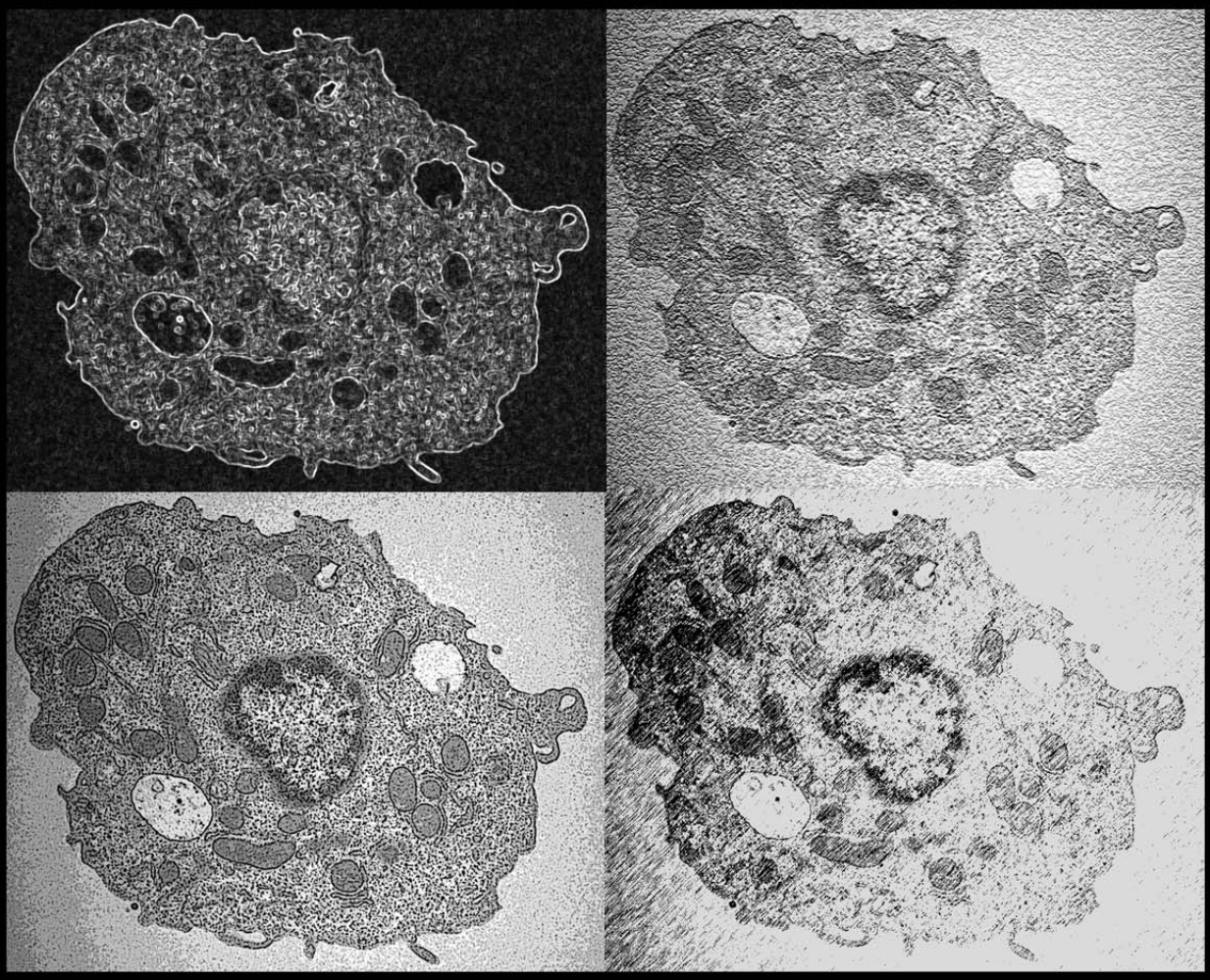
Caracterització de mutacions causants de la malaltia de Gaucher. Aproximació a una teràpia gènica.



Anna Diaz Font

2006

Resultats

CAPíTOL 3

UTILITZACIÓ DE TÈCNIQUES DE REPARACIÓ GÈNICA COM A POSSIBLE TERÀPIA DE LA MALALTIA DE GAUCHER: ELS QUIMERAPLASTS

3.1. Utilització de quimeraplasts com a possible teràpia de la malaltia de Gaucher

Fins al moment s'han desenvolupat diferents tècniques de teràpia gènica, però en general tenen moltes limitacions. Per una banda els vectors utilitzats poden generar reaccions immunes (adenovirus) o integracions a l'atzar en el genoma (retrovirus). Per altra banda, el gen forani deixa d'expressar-se després d'un cert temps. Per tot això és lògic que s'hagin desenvolupat noves estratègies per a la teràpia de malalties genètiques. Una d'aquestes aproximacions és l'utilització de quimeraplasts, molècules quimèriques de RNA-DNA capaces de promoure la correcció d'una mutació mitjançant els mecanismes de reparació de la cèl·lula. En la malaltia de Gaucher una de les mutacions més freqüents és la L444P. Hem intentat corregir aquesta mutació utilitzant quimeraplasts en fibroblasts d'un pacient de la malaltia de Gaucher homozigot per la mutació L444P.

