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IGF1 gene transfer into skeletal muscle using recombinant adeno-associated virus in a rat model of liver cirrhosis

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Systemic administration of recombinant IGF1 at low levels has been shown to improve hepatic function, nutritional status and testicular atrophy in rats with CCl_4-induced cirrhosis. We have developed a recombinant adeno-associated (rAAV) viral vector containing the cDNA for rat IGF1 and confirmed the expression of IGF1 after intramuscular injection of this vector in a rat model of liver cirrhosis. Although weight of injected muscles was significantly increased in rats with mild cirrhosis, this was not the case in rats with advanced, de-compensated cirrhosis. Furthermore, we found no significant amelioration of liver damage in treated rats at any stage of liver cirrhosis. Our results suggest that IGF1 gene transfer into muscle results in a local effect, at least at the vector dose employed here.

Keywords: Adeno-associated virus, Cirrhosis, Insulin-like growth factor 1.

The development of liver cirrhosis involves processes that ultimately compromise organ function and lead to complications derived from hepatic insufficiency. Chronic oxidative damage resulting from sustained ethanol consumption or other factors leads to cell necrosis, hepatocyte proliferation and TGF-beta induced fibrogenesis, with loss of tissue architecture and liver insufficiency in the final stages of the disease (1, 13-15, 21).

It has been shown that low doses of Insulin-like Growth Factor 1 (IGF1), a

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growth factor synthesised primarily in the liver, improves nutritional status, osteopenia, testicular atrophy and liver function in rats with hepatic cirrhosis induced by chronic exposure to CCl_4, an organic compound which shows strong hepatic toxicity (2-6, 18). Protein infusion, however, requires substantial amounts of the recombinant protein, which must be administered as a bolus twice a day. These factors limit the potential application of this strategy to cirrhotic patients. Release of constant, low levels of IGF1 by means of gene transfer is an interesting alternative strategy.

Gene transfer into skeletal muscle has been used to achieve local production of a variety of therapeutic proteins, using different vector systems. The long life-span of mature muscle fibres affords long lasting transgene expression from transduced cells, even when non-integrating vectors like plasmid DNA are used (11, 23, 24). Furthermore, the muscle is highly vascularized and can secrete expressed proteins into the bloodstream for systemic distribution. Of the several vectors available for gene transfer into skeletal muscle, recombinant adeno-associated virus (rAAV) is one of the most attractive because of its low inmunogenicity, its integrative potential and its high transduction efficiency into mature muscle fibres (7, 8, 10, 25).

In this work we describe the development and characterisation of a rAAV vector containing an expression cassette with the cDNA for rat IGF1. In order to achieve sustained production of IGF1 and evaluate its therapeutic potential in liver cirrhosis, this vector was injected into skeletal muscle of cirrhotic rats. Although local expression of IGF1 was detected in injected muscles, this was not enough to increase serum IGF1 to levels that could be therapeutic in this animal model. We discuss the factors that limit the efficacy of this approach and propose alternative strategies.

Materials and Methods

rAAV production and characterisation.- The IGF1 expression cassette includes rat IGF1 cDNA (kind gift from P. Rotwein) driven by the CMV promoter, the bovine growth hormone polyadenylation site from pCDNA3 (Invitrogen) and the myosin light chain enhancer 1/3 (12, 17). Plasmid pAAVIGF1 contains the IGF1 expression cassette flanked by AAV ITRs, and was created by ligation of the expression cassette into Sal I-digested pAAV.LucA (kind gift from I. Maxwell). The helper plasmid encoding *rep* and *cap* genes necessary for rAAV production (pSPRC) and the plasmid encoding all necessary adenovirus helper functions (SuperCos13) were generous gifts from P. Moullier, and have been described elsewhere (22). AAV production was carried out as previously described (22) in the facilities of the Laboratoire de Therapie Gènique of the CHU de Nantes, France. The presence and titre of physical particles of AAVIGF1 was determined by a standard dot-blot method and densitometric analysis using standards of known copy number of plasmid molecules. The titre of physical particles is expressed as DNAse-Resistant Particles per millilitre (DRP/ml). The titre of infective particles in rAAV preparations was determined by a modification of the classical replication-centre assay, as described in (22), and is expressed as infectious units per ml.

