1. Introduction

In recent years, one of the main focuses of the effort to improve cancer treatment and patient prognosis has been gene therapy. Furthermore, the complex nature of cancer has meant that a variety of therapeutic strategies have been developed along two main avenues: local gene therapies and systemic gene therapies (Table 1). The strategies for local cancer gene therapy include suppression of an oncogene, activation of a tumor suppressor gene, and introduction of a suicide gene into cancer cells (McCormick 2001). Unfortunately, delivering a therapeutic gene to every individual cancer cell in a patient with metastatic cancer has so far proven to be an insurmountable task. It has not been possible to treat all cancerous lesions, which can include undetectable ones such as individual cancer cells and micrometastases. In addition, it is difficult to selectively target cancer cells without affecting normal cells. On the other hand, systemic cancer gene therapy such as immunotherapy appears more promising for both inhibiting tumor growth and preventing metastasis. This chapter will summarize the utility of viral vector-mediated systemic cancer gene therapy in the treatment of human malignancies, focusing on the powerful and promising approach of recombinant adeno-associated virus (AAV)-based systemic anti-angiogenic cancer gene therapy.

2. Muscle-directed systemic cancer gene therapy

2.1. Why gene therapy?

Because a neovasculature is essential for tumor growth and metastasis (Folkman 1971), inhibiting the development of the tumor vasculature using anti-angiogenic agents has emerged as an attractive new strategy for the treatment of cancer (Kerbel & Folkman 2002). For many types
of cancer, targeted biological therapies that selectively interfere with tumor angiogenesis have the potential to improve survival among patients, and a number of angiogenesis inhibitors are currently being tested in clinical trials (Shojaei 2012). In addition, preclinical studies using purified anti-angiogenic factors have indicated the ability of anti-angiogenic compounds to minimize the size of established tumors. However, although they are capable of significantly inhibiting tumor cell growth in animal models, the clinical efficacy of administering purified anti-angiogenic factors would likely be limited by the peptides’ short half-life. On the other hand, synthesis and secretion of anti-angiogenic factors following gene transfer may overcome that limitation. If so, gene therapy could play an important role in this field, as anti-angiogenic factors need to be delivered for long periods to control the progression of tumors.

| Strategy                      | Target cells                      |
|-------------------------------|-----------------------------------|
| 1. Local gene therapy         |                                   |
| 1) Suppression of oncogenes   | RNAi, antisense etc.              |
| 2) Activation of tumor suppressor genes | p53 etc.                  |
| 3) Suicide gene therapy       | HSV-TK                            |
| 2. Systemic gene therapy      |                                   |
| 1) Immuno gene therapy        | Cytokine genes etc.              |
| 2) Anti-angiogenic gene therapy | Endostatin etc.            |

Table 1. Strategy of cancer gene therapy

2.2. Why anti-angiogenic?

Anti-angiogenic gene therapy has several advantageous features (Table 2). As mentioned above, this strategy can potentially suppress both the main tumor and small metastatic tumors. Moreover, since angiogenesis is essential for the development of all tumors, this strategy could be applied to a wide variety of cancers. In contrast to genetic therapies targeting tumor cells directly, anti-angiogenic gene therapy does not require the targeting and transduction of cancer cells. Instead, systemic levels of anti-angiogenic factors may be achieved by targeting non-tumor cells, which provide a stable platform for transgene expression and subsequent secretion of the translated proteins. Finally, cancers do not develop resistance to anti-angiogenic therapy, and the patients experience no side effects or side effects that are much milder than those associated with conventional anti-cancer therapies.

| Advantage                           |
|-------------------------------------|
| 1. Effective for almost all cancers |
| 2. Inhibition of tumor growth and prevention of metastases |
| 3. Targeting gene transfer is unnecessary |
| 4. No resistance                     |
| 5. No or mild side-effect            |

Table 2. Advantages of anti-angiogenic Cancer Gene Therapy
2.3. Why muscle directed?

To express therapeutic proteins, skeletal muscles are considered an attractive target for gene delivery because they are large, have a good capacity for protein synthesis and are easily accessible for intramuscular injection. In addition, muscle fibers are capable of expressing and secreting biologically active gene products that they do not normally synthesize (Arruda et al. 2001). Thus, direct injection of viral vectors into muscles has been widely used for both the treatment of muscular disorders and for expression of therapeutic proteins used for the treatment of metabolic disease, genetic bleeding disorders and malignant diseases (Liu et al. 2004; Noro et al. 2004; Yan et al. 2005).

