First National Gene Transfer Safety Symposium:
Internally Deleted, Helper-Dependent Adenoviral Vectors
March 8, 2000

INTRODUCTION

In March 2000, as part of ongoing efforts to enhance the protection of research participants in gene transfer clinical trials, the Department of Health and Human Services announced the initiation of National Gene Transfer Research Safety Symposia.[1] The safety symposia are forums for expert review and public discussion of emerging scientific, medical, ethical, and safety issues in gene transfer clinical research. The exchange of information and in-depth discussions are intended to increase understanding of the safety and toxicity issues in gene transfer, maximize safety of research participants, enhance the development of gene transfer clinical trials, and optimize informed consent processes.

The first Gene Transfer Safety Symposium was convened on March 8, 2000 during the course of the meeting of the National Institutes of Health (NIH) Recombinant DNA Advisory Committee (RAC) and focused on "Internally Deleted, Helper-Dependent Adenoviral Vectors."[2] The internally deleted, helper-dependent vectors comprise a new generation of adenoviral (Ad) vectors. The vectors, also referred to as "mini-Ad" or "gutless" vectors, have been extensively modified and, because they do not express viral proteins, are believed by some investigators to be safer than previous generations of Ad vectors. Notwithstanding the lack of viral protein expression, mini-Ad vectors raise safety issues particularly in regard to their production and clinical application.

The Safety Symposium included discussion of the first protocol proposing clinical administration of mini-Ad vectors, *Phase1, Single Dose, Dose Escalation Study of MiniAdFVIII Vector in Patients with Severe Hemophilia*.[3] Presentations and discussions focused on the preclinical studies to evaluate the difference in the toxicity profile between earlier Ad vectors and the new mini-Ad vector systems. The questions that were addressed focused on whether the proposed systemic administration, dosage range, and patient population were appropriate in light of preclinical findings and toxicity profiles.

The Safety Symposium was co-chaired by RAC members, Drs. Estuardo Aguilar-Cordova and Dale Ando. Two leading experts in the field of adenoviral vectors, Dr. Frank Graham,

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[1] See press release at http://www.hhs.gov/news/press/2000pres/20000307A.html.

[2] The safety and toxicity of earlier generation adenoviral vectors had been the subject of a previous symposium sponsored by the NIH on Dec. 8-9, 1999. This symposium highlighted the value of convening scientific meetings on emerging safety issues and served as a model for the HHS initiative.

[3] The protocol was submitted to the NIH Office of Biotechnology Activities (OBA) and registered as protocol number 0001-372. Although the protocol served as the focal point of the Safety Symposium, the discussion did not constitute formal public discussion by the RAC, a step required for this protocol due to the novel questions it raised. The RAC review of the protocol itself occurred in September 2000.

McMaster University and Dr. Jeffery Chamberlain, University of Michigan, were invited to participate. Dr. Gilbert White II, Principal Investigator, Hemophilia Treatment Center, University of North Carolina School of Medicine, and Dr. Wei-Wei Zhang, GenStar Therapeutics Corporation/Urogen Corporation gave presentations about the vector and the design of the clinical trial. Drs. Chamberlain, Ando, Aguilar-Cordova, and Dr. Anne Pilaro, CBER, FDA, presented more general information about the vector system and the safety data. This report provides background on the development of mini-Ad vectors and their proposed clinical application for hemophilia A and summarizes the vector and clinical issues highlighted during the Safety Symposium.

BACKGROUND

Development and Characteristics of the Mini-Ad Vector

Ad vectors in general have several advantages as gene transfer vectors including high titers and efficient transduction of a broad range of cell types including non-dividing cells. However, Ad vectors also have a major drawback over other vectors. Ad vectors are highly immunogenic in humans. The capsid proteins encoating the vector elicit humoral immune responses resulting in the generation of anti-Ad vector antibodies. In addition, the cellular immune response to the Ad proteins expressed by the vector activates cytotoxic T-lymphocytes leading to elimination of the transduced target cells. In the earliest generation Ad vectors, the E1 gene required for activation of most of the other viral genes was deleted to inhibit viral protein expression. Despite this and the deletion of a few other viral genes from later generation vectors, some low level leaky expression of viral proteins still occurred in the target cells. In an effort to improve the safety profile of Ad vectors, systems have been developed recently that produce completely attenuated vectors.