Virus activity assay.- In order to analyse the activity of the rAAV, 293 cells were infected with various dilutions of rAAVIGF1 and incubated for 48 hours in

serum-free DMEM at 37 °C and 5%CO2. Supernatants were harvested and concentrated 20-fold using Centricon-3K concentrators (Amicon). Total RNA was isolated from the cells by extraction with Ultraspec (Biotex). Presence of IGF1 mRNA was detected by RT-PCR using primers specific for rAAV-encoded IGF1 and Amplitaq Gold (Perkin-Elmer). Additionally, concentrated supernatants were analysed for IGF1 by RIA, and normalized for total protein as determined with the BCA method (Sigma). As a positive control for the activity of the vector, 293 cells were transfected with 9 µg of the original plasmid pAAVIGF1 using Lipofectamine (Life Technologies) according to manufacturer instructions.

Induction of liver cirrhosis.– All animals were housed in a pathogen free environment during the study, according to Institutional guidelines. Three week-old male Wistar rats were selected for this study. Liver cirrhosis was induced by a combination of phenobarbital (Luminal, Bayer) in the drinking water (400 mg/L) and chronic CCl_4 inhalation. After an initial period of one week of phenobarbital administration, which was then maintained throughout the duration of the study, rats were exposed twice a week to a CCl_4 atmosphere, increasing the time of exposure during a period of nine weeks. Once the maximal dose was reached the treatment was maintained for another two weeks, after which the rats were left only with phenobarbital for one week. At this stage, twenty rats were randomly selected for experimental protocol 1, including rats with compensated cirrhosis. Under this protocol, control animals received an injection of 100 µl of PBS in each tibialis anterior muscle whereas treated rats received 100 µl of rAAV stock in each tibialis anterior muscle. This is equivalent to 1.5×10^9 DRP/muscle or 3.1×10^7 ip/muscle. Rats were given no further treatment for four weeks, and blood samples were obtained at weekly intervals.

Another group of twenty four rats received a single maintenance exposure to CCl_4 once a week for a further period of nine weeks, after which the rats were considered to have developed a de-compensated cirrhosis and included in experimental protocol 2. Under this protocol, control animals received an injection of 100 µl of PBS in each tibialis anterior muscle and treated rats received 100 µl of rAAV stock in each tibialis anterior muscle. This is equivalent to 1.5×10^9 DRP/muscle or 3.1×10^7 ip/muscle. After treatment, rats received a weekly maintenance dose of CCl_4 for a period of four months and serum samples were obtained monthly. No further rAAV injections were administered during this period.

Biochemical and histopathological assays.– Blood samples were obtained from all rats from the retro-orbital plexus before treatment and at different time points during the course of the study. At the end of each protocol a final blood sample was obtained, all rats were sacrificed and samples of tibialis anterior muscle, gastrocnemius muscle, heart, spleen, brain, liver, testes, tibial bone and jejunum were stored for histopathological or biochemical analysis. Serum levels of total protein, bilirubin, alkaline phosphatase and aminotransferases (AST and ALT) were determined by routine laboratory methods using a COBAS-MIRA autoanalyser (Roche). Total IGF1 present in the serum was determined with a total rat IGF1 RIA (DSL) following manufacturer instructions. Free serum IGF1 was isolated by the method of FRYSTYK *et al.* (9).

Histological preparations of liver, testes and tibialis anterior muscles were prepared after fixation in 4% formaline and inclusion in paraffin. Histology of muscle and testes samples was evaluated by hematoxilin-eosin staining and liver fibrosis was assessed by Gomori's tricromic staining and semi-quantitative scoring of collagen content by two independent observers.

