In this paper, Oberhammer et al. report that TGF-β1 is involved in the initiation of hepatocyte apoptosis, both *in vitro* and *in vivo*. They assessed the presence of apoptosis by means of light and electron microscopy and used a fluorescent DNA-binding dye to determine the proportion of nuclei with condensed chromatin and fragmentation. Compared with cultures without TGF-β1, hepatocyte cultures maintained in medium containing TGF-β1 showed, after 48 hr in culture, a 50% decrease in total number of nuclei and an increase in the proportion of nuclei containing condensed chromatin. To test the effect of TGF-β1 on apoptosis *in vivo*, rats were given cyproterone to induce liver hyperplasia and killed 2 days after the last administration of the drug. Animals that received TGF-β1 during this period showed a fivefold higher number of apoptotic bodies, leading the authors to conclude that TGF-β1 can trigger hepatocyte apoptosis *in vivo*. Not surprisingly, they also report that injections of TGF-β1 latent complexes were without effect. These complexes do not bind to TGF-β1 receptors and are probably not activated by normal liver cells (4).

Despite these interesting results, more data are required before definitive conclusions about the role of TGF-β1 in hepatocyte apoptosis and the regulation of liver size can be reached. Oberhammer et al. measured hepatocyte apoptosis only with morphological methods but apparently could not demonstrate the formation of DNA ladders. Although they state that ladders may not be found in apoptotic cell death of epithelial cells, ladder formation has been described in apoptosis of hepatocytes and other epithelial cells (5). The morphological criteria used by the authors are not always entirely clear to readers who do not have access to the many photographs the authors must have used to prepare the manuscript. Thus it is not always easy to understand distinctions made between various stages of chromatin condensation or fragmentation described in the paper. Similarly, it is not clear what the categories "apoptotic body with and without chromatin" used for scoring hepatocyte apoptosis *in vivo* may mean because these morphological patterns are not illustrated.

The mechanisms of the TGF-β1 effect must also be explored. Although the authors' main conclusion is that TGF-β1 is involved in the initiation of hepatocyte apoptosis *in vivo* and *in vitro*, it is difficult to determine whether TGF-β1 has a primary or secondary role in the process. For instance, in the *in vivo* experiments, livers were regressing in mass because of the withdrawal of cyproterone. TGF-β1 accelerated the course of regression but may have not initiated it. Furthermore, TGF-β1 is not normally made by hepatocytes *in vivo* and *in vitro*, and it would be important to establish whether good correlation exists between presence of TGF-β1 and apoptosis in individual cells and what the sources for TGF-β1 in apoptotic hepatocytes might be. Finally, one may ask whether a potential role of TGF-β1 in hepatocyte apoptosis is in any way related to the role of the factor as an inhibitor of hepatocyte replication. The culture experiments were performed by placing hepatocytes in collagen gels maintained in the absence of growth factors. What would be the relationship between cell replication and apoptosis in this culture system? If TGF-β1 blocks cell replication and can also induce apoptosis, how does the hepatocyte distinguish between a kiss that kills from one that merely restrains continued cell proliferation?

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DNA/PROTEIN COMPLEXES DELIVERED IN CONJUNCTION WITH ADENOVIRUS GENERATE HIGH-EFFICIENCY *IN VITRO* TRANSFECTION: A POWERFUL TRANSFECTION REAGENT WITH POTENTIAL BROAD APPLICATIONS

Cristiano RJ, Smith LC, Woo SLC. Hepatic gene therapy: adenovirus enhancement of receptor-mediated gene delivery and expression in primary hepatocytes. Proc Natl Acad Sci USA 1993;90:2122-2126.

ABSTRACT

We have combined a receptor-mediated DNA delivery system with the endosomal lysis ability of adenovirus and shown that DNA can be delivered into primary hepatocytes, resulting in a high level of gene expression. When asialoorosomucoid conjugated with poly(L-lysine) was used to deliver the *Escherichia coli* β-galactosidase gene into primary hepatocytes through binding with the hepatic asialoglycoprotein receptor, only a low level of β-galactosidase was detectable, with less than 0.1% of the hepatocytes being transfected. This level of activity can be greatly enhanced by the cointernalization of the DNA protein complex with a replication-defective adenovirus, resulting in 100% of the hepatocytes staining blue with 5-bromo-4-chloro-3-indolyl β-D-galactoside. Quantitative analysis of β-galactosdase expression also showed a 1000-fold enhancement of activity. To test the applicability of this DNA delivery system for the correction of phenylketonuria, a metabolic disorder that causes severe mental retardation in children, we have delivered the human phenylalanine hydroxylase (PAH) gene to hepatocytes derived from a PAH-deficient mouse strain and dem-

onstrated complete reconstitution of enzymatic activity. This method shows great promise for efficient gene delivery to the liver for correction of hepatic disorders.

