expression was eliminated. This study suggests that the use of the PILP technology and liver-specific promoter enables efficient and liver-specific expression of an exogenous gene.

Hepatocellular carcinoma (HCC) accounts for over 90% of all primary liver cancers and leads to nearly one million deaths annually because HCC responds poorly to conventional chemotherapy (Bosch et al., 2004; Roberts and Gores, 2005) and frequently recurs shortly after surgical or nonsurgical treatments (Venook, 1994; Chen et al., 1994). To overcome these clinical problems, newly established treatments, such as gene therapy, can be combined with the conventional treatments.

In order to succeed in cancer gene therapy, the efficient delivery of therapeutic genes to a target site is a major challenge. We have developed a novel and efficient non-viral gene delivery system—pegylated immuno-lipopolyplexes (PILP), which is a ternary complex formed with anionic liposomes, cationic polymer and DNA (Hu et al., 2010). This is the second generation of non-viral gene delivery vectors that can improve gene transfer compared to the first generation of non-viral gene delivery vectors represented by lipoplexes and polyplexes. The plasmid DNA is compacted by PEI in the particle. The surface of the particle (lipopolyplexes) is decorated with strands of polyethylene glycol (PEG) to promote stabilization in the bloodstream and the tips of the PEG strands are conjugated with a targeting monoclonal antibody (MAb). This peptidomimetic MAb triggers receptor-mediated endocytosis into tumor cells. The pattern of gene expression *in vivo* is determined by the receptor specificity of the targeting MAb. Intravenous administration of the PILP resulted in the highest gene expression of the reporter genes enhanced green fluorescent protein (EGFP) and luciferase in liver and tumor, and the second highest gene expression in spleen and measurable gene expression in lung and brain (Hu et al., 2010).

For liver cancer gene therapy, expression of therapeutic genes in non-liver organs is not expected and may be eliminated by the use of a liver-specific promoter such as apolipoprotein A-I (ApoAI) (Higuchi et al., 1988). Furthermore, ApoAI promoter was reported to associate with low hepatotoxicity, much more stable transgene DNA levels, and an absence of promoter attenuation (De Geest et al., 2000, 2001). However, so far liver-specific gene expression induced by ApoAI promoter has not been validated yet *in vivo* by either a viral or non-viral gene transfer approach. Therefore, the purpose of the present study is to test the hypothesis that the expression of an exogenous gene can be restricted to the liver *in vivo* with the use of both the PILP gene targeting technology and a liver-specific promoter.

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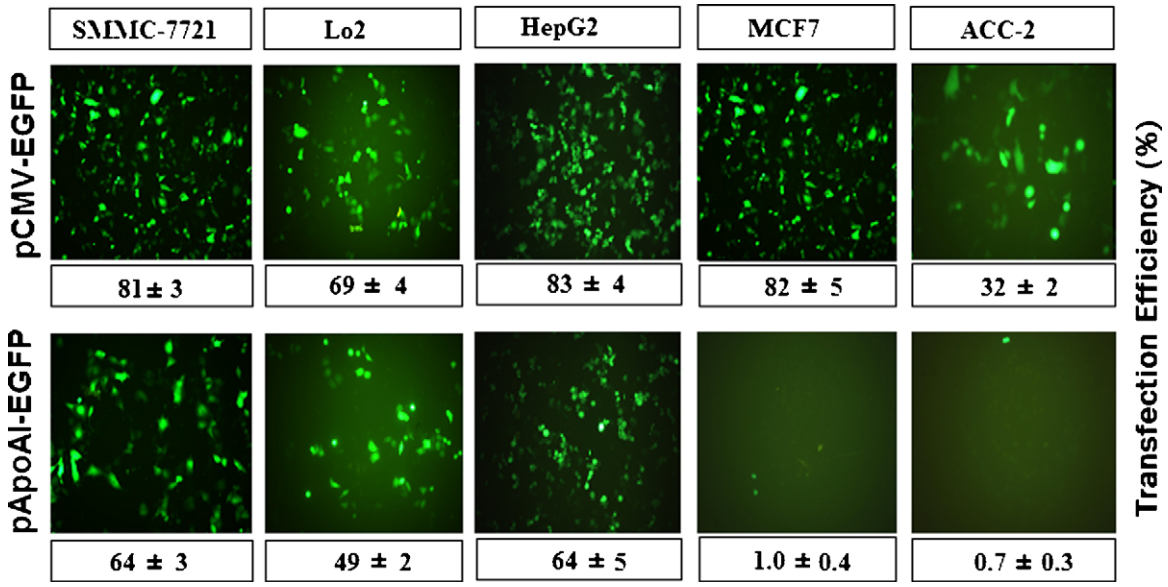