Statistical analysis.– All variables in the study were analysed as independent samples except those that implied a follow-up in time (body weight and all data from serum samples obtained during the course of the protocols), which were treated as a repeated measures design. Normality was assessed by the Shapiro-Wilks and Kolmogorov-Smirnov (var. Lilliefors) tests, considering normal distributions when both tests gave a $p>0.05$, and proceeding with either parametric or non-parametric analysis according to this result or to the nature of the variable. A result was considered significant when its p-value was less than 0.05. For comparison of the variables in which the result of an improvement in hepatic function was known, one-tailed probability was used in the direction of the expected change. Otherwise, two-tailed probability was used. All calculations were performed using SPSS v7.5.

Results

Production and biological activity of rAAVIGF1.– We obtained rAAVIGF1 stocks with titres of $1.5x10^{10}$ DRP/ml (physical particles) and $3.10x10^{8}$ ip/ml (infective particles). This represents a physical-to-infective particles ratio of 40. Contaminating adenovirus was not present and rep^{+} AAV titre was $1.5x10^{5}$ ip/ml. The specific transcript derived from rAAVIGF1 was detected by RT-PCR in 293 cells infected with the virus and was also present in rAAVIGF1-injected muscles, but not in control muscles (Fig. 1). The amounts of IGF1 detected by RIA in the supernatants of 293 cells infected with rAAVIGF1 or transfected with pAAVIGF1 are also shown in Figure 1. These results indicate that the rAAV is capable of infecting cells and expressing the protein in vitro without adenoviral helper functions, and that injected muscles are also expressing IGF1 *in vivo*.

Liver function in cirrhotic rats.– In protocol 1, a compensated cirrhosis was induced and then CCl_4 administration was discontinued for the rest of the study. Table I summarises all the variables stud-

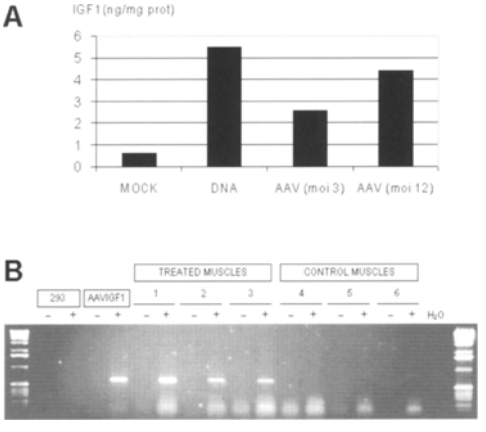

Fig. 1. **A.** IGF1 levels from 293 cells mock-transfected (MOCK), transfected with pAAVIGF1 (DNA) or infected with rAAVIGF1 at different multiplicities of infection (moi). Cell culture supernatants were concentrated and rat IGF1 levels were assayed as described in Methods. **B.** RT-PCR detection of the specific IGF1 mRNA encoded by rAAVIGF1 in 293 cells either mock-infected (293) or infected with the recombinant virus (HAAVIGF1), and in representative muscles from rats injected with the virus (treated muscles) or injected with PBS (control muscles). Two lanes are shown for each sample, representing non-retrotranscribed RNA (-) and cDNA (+).

Table I. *Data of liver function (total protein, AST, ALT, ALP), body and muscle weight (g) and serum IGF1 obtained at different time points during Protocol 1 in control (PBS) and AAVIGF1 treated animals.* Values are shown as Mean ± SEM (n=10 in both groups). Statistical differences ($p < 0.05$) are depicted by *. ND= Not Done. N/A=Not Applicable.

	Day 0		Day 15		Day 31	
	PBS	AAVIGF1	PBS	AAVIGF1	PBS	AAVIGF1
T. protein (g/dl)	6.87±0.07	6.82±0.10	6.94±0.06	6.96±0.13	6.94±0.07	6.94±0.11
AST (u/l)	114.5±15.7	141.1±27.8	106.3±9.8	110±15.8	107.2±13.6	98.7±10.3
ALT (u /l)	76.5±6.0	85.8±8.9	53.5±5.3	62.2±8.5	53.8±4.6	63.9±10.2
ALP (u /l)	118.1±13.5	128.3±16.5	96.5±8.5	106.2±12.6	96.6±7.7	97.4±11.1
Body w.	417.0±6.93	431.6±6.31	ND	ND	474.2±13.47	480.5±9.3
Tibialis Ant. w.	N/A	N/A	N/A	N/A	0.74±0.01	0.82±0.02*
Gastrocn. w.	N/A	N/A	N/A	N/A	2.47±0.09	2.13±0.19
IGF1 (ng/ml)	ND	ND	ND	ND	1577.8±154.5	1593.2±96.7

