further intermolecular recombinations through the ITRs would not occur. In the present study, we further investigated the "dog bone" genomes as a tool to understand the mechanisms of AAV-ITR hairpin opening and rAAV vector genome recombinations in mice. Consequently, we have elucidated the following: (1) "dog bone" genomes emerged in scid liver only when rAAV8 vectors were injected at doses of 1.8 x 10e12 or higher, at which vector copy numbers in the livers were > 800 ds-vg/dge (ds vector genomes per diploid genomic equivalent); (2) rAAV vectors delivered to scid liver by rAAV2 vectors never resulted in accumulation of "dog bone" genomes no matter how much vectors were injected; (3) in non-hepatic scid tissues such as heart, muscle and kidney, "dog bone" genomes accumulated at a much lower vector genome load (~2 ds-vg/dge); (4) hepatocyte cell cycling recruited a supplementary AAV-ITR hairpin opening activity that did not exist in a quiescent state, facilitating vector genome recombinations through the AAV-ITRs. These observations demonstrate that AAV-ITR hairpin opening in non-hepatic tissues largely depends on the DNA-PKcs-associated cellular endonuclease activity, while in the liver, the pathways of AAV-ITR hairpin opening are redundant and cell cycle dependent. Based on the recent discovery of DNA-PKcs/Artemis pathway for DNA double-strand break repair together with our observations, we propose that a cellular endonuclease, presumably Artemis, plays a crucial role in AAV-ITR hairpin opening and subsequent vector genome recombinations in concert with DNA-PKcs. We are currently investigating the role of Artemis in this process using Artemis deficient cells.

6. AAV8-Mediated Transgene Expression in Mice and Non-Human Primates

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Vectors using AAV8 capsid have shown remarkable results in liver-directed gene transfer in mice. However, the utility of AAV8 vectors in larger animal models have scarcely been described. Here we report our results with mice and non-human primates (cynomolgus macaque) to test the usefulness of AAV8 vectors for human applications. As a transgene, we chose macaque coagulation factor IX (FIX) with a mutation at position 262 (macFIXT262A). Based on our previous study, this molecule carries minimal alteration and can be detected with a monoclonal antibody against human FIX, and an assay system utilizing this antibody has been established to quantitate macFIXT262A even in the presence of macaque FIX (J of Thromb and Haemost 2: 275-80, 2003). A stock of AAV8 vector encoding macFIXT262A driven by human alpha1-antitrypsin (hAAT) promoter with a liver specific enhancer was prepared. When the vector was injected into C57BL/6 mice intraportally at 1×10^{10} vg/body, plasma concentration of the transgene showed more than 100 % of the normal level. When the same vector was injected into a young adult male macaque at a dose of 1×10^{12} vg/kg, plasma concentration of the transgene was detectable throughout the observation period, but not recognizable as therapeutic level (< 0.1%). The efficacy was again tested in another male at a higher titer of 1×10^{13} vg/kg, and resulted in a similar outcome with a slightly higher level. Macaques were extensively immunosuppressed with FK506 and cyclophosphamide until 8 weeks after injection. To better understand these results, potential factors affecting transgene expression were analyzed. Neutralizing antibody against AAV8 capsid was not detectable before injection. Antibody against transgene product was not recognizable. At present, none of the factors inhibiting transgene expression is identified, implying a species-specificity of the efficacy of AAV8 vectors.

7. Adeno-Associated Virus Type 2 (AAV2) Contains an Integrin alpha5beta1 Recognition Sequence Essential for Viral Cell Entry

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Integrins have been implicated as co-receptors in the infectious pathways of several non-enveloped viruses. For example, adenoviruses are known to interact with several alphaV integrins by virtue of a high affinity arginine-glycine-aspartate (RGD) domain present in the penton base of the capsid. In case of adeno-associated virus type 2 (AAV2), which lacks this RGD motif, integrin alphaVbeta5 has been identified as a co-receptor for cellular entry. However, the molecular determinants of AAV2 capsid-integrin interactions and the potential exploitation of alternative integrins as co-receptors by AAV2 have not been determined thus far. In this report, we demonstrate that integrin alpha5beta1 serves as an alternative co-receptor for AAV2 infection in human embryonic kidney 293 cells. Such interactions are mediated by a highly conserved asparagine-glycine-arginine (NGR) motif known to bind alpha5beta1 integrin with moderate affinity. Mutation of this domain reduces transduction efficiency by 10-fold compared with wild type AAV2 in vitro and in vivo. Further characterization of mutant and wild type AAV2 capsids using cell surface and solid phase binding assays, peptide inhibition studies, and transduction assays in cell lines lacking specific integrins confirmed the role of the NGR motif in promoting AAV2-integrin interactions. Molecular modeling studies suggest that the NGR domain forms a surface loop located at the three-fold axis of symmetry adjacent to residues previously implicated in binding heparan sulfate, the primary receptor for AAV2. Based on the aforementioned results, we propose that cellular internalization of AAV2 follows a "click-to-fit" mechanism that involves co-operative binding of heparan sulfate and alpha5beta1 integrin receptors by the AAV2 capsid.

8. Adeno-Associated Virus 2-Mediated Gene Transfer: A Complex Interaction between Epidermal Growth Factor Receptor Protein Tyrosine Kinase Signaling and Ubiquitin/ Proteasome Pathway in Transgene Expression

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The transduction efficiency of AAV vectors varies greatly in different cells and tissues in vitro and in vivo. We have documented that a cellular protein, FKBP52, inhibits the viral second-strand DNA synthesis, and consequently, transgene expression (J. Virol., 75: 9818, 2001). FKBP52 is phosphorylated at tyrosine residues by epidermal growth factor receptor protein tyrosine kinase (EGFR-PTK), which inhibits AAV second-strand DNA synthesis by greater than 90% (J. Virol. 72: 9835, 1998; J. Virol., 75: 9818, 2001). Tyrosine-phosphorylated FKBP52 is dephosphorylated by T-cell protein tyrosine phosphatase (TC-PTP), which negatively regulates EGFR-PTK signaling, and leads to a significant increase in AAV transduction (J. Virol. 77: 2741, 2003). We have documented high-

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