Fig. 1. Comparison of EGFP transfection efficiency in different cell lines. The cells were seeded in 6-well plates and incubated in DMEM or 1640 medium supplemented with 10% serum at 37 °C. pCMV-EGFP or pApoAI-EGFP plasmid was transfected into different wells of cells with ExGen 500 at a dose of 3 μg of DNA per well. Forty-eight hours later fluorescent microscopy was performed to visualize EGFP expression, and percent transfected cells (transfection efficiency) were measured using a FACS Calibur flowcytometer. Transfection efficiency was expressed as mean ± SE of five measurements.

The vector pApoAI-EGFP, the EGFP expressing plasmid under the control of liver-specific promoter ApoAI was constructed as described previously (Ren et al., 2009). Briefly, the −290 to +209 bp region of ApoAI promoter (GenBank Accession number: J04066) was PCR amplified from the ApoAI promoter harboring vector pAI-W, and was then inserted into pCMV-EGFP vector to replace the CMV promoter.

Five cell lines were used in the *in vitro* transfection experiment. HepG2 and SMMC-7721 are cancer cell lines derived from human liver carcinomas. MCF7 and ACC-2 are two cancer cell lines of non-liver origin, and Lo2 is a normal liver cell line. HepG2 and Lo2 cells were cultured in DMEM with 10% heat-inactivated fetal bovine serum. MCF7, SMMC-7721 and ACC-2 cells were grown in RPMI 1640 supplemented with 10% FBS. All aforementioned cells were seeded in 6-well plates at a density of 2.0×10^5 cells/well. When the cells reached about 70–80% confluence, pCMV-EGFP and pApoAI-EGFP were transfected into different wells of cells with ExGen 500 (Glen Burnie, MD, USA) at a dose of 3 μg of DNA per well. After a 48-h incubation at 37 °C, a fluorescent microscope (Leica) was used to qualitatively visualize expression of EGFP. Mean fluorescence intensity per cell and percent transfected cells (transfection efficiency) were measured using a FACS Calibur flowcytometer (BD Biosciences). Transfection efficiency was expressed as mean ± SE of five measurements. The mean fluorescence intensity per positive cell was normalized against the fluorescence intensity of untransfected cells (control) to account for cellular auto-fluorescence. The result (Fig. 1) showed that there was extensive EGFP expression in all the pCMV-EGFP treated cell lines with a transfection efficiency varying from 32% to 83%. The mean fluorescence intensity per cell of SMMC-7721, Lo2, HepG2, MCF7 and ACC-2 is 3200 ± 410, 3100 ± 320, 3400 ± 520, 3300 ± 280, and 2500 ± 280, respectively. In contrast, among the five pApoAI-EGFP treated cell lines EGFP was only expressed in the cell lines of liver origin including liver cancer cell lines HepG2 and SMMC-7721 with a transfection efficiency of 64% and normal liver cell line Lo2 with a transfection efficiency of 49%, but there was no EGFP expression in cell lines of non-liver origin including MCF7 and ACC-2. The mean fluorescence intensity per pApoAI-EGFP treated cell of SMMC-7721, Lo2, HepG2, MCF7 and ACC-2 is 2400 ± 330, 2200 ± 410, 2200 ± 340, 0, and 0, respectively. This suggests that a plasmid under the control of the liver-specific promoter ApoAI is able to restrict the expression of an exogenous gene in cells of liver origin.