ied in these animals. We found no significant differences in body weight between control and treated animals at any particular time point during the study (repeated measures F-test, $p>0.05$), and all the rats showed good general condition at the end of the study. However, we found a significant increase in muscle weight in rAAV-IGF1-treated animals (independent samples t-test, $p=0.011$) as shown in Table I. Histological examination of the muscles did not show an increase in centre-nucleated fibres, suggesting that the increase in weight was due to hypertrophy rather than hyperplasia. None of the biochemical tests of liver function (AST, ALT, ALP and total protein) showed a significant change in the group treated with rAAVIGF1 (repeated measures test, p>0,05). Histopathological examination of liver and testes showed established liver cirrhosis and little or no gonadal atrophy. Semi-quantitative scoring of liver fibrosis by Gomori's tricromic staining showed no significant differences (Mann-Whitney U test, $p>0.05$) between control and treated animals.

In protocol 2 rats received CCl_4 exposure once a week for a further period of 9 weeks before treatment, and continued receiving this toxin during the entire length of the study. This resulted in a more advanced liver cirrhosis, frequently de-compensated and leading to the development of ascites. We could not detect differences in the evolution of body weight between the two groups during the study (repeated measures F-test, $p>0.05$). Three rats from each group (25%) died during the study indicating strong liver damage and lack of differences in survival between the group treated with rAAV and the control group. At the end of the study most rats showed a poor nutritional status, evident liver damage by macroscopical examination, testicular atrophy and variable amounts of ascites fluid. Normalised weight did not differ for any of the organs analysed, not even in the injected muscles (independent samples t-test and Mann-Whitney U test, $p>0.05$). Biochemical assays showed no differences in AST, ALT, ALP, total protein and bilirubin in the time points analysed: day 0, day 31, day 77 and day 133 (repeated measures F-test, $p>0.05$). Total IGF1 levels in serum were not different between the two groups at an intermediate time point (day 31) and at the end of the study (independent samples *t*-test, $p>0.05$). In

order to exclude that IGF1 is being rapidly secreted to the bloodstream and remains there in a free form, not associated to circulating binding proteins, free serum IGF1 was measured, but no differences were found (independent samples *t*-test, $p>0.05$). Histopathological examination of liver sections showed marked fibrosis in all rats, as measured by Gomori's tricromic staining. No differences were present between the two groups by semi-quantitative scoring of collagen content (Mann-Whitney U test, $p>0.05$). Testicular atrophy was present in some rats with no relation to treatment. Muscle fibres showed no marked hypertrophy or hyperplasia when the two groups were compared.

Discussion

It has been shown that low doses of IGF1 improve the nutritional status, osteopenia, hypogonadism and liver function tests in rats with hepatic cirrhosis induced by chronic exposure to CCl_4 (2-6, 18). Combining the therapeutic potential of recombinant IGF1 in a rat model of liver cirrhosis with the efficiency of rAAV-mediated gene transfer into skeletal muscle could provide an interesting model of gene therapy for liver disease with potential clinical applications. In this report we describe the development of a rAAV intended for transfer of the IGF1 gene into skeletal muscle, and demonstrate that viral stocks are infectious and can transduce 293 cells in vitro (resulting in a secreted form of the growth factor that can be measured by a RIA assay specific for total rat IGF1) and muscle fibres in vivo (shown by the detection of specific mRNA in injected muscles).