The mini-Ad vector is designed to reduce the immunogenicity of the Ad vector by ridding it of all viral genes. Essentially the vector is a plasmid containing the Ad origin of replication and packaging signal and an inserted therapeutic transgene. Production of this vector requires the use of a helper virus to supply the viral proteins necessary for replication and encapsidation. In the helper virus, the packaging signal is partially deleted and the adenoviral E1 gene is replaced by a reporter transgene rendering the helper virus replication incompetent. The vector plasmid and helper virus are introduced into packaging cells that provide the E1 functions required for viral propagation. A mixture of mini-Ad vector and helper virus will be produced, but the vector can be separated from helper by differences in density on gradients.

Theoretically, the absence of Ad protein expression should improve the safety of the vector over the early Ad vectors. Expression of viral products by the earlier generation vectors induced cellular immune responses that destroyed the vector-transduced cells and caused liver abnormalities. Pre-clinical mini-Ad FVIII vector studies at the same doses did not induce liver abnormalities in the any of the animal models (mouse, rat, dog and non-human primates). In addition to less toxicity, the mini-Ad vector expressed the transgene at higher levels and for a longer duration (up to one year). A further advantage of the mini-Ad is its increased capacity (up to 36 kb) allowing for insertion of larger transgenes and gene or tissue-specific regulatory

elements to improve control of transgene expression. However, the capsid is still intact and humoral responses, especially in instances of pre-existing immunity to adenovirus, could occur.

VECTOR PRODUCTION ISSUES

***Replication Competent Adenovirus Contamination*:** The mini-Ad vector system presented at the March 2000 Safety Symposium should theoretically have decreased potential for generation of replication competent adenovirus (RCA) compared to Ad vector systems that use 293 cells. The 293 cells contain considerable Ad sequence that could homologously recombine with vector sequence to generate RCA during vector production. In the mini-Ad vector system, A549 cells are used that have less Ad sequence for minimal overlap with vector sequence.

***Titer*:** In addition to optical density measurements, more stringent methods of vector titering would be useful including real time PCR and visual counting of particles by electron microscopy. Since they contribute to toxicity, the percentage of empty capsids in a vector preparation also should be determined.

***Helper Virus Contamination*:** The vector product is fractionated to separate vector from helper virus. Less than 0.1 % helper virus was detected in the presented vector system preparations. Assays for helper detection include DNA digestion and a biological assay to detect expression of a Lac-Z reporter gene inserted into the helper virus. Quantification of helper virus levels was noted to be difficult because different assays (plaque forming units vs. helper reporter gene transduction vs. quantitative PCR) may detect different levels. Because the helper vector is essentially an earlier generation vector, the effect of contamination on mini-Ad vector expression, duration and immunogenecity will need to be determined.

***Transgene Cassette*:** The higher capacity of mini-Ad vectors permits the inclusion of larger genomic transgenes with tissue specific promoters and regulatory elements resulting in increased levels of expression and duration. Lowered immunogenecity may also be associated with the use of tissue specific promoters as opposed to constitutive promoters due to the lack of expression in antigen presenting cells.

***Stuffer DNA*:** Adenoviruses require a certain genome size for packaging. The deleted mini-Ad vectors are below the minimal size for packaging into the virion. In order to meet this minimal size, mini-Ad vectors must contain "stuffer" DNA. In the vector presented, the transgene cassette contained a large amount of regulatory sequences bringing the vector to sufficient total size, but vectors with smaller transgene cassettes will need the insertion of additional sequence. The source of this stuffer DNA is likely to vary among different vectors. Possible interactions between the stuffer DNA and the transgene cassette need to be considered in vector design. The presence of regulatory elements may interfere with the expression or stability of the transgene product. Because of the need to determine the effect of different combinations of stuffer and transgenes, substitution of a different transgene into a mini-Ad vector will require more extensive testing of expression and toxicity than previously required when switching transgenes in earlier Ad vectors. Since the stuffer DNA or transgene regulatory sequence may contain genomic DNA, there may be increased potential for homologous recombination leading to integration into the

cellular genome. This would raise issues of long term transgene expression and potential for insertional mutagenesis. For the vector system presented, PCR analysis suggested that the vector remained episomal in the target cells.