In order to test the *in vivo* liver-specific gene expression in mice, either pCMV-EGFP or pApoAI-EGFP plasmid was formulated as pegylated immuno-lipopolyplexes (PILP) as described previously (Hu et al., 2010). Female BALB/c mice at age of 6–8 weeks were obtained from Experimental Animal Center of University of Zhengzhou, kept in filter-topped cages with standard rodent chow and water available ad libitum, and a 12 h light/dark cycle. The experiments were performed according to the national regulations and approved by the local animal experiments ethical committee. Subcutaneous hepatocellular carcinoma tumors were induced by inoculation of 1×10^7 mouse hepatocellular carcinoma H22 cells in the flank. When the tumor volume reached 0.5–1 cm^3, groups of female BALB/c mice ($n = 5$) were intravenously administered with saline or 40 μg/mouse of either pCMV-EGFP or pApoAI-EGFP plasmid formulated as PILP. To prepare the PILP, the rat anti-mouse transferrin receptor monoclonal antibody 8D3 was used. Mice were sacrificed 48 h after the injection and pieces of tumor, lung, liver, spleen and brain were removed and frozen in OCT (optimal cutting temperature) medium for qualitative analysis of EGFP expression. Ten micron sections were prepared on a cryostat and fluorescent microscopy was performed using a Leica-SP2 fluorescent microscope with an argon laser for blue light excitation at 488 nm. Percent transfected cells (transfection efficiency) were calculated based on the numbers of transfected and un-transfected cells in five fields under the microscope.

The result (Fig. 2) showed that pCMV-EGFP treatment caused the highest gene expression in liver and tumor with a transfection efficiency of 55% and 64%, respectively, and also detectable GFP signal in spleen, brain and lung with a transfection efficiency of 42%, 18% and 10%, respectively, which is in accordance with our previous report (Hu et al., 2010). In contrast, pApoAI-EGFP treatment only caused high GFP expression in liver and tumor with a transfection efficiency of 40% and 48%, respectively, but there was no detectable GFP expression in spleen, brain and lung. There was no GFP expression in all these organs in the control group. It is well

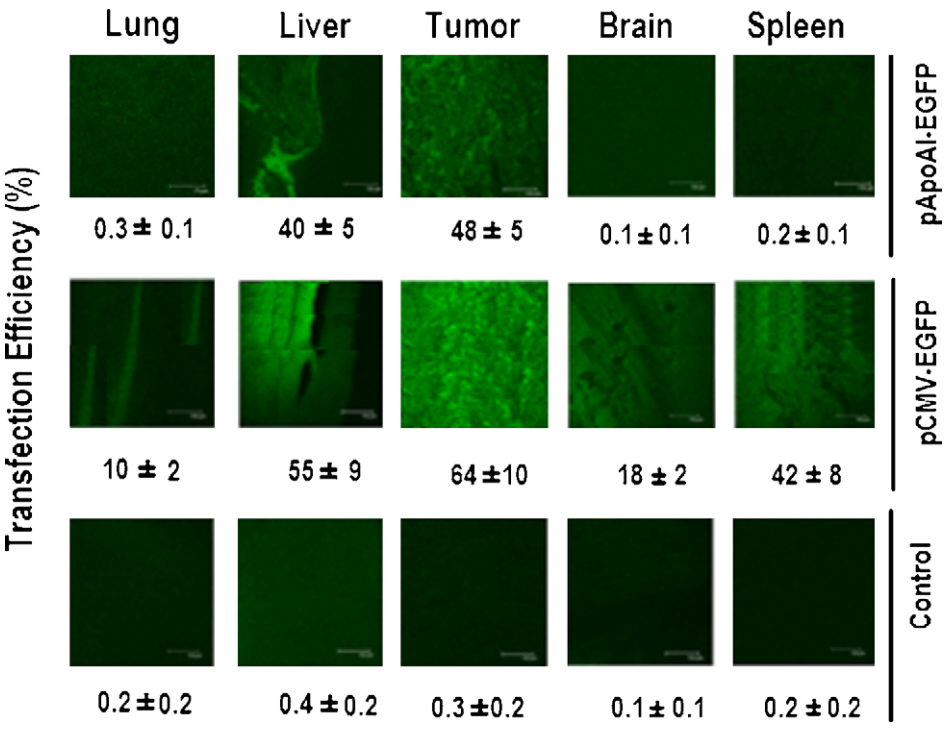

Fig. 2. *In vivo* gene expression. EGFP gene expression in liver, tumor, lung, brain, and spleen was determined by fluorescent microscopy at 48 h after intravenous injection of saline (control), 40 μg/mouse of pCMV-EGFP or pApoAI-EGFP plasmid formulated as PILP to female BALB/c mice ($n = 5$). Scale bars in all panels are 150 μm. Percent transfected cells (transfection efficiency) were calculated based on the numbers of transfected and un-tranfected cells in five fields under the microscope.