Els resultats obtinguts mostren una molt baixa, o nul·la, eficiència de correcció. Recentment s'han publicat diferents treballs on descriuen també resultats negatius per altres malalties utilitzant aquesta tècnica, deixant en entredit l'eficàcia d'aquesta tècnica.

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Unsuccessful chimeraplast strategy for the correction of a mutation causing Gaucher disease

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Abstract

A number of gene therapy approaches have been developed for the treatment of genetic diseases, most of them based on the use of viral vectors. However, in general, they have not been successful and some complications, such as immune reactions induced by adenoviral vectors or random integration of retroviral vectors, have been reported frequently. To overcome these limitations, novel strategies have recently emerged. One of them is chimeraplasty, based on the correction of single-base mutations by mismatch repair mechanisms using chimeric RNA/DNA oligonucleotides, named chimeraplasts. Several papers have reported the use of this method to correct a number of pathological mutations underlying different diseases. In Gaucher disease, the most prevalent spingoliposis, mutation c.1448C->T (L444P), is one of the most common mutations in many populations. We have attempted to correct mutation c.1448C->T in fibroblasts from a Gaucher disease patient using a chimeraplast approach. Although we have shown that the chimeraplast reaches the fibroblast nucleus by colocalization with nuclear structures, no genomic correction was detected. To evaluate whether fibroblast and hepatocyte extracts are able to effect correction in vitro, we followed a cell-free extract assay using *Escherichia coli* cells. Our results show a very low efficiency (if any) of chimeraplast correction. A growing number of negative results for chimeraplast experiments have recently been reported. This promising technique has turned out to be inconsistent and impossible to replicate in most laboratories and many of the first successful results are now being questioned. Our negative data are consistent with this criticism.

Keywords: Gaucher disease; c.1448C->T (L444P) mutation; Chimeraplasts; Gene therapy

Introduction

Current gene therapy approaches for the treatment of genetic diseases are based on the introduction into a cell of an intact copy of a gene that should supply the function not performed by the endogenous mutated gene. The therapeutic gene is usually delivered using viral vectors. However, immune reactions induced by adenoviral vectors and random integration of retroviral vectors have produced several complications. To overcome these limitations, novel strategies have recently emerged. One of them consists of correction of single-base mutations by mismatch repair mechanisms using chimeric RNA/DNA oligonucleotides named chimeraplasts [1]. Several papers reported the use of this method to correct a number of pathological mutations underlying diseases such as sickle-cell anemia [1], hemophilia B [2], or Crigler-Naijar syndrome [3].

However, this promising technique turned out to be inconsistent and impossible to replicate in most laboratories, as recently pointed out by Taubes [4]. He claimed that the difficulties of publishing negative results helped the first few positive results, most of which were from the same laboratory or from related groups, to remain unquestioned.

Gaucher disease (OMIM 230800, 230900, 231000) is a recessively inherited lysosomal storage disorder characterized by decreased hydrolysis of glucocerebroside due to