***Vector Rearrangement*:** Obtaining sufficient titer of the mini-Ad vector requires multiple sequential preparations that may increase the risk of vector rearrangement. Assays to detect rearrangement include specific restriction enzyme digestion of vector followed by gel and southern blot analysis. PCR analysis has also been performed but may be problematic to interpret. A highly sensitive assay that did not require depleting vector stocks during testing would be most useful. While no rearrangements were detected with the vector system presented, rearrangements of vector and helper have been detected after 9-12 serial passages by other investigators. Variability in stability was observed among different vectors.

***Seed Stock*:** The requirement for serial passaging also complicates the issue of seed stock definition. The type of quality control to verify the integrity of the seed stock will need to be determined. If additional passages are required to generate sufficient clinical grade vector from the seed lot, it will be necessary to monitor again for vector rearrangements that may have occurred during those subsequent rounds of replication. Scaling up mini-Ad vector preparations for clinical uses will be more difficult than with the helper independent Ad vectors.

CLINICAL ISSUES

***Capsid Toxicity*:** While the mini-Ad vectors will not express viral genes in the transduced target cell, the administered vector is still encapsidated in the viral coat. In this respect, the mini-Ad vector is identical to the previous vectors. The potential toxicity of the adenoviral capsid in high dosage administration is still of concern. The acute toxicity data accrued with the early vectors may be applicable to the mini-Ad vector.

***Vector-Disease Match*:** Given that no single vector type is appropriate for all diseases, the suitability of a particular vector type to meet specific disease treatment requirements must be considered. Because the duration of gene expression is not long term, adenoviral vectors may not be optimal for treatment of chronic diseases. Adenoviral vectors do not integrate into the host genome, thus expression will be lost from replicating cells. Currently, repeat administration is not possible due to the generation of neutralizing antibodies to the viral capsid protein raised following the initial administration. However, mini-Ad vectors may be more suitable than the earlier Ad vectors. The lack of viral products should decrease the chronic immunogenecity response. For this reason and the inclusion of tissue specific regulatory elements for the transgene, duration of expression has increased to up to one year. Another possible advantage to the mini-Ad vectors is their higher capacity may allow the insertion of the multiple transgenes necessary for treatment of polygenic diseases.

Hemophilia A is considered a suitable disease candidate for gene transfer via a mini-Ad vector for a number of reasons. Hemophilia A, which accounts for about 85 percent of the incidence of hemophilia, is caused by low levels or complete absence of the blood clotting protein factor VIII (FVIII). Treatment currently consists of intravenous infusion of plasma-derived or recombinant

FVIII concentrates at the time of a bleeding episode. Prophylactic treatment has been shown to be beneficial in pediatric patients but is prohibitively expensive in adults. Expression sustained for even one year following gene transfer would still be a significant improvement over FVIII infusions administered only during bleeding episodes.

The severity of hemophilia is related to the amount of clotting factor in the blood. About 70 percent of hemophilia A patients have less than one percent of the normal amount and, thus, have severe hemophilia. A small increase in the blood level of the clotting factor, up to five percent of normal, results in mild hemophilia. Thus, hemophilia A is considered a suitable target for gene transfer because the amount of FVIII expression needed to achieve a therapeutic effect is very low. Also neither tissue specific nor precise levels of FVIII expression are required. Since hemophilia A treatment does not require high levels of trangene expression, it may be treatable with doses of mini-Ad vector low enough to avoid toxicity. However, due to the toxicity profile of adenoviruses, any type of Ad derived vector may not be suitable to treat diseases of the liver or bleeding disorders.