COMMENTS

The concept is extremely enticing. Couple a receptor ligand to polylysine and electrostatically complex these proteins to plasmid DNA, generating an *in vivo* transfection reagent that targets specific cell-surface receptors. The first DNA/protein complex system developed utilized asialoorosomucoid coupled to polylysine to target the asialoorosomucoid receptor on HepG2 cells and hepatocytes (1). The initial *in vitro* transfection studies reported efficiencies comparable to that of calcium phosphate. First with the CAT reporter gene and then with therapeutic genes, DNA/protein complexes have been used to target liver cells (2, 3). *In vivo* transfection of liver cells was demonstrated first for several days after intravenous infusion and then for several months when complex was delivered in combination with partial hepatectomy (2). These studies used high-solubility complexes.

Extensive studies have been performed targeting the transferrin receptor with transferrin polylysine conjugates complexed to plasmid DNA (4). These studies have used lower-solubility complexes of less than 60 μg/ml. Replication-deficient adenovirus in combination with DNA/protein complex has been shown to increase transfection efficacy (5). The putative mechanism is endosomal lysis, although DNA/protein complexes lacking receptor ligands generate a noteworthy background transfection efficiency in combination with adenoviruses. In competition studies using free ligand, it is possible to reduce but *not* completely compete off adenoviral-augmented transfection. These transfection controls imply that adenoviruses affect more than just endosomolysis; they also facilitate entry of DNA/protein complexes into cells other than through their specific receptor target. Subsequent experiments have shown that direct conjugation of adenovirus to complex leads to further increases in complex efficiency (6).

This study demonstrates the dramatic effect of replication-defective adenovirus in augmenting transfection produced by asialoorosomucoid-polylysine DNA/protein complexes and the delivery to primary hepatocyte cultures of a potentially therapeutic gene, the human phenylalanine hydroxylase gene. In the absence of adenovirus, the DNA/protein complexes used in this study functioned at low levels of efficiency. When complex and adenovirus were delivered simultaneously, transfection efficiency improved dramatically, more than 1,000-fold. At optimal efficiency, virtually every hepatocyte in culture was transfected, as judged on the basis of cytochemical β-galactosidase staining.

The authors went on to demonstrate that the synthesized complexes, in the presence of adenovirus, were capable of delivering a therapeutic gene to hepatocytes deficient in phenylalanine hydroxylase by reconstituting enzymatic activity. The applications of an *in vitro* complex transfection system to the treatment of genetic disease are limited. After further refinement in complex composition, DNA/protein complexes coupled to adenovirus should confer high-efficiency *in vivo* transfection. The importance of linking the endosomolysis reagent directly to the DNA/protein complex becomes more important when the complexes are introduced into hepatocytes *in vivo*. Complex and adenovirus theoretically should be internalized in the same endosome if endosomolysis is to increase gene-transfer efficiency. The probability of complex and adenovirus targeting the same endosome is obviously increased if adenovirus and complex are chemically linked. Ultimately, more simple complexes incorporating a protein for endosomal lysis, as opposed to an entire adenovirus, will be developed, potentially increasing efficacy and specificity.

Defining the reaction conditions for conjugating receptor ligand to polylysine and the formation conditions for DNA/protein complex synthesis is technically challenging because of the large number of possible reaction conditions. The exact biochemical difference between the low-solubility complexes reported in this study and the high-solubility complexes previously reported is not clear. This evident discrepancy underscores the potential for multiple reaction products from these conjugation reactions. The fastidious removal of unreacted free polylysine during the purification steps following conjugate synthesis appears to be one parameter essential to the generation of higher-solubility complexes, whereas it would appear that the optimal biochemical composition of high-solubility DNA/protein complex remains to be defined (7).

There are several potential advantages of the DNA/protein complex system compared with the direct production of recombinant adenoviral vectors. DNA/protein complexes may accommodate large cDNAs; plasmids as large as cosmids have been successfully transfected by means of transferrin complexes (8). Recombinant adenoviruses are limited by the size constraints of packaging the adenoviral genome into particles. In addition, the mechanism of persistence of the constructs also differs if they are delivered as recombinant adenoviruses or DNA/protein complexes. For investigational purposes, internal transfection controls may also be run, utilizing the ability of DNA/protein complexes to deliver two plasmids simultaneously. Finally, for research purposes it may be possible to express a large number of plasmids with one synthetic lot of adenovirus-enhanced DNA/protein complexes, eliminating the need and expense of producing unique recombinant adenoviruses for each clone.

DNA/protein complexes in conjunction with an endosomal lysis reagent represent a substantial advance in transfection technology compared with calcium phosphate or lipofectin. The power of a transient *in vitro* transfection system may be limited in terms of gene therapy, but the ability to express a gene in every hepatocyte in primary culture has tremendous potential research applications. Many experiments requiring high levels of transient expression in hepatocytes that were

not previously feasible will be readily accomplished as the technology outlined in this paper is disseminated. Transient transfections may be further extended to produce high-efficiency–selected, stably transfected cell lines for the production of proteins or viral packaging cell lines for investigational and therapeutic use. As the potential exists to further refine their constitution, DNA/protein complexes should fulfill their full potential as a gene therapy modality.

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