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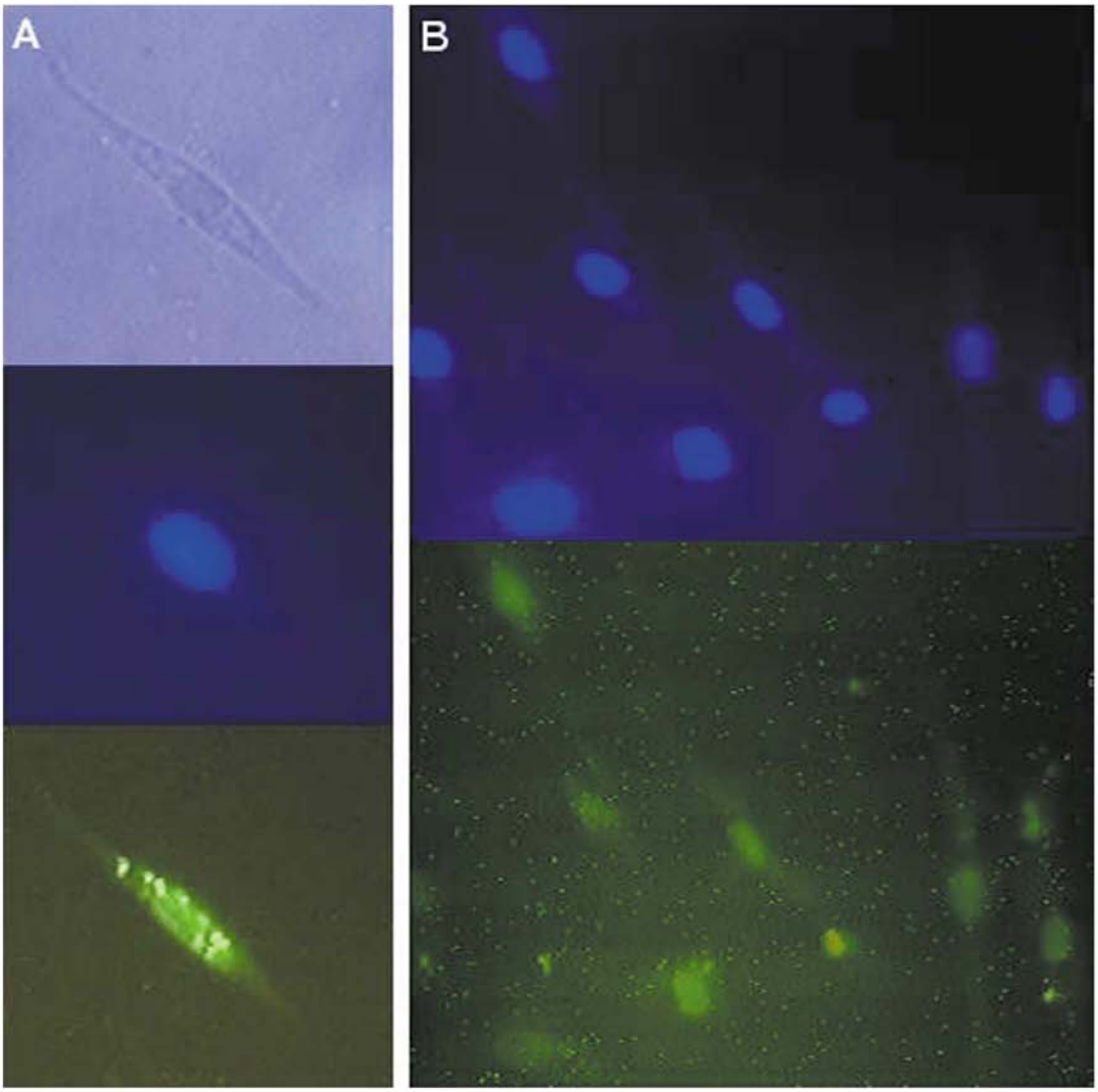

Fig. 1. Cytofectin-mediated internalization of a corrector chimeraplast into the nucleus of primary fibroblasts from a Gaucher disease patient. (A) Upper panel, bright field image of a fibroblast cell; middle panel, DAPI staining of the nucleus; lower panel, FITC-labeled chimeraplast. (B) The comparison between DAPI staining of fibroblast cells (upper panel) and FITC-labeled chimeraplasts (lower panel) shows that the nuclear uptake of chimeraplasts is highly efficient.

deficient activity of glucocerebrosidase (D-glucosyl acylsphingosine glucohydrolase, EC 3.2.1.45). It is mainly caused by mutations in the gene encoding glucocerebrosidase (GBA), located on 1q21. The severe mutation c.1448C->T (L444P), associated with a neurological phenotype, is one of the most common mutations in most non-Ashkenazi Jewish populations studied. Here we report an attempt to correct mutation c.1448C->T in fibroblasts from a Gaucher disease patient who was homozygous for this mutation, using a chimeraplast approach.

Results and discussion

The sequence of the 68-mer chimeraplast designed to correct the c.1448C->T mutation was 5′-AGAAGAACGACCTGGACGCAGTGGCTTTTgccacugcguCCAGGucguucuucuGCGCGTTTTCGCGC-3′ (synthesized by Cruachem Ltd., Glasgow, UK), where lower case indicates 2′-*O*-methyl ribonucleosides and upper case corresponds to DNA. To study the uptake and nuclear localization of the chimeric oligonucleotide in the fibroblasts, a 5′ fluorescein isothiocyanate (FITC)-con-