known that CMV promoter does not have tissue specificity and the pattern of gene expression *in vivo* mediated by PILP is determined by the receptor specificity of the targeting MAb. In this study, the targeting MAb was rat anti-mouse transferrin receptor monoclonal antibody 8D3. Mouse transferrin receptor was over-expressed in tumors and highly expressed in liver, spleen, and brain (Calzolari et al., 2007; Shi et al., 2001). That is why pCMV-EGFP treatment caused different levels of gene expression in these organs. The net charge of the PILP was positive (Hu et al., 2010). The positively charged DNA complexes can be rapidly sequestrated by lung after intravenous administration (Tros de Ilarduya et al., 2003). Therefore there was also GFP expression in lung in the pCMV-EGFP treated mice.

Apolipoprotein A-I is the major protein component of high density lipoproteins (HDL), which play a key role in reverse cholesterol transport (RCT). RCT is a very important antiatherogenic process that results in the removal of cholesterol (Chol) excess from peripheral cells, and its transport toward Chol metabolizing organs as liver or steroidogenic tissues (Gonzalez et al., 2008; Groen et al., 2004). ApoAI gene is expressed primarily in the liver and small intestine (Jones and Leffak, 1999; Ge et al., 1994). The −256 to −41 bp DNA region upstream from the transcription start site (+1) of the human ApoAI gene contains regulatory elements which are necessary and sufficient for expression in hepatoma (HepG2) cells and in the livers of transgenic mice but are not sufficient for expression in intestinal carcinoma (Caco-2) cells and the intestines of these animals (Higuchi et al., 1988; Sastry et al., 1988; Walsh et al., 1989). It was reported that transcriptional activity of the ApoAI gene in liver cells is mediated by a powerful hepatocyte-specific transcriptional enhancer located between nucleotides −222 and −110 bp in the ApoAI gene 5′-flanking region (Widom et al., 1991). This enhancer contains three sites, A (−214 to −192 bp), B (−169 to −146 bp), and C (−134 to −119 bp), each of which binds one or more nuclear proteins in HepG2 cells. Sites A, B, and C have no significant activity on their own even when they are individually multimerized. However, their combination, as it occurs in the ApoAI gene enhancer, results in very high levels of activity (Widom et al., 1991). In this study we chose the −290 to +209 bp region to be cloned by PCR as ApoAI promoter. Our results confirmed that ApoAI promoter enabled liver-specific gene expression *in vivo*. However, we noted that the gene expression level induced by ApoAI promoter was significantly lower than that induced by CMV promoter, which is in agreement with previous reports (De Geest et al., 2000, 2001). CMV, SV40 or apolipoprotein E enhancers may be incorporated to increase the gene expression level and persistence (Xu et al., 2001; De Geest et al., 2000, 2001).

Our PILP gene delivery system has a targeting monoclonal antibody 8D3, which is a rat anti-mouse transferrin receptor monoclonal antibody (MAb). This peptidomimetic MAb triggers receptor-mediated endocytosis into cells that express transferrin receptor. It was reported that hepatocytes have more than three times more transferrin receptors than do nonparenchymal cells (Vogel et al., 1987), which may be favorable for hepatocytes to get more transgenes. Furthermore, ApoAI was reported to be only produced in liver parenchymal cells (Pape et al., 1991). Therefore, the liver expression may be restricted to hepatocytes, which needs to be confirmed by further experiments.

For possible application of this novel method in treatment of human liver cancer, the human insulin receptor monoclonal antibody 8314 may be used to target the transgene to liver since insulin receptor is substantially expressed on human (including Old World primates like rhesus monkeys) liver cells as well as the blood vessels that perfuse the liver cells (Zhang et al., 2003). A smaller particle size should be favorable for the particles to pass through the liver sinusoidal fenestrae, which could result in higher gene transfection efficiency. Wisse et al. (2008) reported a smaller size of liver fenestrae in humans than that in rodents. The size of the current particle is about 135 nm, which is a little bit larger than the reported human liver fenestrae size 107 ± 1.5 nm (Wisse et al., 2008). Therefore, further evaluation of the novel gene delivery system in non-human primates like rhesus monkeys may be necessary.

Our findings indicate that with the PILP technology an exogenous gene can be efficiently delivered to liver and tumor and that with the use of liver-specific promoter the gene expression in non-liver organs can be eliminated.

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