Using a rat model of liver cirrhosis induced by chronic exposure to CCl_4, we have used this rAAV vector in two different experimental settings in which the administration of exogenous IGF1 has been shown to be beneficial (2-6, 18). One of the experimental protocols included rats with mild liver damage in which the toxin was discontinued after the administration of the rAAV, with a view to ascertain whether IGF1 released from the muscle could speed up the recovery from the initial toxic insult. The second experimental protocol was designed to study the effects of the rAAV in a group of rats with advanced liver cirrhosis in which the toxin was maintained after the administration of the vector, in order to see whether the injection of the virus could either stop the progression of the disease or improve established liver damage.

IGF1 expression in injected muscles was confirmed by RT-PCR, and treatment with rAAV increased the weight of vector-injected muscles in rats with mild compensated cirrhosis. This effect was specific for rAAVIGF1-injected muscles, as such a difference was not found in control muscles (gastrocnemius) from the same animals. An increase in muscle weight has been reported in transgenic mice that over-express IGF1 in skeletal muscle (16, 19, 20), supporting the notion that, in our experimental setting, IGF1 is being expressed from injected muscles and effecting an autocrine or paracrine action, or a combination or both. Interestingly, this effect was not reproduced in our rat model of advanced cirrhosis (Protocol 2). This is most likely due to the poor nutritional status and overall loss of weight in these animals, so that generalised loss of muscle mass overcomes any potential hypertrophy resulting from local IGF1 production.

Although local expression of IGF1 was confirmed, we did not observe any systemic effect in cirrhotic animals, and IGF1 serum levels were not significantly increased in rats treated with the rAAV. Several factors could account for the lack of increased serum levels of IGF1 in treated rats. First, the growth factor is probably being produced in small amounts in muscle and then rapidly secreted to the bloodstream, where it is completely bound by the various circulating IGF1-binding proteins. Second, skeletal muscle contains large numbers of IGF1 receptors, so that fast recapture of newly synthesised IGF1 by these receptors could further result in undetectable levels of the growth factor in serum (Dr. K. Lund, personal communication). Furthermore, the dose of rAAV used in this study was probably not high enough to show systemic effects. In this regard, it is worth noting that bolus administration of recombinant IGF1 probably overcomes these limiting factors because the injected protein reaches a peak of free IGF1 in serum and then decreases rapidly, after it is taken up by peripheral receptors. This could explain the increased therapeutic benefit observed with recombinant IGF1 administration as compared to IGF1 gene transfer into muscle at the vector dose employed here.

Our results also raise interesting questions concerning the general strategy of muscle gene transfer, suggesting that its efficacy will be highly dependent on the therapeutic protein being expressed. Whereas this approach has proved successful for a long list of therapeutic proteins, our results suggest that some molecules could require higher vector dose in order to achieve systemic effects, depending on factors such as local density of receptors, half-life in blood or abundance of binding proteins.

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M. ZARATIEGUI, I. CASTILLA-CORTÁZAR, M. GARCÍA, J. QUIROGA, J. PRIETO y F. J. NOVO. *Transferencia génica de IGF1 al músculo esquelético mediante vectores virales adeno-asociados en un modelo de cirrosis hepática en rata.* J. Physiol. Biochem., **58** (3), 169-176, 2002.

La administración sistémica de IGF1 a bajos niveles provoca mejoría de la función hepática, el estado nutricional y la atrofia testicular de las ratas con cirrosis hepática inducida por CCl_4. En este trabajo, se ha desarrollado un vector viral adeno-asociado (rAAV) que contiene el cDNA de IGF1 de rata y se ha confirmado la expresión de IGF1 tras la inyección intramuscular de dicho vector en un modelo de cirrosis hepática en rata. Aunque el peso de los músculos tratados aumenta significativamente en ratas con cirrosis incipiente, este efecto no se reproduce en ratas con cirrosis avanzada y descompensada. Por otra parte, no se puede objetivar una mejoría del daño hepático en las ratas tratadas, independientemente del estado de la cirrosis que presentan. Nuestros resultados sugieren que la transferencia génica de IGF1 al músculo provoca un efecto local, al menos a las dosis de vector empleadas en este estudio.

Palabras clave: Virus adeno-asociado, Cirrosis, IGF1.

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