2.4. Why an AAV vector?

The potential of anti-angiogenic gene therapy in cancer is currently being evaluated using viral and non-viral vectors. The development of an effective gene delivery system is absolutely critical to the effectiveness and safety of gene therapy. Among the several gene transfer strategies being considered at present, the AAV vector appears the most promising, in view of its lack of pathogenicity, wide tropisms and long-term transgene expression in vivo. Gene therapy studies using different serotypes of recombinant AAVs as delivery vehicles have demonstrated that AAVs are an effective modality for cancer gene therapy that meet the requirements necessary for anti-angiogenic therapy.

2.5. A concept of muscle-directed systemic cancer gene therapy

Figure 1 illustrates the concept of muscle-directed systemic cancer gene therapy. First, an AAV vector encoding anti-angiogenic agents is injected into a muscle. After a single injection, secreted anti-angiogenic agents are circulating throughout the entire body. These circulating factors suppress both the primary tumor and undetectable metastatic lesions through inhibition of tumor angiogenesis. If the gene therapy alone is not sufficient to suppress all the tumors, other therapies such as radiation, chemotherapy or immunotherapy can be added to enhance the effect.

Figure 1. A concept of muscle-directed systemic cancer gene therapy
3. Applications of systemic cancer gene therapy

3.1. Systemic anti-angiogenic cancer gene therapy

To assess the feasibility of anti-angiogenic gene therapy using an AAV vector, we constructed an AAV vector encoding murine endostatin (AAV/mEnd) (Figure 2A), which is a tumor-derived angiogenesis inhibitor and is the first endogenous inhibitor of angiogenesis to be identified in a matrix protein (O’Reilly et al. 1997). We then attempted to use the vector to treat pancreatic cancer in an orthotopic model. When PGHAM-1 derived from chemically induced hamster pancreatic cancer cells is injected into the pancreas of hamsters, a ductal adenocarcinoma develops that closely resembles the human disease and, like its human counterpart, it frequently metastasizes to the liver (Matsushita et al. 2001; Yanagi et al. 2000). After AAV/mEnd (5x10^10 vector genomes) was intramuscularly injected into the left quadriceps, we assessed the ability of AAV-mediated systemic delivery of endostatin to suppress metastatic pancreatic cancer. We found that intramuscular injection of the AAV/mEnd vector increased serum endostatin levels, as compared to a control AAV vector encoding GFP (Figure 2B). In addition, the size of the primary pancreatic tumors and the sizes and number of liver metastases were all reduced in the treated animals (Figure 2C). This suggests that AAV-mediated systemic delivery of endostatin represents a potentially effective treatment for pancreatic cancer and liver metastases.

Figure 2. (A) Construction of recombinant AAV vector plasmids expressing murine endostatin (AAV/mEnd) and control GFP expressing vector (AAV/GFP). ITR: inverted terminal repeats, CAGp: CAG promoter, B19p: B19 promoter, TKp: thymidine kinase promoter, neoR: neomycin resistance gene (B) Serum levels of endostatin after intramuscular injection of AAV/End (42 days after vector injection). Intramuscular injection of AAV/End increased the serum endostatin level. (C) Effects of AAV-mediated endostatin expression on pancreatic tumor growth and liver metastasis.
To achieve the anti-angiogenic state in the model animals used in this experiment, we used classical AAV serotype 2 vectors (Noro et al. 2004). One problem with that vector is that it takes several weeks to reach the maximal level of transgene expression. Therefore, to evaluate the efficacy of cancer gene therapy in a transplantation model animal, the AAV vector must be injected before tumor inoculation. Recently, several different AAV serotypes have been characterized, and we investigated which AAV serotype would maximize the efficiency of the gene transfer into muscle.

3.2. The best AAV serotype

A number of novel AAV serotypes have been isolated from nonhuman primates (Gao et al. 2002), and we endeavored to determine which AAV vector most efficiently mediates muscle expression of anti-angiogenic proteins useful for treating cancer. Included among these were AAV serotypes 1, 7 and 8, which, when developed as vectors, mediate gene transfer into various tissues much more efficiently than those based on previously described serotypes. Therefore, to determine the AAV serotype that most efficiently mediates muscle expression of antiangiogenic proteins, we injected 4 different AAV vector serotypes (AAV1, AAV2, AAV7 and AAV8) encoding mEnd together with GFP into a quadriceps muscle in C57BL/6 mice. The highest GFP expression and plasma mEnd levels were observed one week after injection in mice administered AAV8 (8>7>1>2) (Figure 3A). Moreover, expression of mEnd was sustained for at least 6 months. We then confirmed the transduction efficiency into the muscle using an AAV vector harboring the luciferase gene (AAV/Luc). In vivo imaging showed that the greatest expression occurred in mice administered AAV8 (Figure 3B). Taken together, these results clearly demonstrate that AAV8 is able to efficiently mediate gene transfer into muscle tissue, leading to prolonged expression and secretion of the gene product (Isotani et al. 2011).