taining chimeraplast was used. Patient fibroblasts were cultured on glass coverslips in a 6-well plate, in MEM medium (Gibco-BRL) containing 100 U/ml penicillin/streptomycin (Life Technologies, Gaithersburg, MD), and 10% fetal calf serum (FCS) at 37 °C and 5% CO_2. For lipoplex-mediated chimeraplast transfection, the cells were grown at 60–70% confluency. Prior to transfection, the medium from the cells was replaced with 400 μl of fresh MEM medium. A 100-μl MEM solution with Cytofectin GSV™ lipoplexes (25 μg/ml, Glen Research) and chimeraplast (1.25 μM) was added dropwise to the cells. Subsequently, the plates were centrifuged for 5 min at 280*g* and returned to the CO_2 incubator. Four hours after transfection, the wells were filled with 500 μl/each of serum-containing medium and incubated for 24 h at 37 °C and 5% CO_2. To increase chimeraplast uptake into the cell nuclei, the lipofection was performed in the presence of 8 μg/ml polybrene [5]. For microscope examination, the cells were washed twice in PBS and fixed for 5 min in 4% paraformaldehyde at RT, and the nucleus was stained with DAPI (Sigma) 1:200 in PBS for 15 min at RT. The coverslips were mounted in water-soluble glycerolgelatin mounting medium and studied under a fluorescence microscope. To quantify the extent of correction, genomic DNA was isolated from the transfected fibroblasts. A PCR amplification of a 249-bp DNA fragment of the *GBA* gene containing the c.1448C->T mutation was performed, and the presence of the corrected allele was tested by *Nci*I restriction analysis (forward primer: 5′-TATGACTTAAGCCGGGAGAGCCAGGGCAGAGCCTC-3′, reverse primer: 5′-TTTAGCACGACCACAACAGC-3′). The mutation creates a *Nci*I site, and a second *Nci*I site was introduced in the forward primer as an internal control of digestion.

The colocalization of chimeraplasts with nuclear structures (Fig. 1) confirmed that the DNA/RNA oligonucleotide reached the cell nucleus. However, PCR-RFLP (Fig. 2) did not detect any wild-type band, indicative of correction, in the patient transfected fibroblasts. The lowest ratio of WT/mutant allele detected was 3%, when the method sensitivity was tested (not shown). Thus, these results indicate that either there is no correction or the level of correction is below 3%.

These negative results prompted us to evaluate whether fibroblast extracts effect correction in vitro, following the cell-free extract assay described by Cole-Strauss et al. [6]. Results on the use of chimeraplasts on fibroblasts are scarce and controversial. Tran et al. [7] have recently shown a low, although detectable, level of correction in rodent fibroblasts only if the mutation was present in a plasmid but not in a cell chromosome. However, Thorpe et al. [8,9] failed to find any correction of an episomal mutation in COS7, a monkey kidney fibroblast cell line. Finally, Gamper et al. [10] reported a successful in vitro correction using extracts from mouse embryonic fibroblasts, with an approach similar to the one we describe here [6].

The cell-free extract system is based on the correction of a mutant kanamycin resistance gene contained in a plasmid (pK^sm4021). The corrector chimeraplast (Kan4021C) and the plasmid are incubated together with a mammalian cell-free extract. The ratio of kanamycin/ampicillin resistant colonies after transformation of *Escherichia coli* cells is indicative of the level of correction. Cell-free extracts from hepatocytes were reported to correct efficiently [6]. Thus, extracts from the human hepatoma cell line HepG2 were used as control. The pK^sm4021 plasmid was kindly provided by Dr. E. Kmiec (University of Delaware, Newark, DE, USA) and the Kan4021C chimeraplast and HepG2 cells by Dr. Josep Aran (Centre de Genètica Mèdica i Molecular, Institut de Recerca Oncològica). Cell-free extracts were prepared as described [6]. We obtained negative results for both the hepatocyte and the fibroblast cell extracts. Nine independent experiments with varying amounts of hepatocyte protein extract (10 to 50 μg) were performed. The few kanamycin resistant colonies found (1–5 out of 1000–70,000) were not the result of chimeraplast correction, as they bore no plasmid. These results show a very low efficiency (if any) of chimeraplast correction, consistent with the growing number of negative results recently reported [4], which increase skepticism and question the validity of reports of the success of this technique.

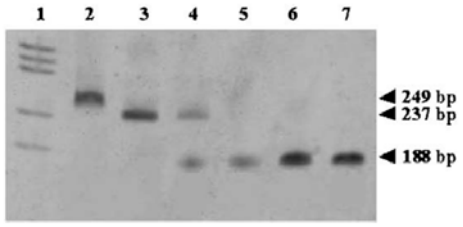

Fig. 2. *Nci*I restriction analysis of PCR products from genomic DNA of fibroblasts transfected (and nontransfected) with a c.1448C->T corrector chimeraplast. Lane 1: molecular weight marker (ϕX174 *Hae*III); lane 2: undigested amplicon (249 bp); lane 3: +/+ amplicon digested with *Nci*I (237 bp); lane 4: +/c.1448C->T amplicon digested with *Nci*I; lane 5: c.1448C->T/c.1448C->T amplicon digested with *Nci*I (188 bp); lanes 6 and 7: *Nci*I-digested amplicons from chimeraplast-transfected c.1448C->T/c.1448C->T fibroblasts (independent experiments).

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