***Preclinical Animal Model Results*:** Studies were performed using C57BL/6 and Balb/c mice, cotton rats, and hemophiliac mouse and dog models. Long-term expression at therapeutic levels was seen in three groups of C57BL mice out beyond six months post vector injection. A linear dose response was also seen. In the hemophiliac mice, phenotypic correction of hemophilia was observed as measured by reduced blood flow rate, and clotting times. As of an update at the September 26, 2000, meeting of the RAC, expression had been seen out to 500 days in one mouse. In a larger safety study utilizing 240 mice, doses that cause elevated liver enzymes or death with the early Ad vectors did not affect liver enzyme profile. At the highest dose (4.3×10^{12}vp/kg), there was transient decrease in platelet counts and mild elevation of liver enzymes. Biodistribution studies by a PCR based assay showed that over 80% of vector was localized in the liver and persisted in episomal form for over one year. Studies in hemophiliac dogs showed no toxic effects at highest dose (7.6×10^{11}). Preliminary data from a study of eight young adult male cynomolgus monkeys presented in September 2000 indicated that therapeutic levels of FVIII could be obtained with vector doses that did not induce significant toxicity. Minor elevation of liver transaminases and alterations in white blood and platelet counts were observed transiently at the highest dose (4.3×10^{12}vp/kg).

***Dosage*:** Studies with earlier generation Ad vectors indicated the existence of a dosage threshold effect for both transgene expression and toxicity. Identification of the threshold level is complicated by variability among animal models and even within the patient population. As with all Ad vectors, predictors of susceptibility should be determined to identify patients most sensitive to toxic effects. Due to the immune response to Ad vectors, repeat dosing is not currently possible; therefore, patients receiving low doses in safety trials would be precluded from receiving possibly therapeutic doses later. Also the possible pre-existence of anti-Ad antibodies in patients may potentially interfere with vector efficacy.

CONCLUSIONS

The mini-Ad vectors incorporate a number of safety modifications that may improve the benefit/risk ratio to be considered for the use of Ad vectors in clinical trials. Nonetheless, because they share features of the earlier Ad vectors, they raise many issues that still need to be addressed. The following issues with respect to vector production were noted:

$ Quantitative and qualitative assays are needed for the titration of vector, defective vector particles and RCA.

$ In this system, the titer of helper vector must also be measured and the effect of contamination with helper, essentially first generation vector, on the expression and toxicity of mini-Ad preparations will have to be determined.

$ The issue of possible vector rearrangement will be of greater concern with the mini-Ad vectors due to the need to serially propagate them to achieve sufficient titer.

$ An advantage of the mini-Ad vectors is their greater capacity allowing the insertion of larger genes, and possibly multiple genes. The ability to insert more regulatory sequences appeared to improve the level and duration of transgene expression, and allowed the use of tissue specific promoters.

$ In many vectors, however, additional stuffer DNA will need to be inserted to increase the vector size for efficient packaging. The effect of particular stuffers on transgene expression, toxicity, and potential for genomic recombination will need to be determined for individual vectors.

Among the salient issues related to clinical application of mini-Ad vectors were the following:

$ While the preclinical data suggests that removal of all viral genes from the mini-Ad vector decreased the cellular immune response, the toxicity of the adenoviral capsid proteins is still an issue with these vectors. Initial dosage and dose escalation regimens should be determined with safety of primary concern.

$ Because of the anti-Ad antibody response, mini-Ad vectors cannot be readministered. Therefore, the use of a non-integrating vector incapable of long term transgene expression for the treatment of chronic diseases was called into question particularly if it precluded future treatment for the research participant.

$ Hemophilia A may still remain a suitable candidate disease for gene tranfer with mini-Ad vectors because it does not require tissue or dose specific expression and even if the duration of transgene expression is limited to one year, this may offer superior treatment over intermittent infusions of FVIII during bleeding episodes.

Further preclinical studies and clinical trials should determine whether the mini-Ad vector offers an improved benefit/toxicity ratio through increased transgene expression and decreased toxicity. NIH OBA and the RAC will follow the progress of this vector system and provide updates as appropriate.