3.3. Melanoma differentiation-associated gene-7/interleukin-24 (mda-7/IL24)

Another candidate gene for systemic cancer gene therapy is melanoma differentiation-associated gene-7/interleukin-24 (mda-7/IL24), which has anti-angiogenic properties (MDA-7/IL24 bioactivity was 20- to 50-fold greater than endostatin or angiostatin) as well as several other features useful for cancer gene therapy. Mda-7/IL24 selectively induces apoptosis in cancer cells without harming normal cells, and it exerts both immunomodulatory effects and potent antitumor bystander effects. These multifunctional tumor-specific cytotoxic effects of MDA-7/IL24 make this molecule a promising gene-based therapeutic agent for the treatment of cancer. To assess the in vivo effects of AAV-mediated systemic delivery of MDA-7/IL24, we constructed an AAV vector encoding MDA-7/IL24 (AAV/IL24). A single intravenous injection of AAV/IL24 (2.0 x 10^{11} vector genomes) into a subcutaneous tumor induced by injecting Ehrlich ascites tumor cells into the dorsum of DDY mice significantly inhibited tumor growth and increased survival among the AAV/IL24-treated mice (Figure 4). In addition, TUNEL and immunohistochemical analyses showed significant induction of tumor cell-specific apoptosis and a reduction in microvessel formation within the tumors (Tahara et al. 2007). These results clearly demonstrate that continuous systemic delivery of MDA-7/IL24 can serve as an effective treatment for cancer.
Figure 3. Expression of transgenes following intramuscular administration of AAVs. Following injection of the respective AAV serotypes of the AAV/mEnd vectors, which express murine endostatin together with GFP, into quadriceps muscles, expression of GFP and plasma concentrations of mEnd were analyzed (A). Four AAV serotypes of AAV/Luc, which express luciferase, were injected into the quadriceps muscles of DDY mice. Four weeks after injection, these mice were analyzed by in vivo imaging system.

To then assess the feasibility of AAV8-mediated muscle-directed cancer gene therapy using MDA-7/IL24, we established mixed-lineage leukemia (MLL)/AF4 transgenic (MLL/AF4 Tg)
mice (Tamai et al. 2011). These mice developed pro-B cell (CD45R/B220⁺CD19⁺CD43⁺) lymphoma as well as leukemia with high-level HOXA9 expression by 12 months of age, at which time lymphoma cells had infiltrated the liver, lung and spleen. In addition to multiple sites of tumor infiltration, a non-solid hematological malignancy, leukemia, was also present, making local gene therapy entirely impractical. This model is therefore well suited to analyze the utility of AAV-mediated systemic gene therapies. So far, we have observed that after a muscle-directed single AAV/IL24 injection, infiltration of tumor cells into all organs was suppressed (Tamai et al. 2012). Thus, AAV vector-mediated systemic delivery of MDA-7/IL24 represents a potentially important new approach to anticancer therapy.

4. Summary and future developments

Here we present evidence of the utility of AAV-mediated muscle-directed systemic cancer gene therapy using anti-angiogenic agents together with MDA-7/IL24. This new approach is safe and non-invasive, and could be used to treat primary tumors as well as undetectable metastatic tumors and hematological non-solid tumors without serious side effects. The potential utility of anti-angiogenic gene therapy in cancer is currently being evaluated in combination with radiation, chemotherapy or immunotherapy, which appear to provide a synergistic effect. Moreover, systemic cancer gene therapy may enable reduction of the dose of radiation, chemotherapy or immunotherapy needed to be effective, thereby reducing such side effects as bone marrow suppression. Thus, the combination of gene therapy with conventional anti-cancer therapy may overcome serious problems currently associated with cancer treatment.

Acknowledgements

We thank Dr. James Wilson at the University of Pennsylvania for providing AAV packaging plasmids (p5E18RXC1, p5E18-VD2/7 and p5E18-VD2/8). We also thank Takuya Noro, Ichiro Tahara, Noriko Miyake, Mayu Isotani and Hayato Tamai for helpful discussion and technical assistance. This work was supported in part by grants from the Ministry of Health and Welfare of Japan and the Ministry of Education, Science and Culture of Japan.

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