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Retroviral-mediated gene therapy for the treatment of citrullinemia. Transfer and expression of argininosuccinate synthetase in human hematopoietic cells

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Abstract. Citrullinemia is a recessive genetic disease caused by a deficiency in argininosuccinate synthetase (AS). Retroviruses were used to transduce the human AS gene into cultured human cells. Using amphotropic viruses with high titer ($>10^6$ cfu/ml), we were able to correct the defect in cultured fibroblasts from citrullinemic patients. Retroviral transduction of the human AS gene into human bone marrow cells was also studied. Co-cultivation was used to infect the cells and up to 80% of progenitor cells were found to be carrying and expressing the AS retrovirus after infection. When the infected cells were kept in culture, integration and expression of the retrovirus was observed. Retroviral sequences were present and expressed in the cultured bone marrow-derived cells for up to 10 weeks.
Key words. Gene therapy; retrovirus; citrullinemia; human.

Somatic gene transfer offers the possibility of a new approach in the treatment of human genetic diseases. Recombinant defective retroviral vectors allow gene transfer into virtually any cell type (see reviews by Demarquoy et al.[1], Cournoyer et al.[2] and Friedman[3]). Hematopoietic cells provide the best model available at present for such gene transfer methodologies. These cells can be cultured ex vivo and transplanted back into the host.

Citrullinemia is a disease of urea synthesis associated with a deficiency in argininosuccinate synthetase (AS, EC 6.3.4.5). In the liver of ureothelic animals AS catalyses the condensation of citrulline and aspartic acid into argininosuccinic acid (ASA) as shown below:

$$\text{citrulline} + \text{aspartate} + \text{ATP} \longrightarrow \text{argininosuccinate} + \text{AMP} + \text{PPi}$$

ASA is subsequently converted into arginine and fumaric acid by argininosuccinate lyase (AL). The reaction catalyzed by AS is part of the urea cycle and plays an important role in nitrogen metabolism. Patients with citrullinemia show large amounts of citrulline and amonia in the circulating blood, which result in severe neurological damage or death unless treatment is initiated immediately after birth. Reviews of the clinical aspects of the disease are available[4,5]. The AS locus has been extensively studied. The AS gene has been cloned and the cDNA sequences are known for the human[6], rat[7] and bovine[8] enzymes. The human gene, which maps on chromosome 9q34[9], contains at least 13 exons, and several pseudogenes have been described[10]. Citrullinemia is mostly due to single base mutations within the cDNA, but small deletions of the coding sequence have also been described[11]. Currently, strict dietary regimens and drugs that facilitate nitrogen excretion are the only means of therapy, and despite treatment continued episodes of hyperammonemia may occur, leading to considerable mental impairment.

The AL gene is expressed in virtually all tissues, and citrulline is continually available from the blood supply. Although transduction of AS activity into hepatocytes would be the appropriate therapeutic approach, high levels of AS activity in bone marrow-derived cells or skin fibroblasts might allow elimination of the excess of citrulline and ammonia, and thus would bring about a partial correction of the disease. Despite the lack of animal model citrullinemia provides some attractive technical and biological features as a system for developing gene therapy. Hematopoietic cells and many cultured cell lines do not normally express AS but express AL. This provides a selection for the expression of AS in human citrullinemic cells or rodent cell lines, by growing cells in a medium in which citrulline has been substituted for arginine. Furthermore, ^{14}C citrulline incorporation into protein can be used to monitor gene transfer of AS activity into cultured cells. This allows producer cell clones and infected cells to be screened for AS expression.

Materials and methods

Viral production: The retroviral vector pΔNN2AS was used[12]. The vector was co-transfected with pSVneo[13] (10:1), using calcium phosphate transfection, into the amphotropic packaging cell line GP + envAm12[14] and the ecotropic packaging cell line GP + E-86[15]. Cells were cloned and the titer was determined using 3T3 cells, which do not express the AS gene in a medium with citrulline substituted for arginine[12]. The titer was determined several times, and the average was found to be 3.5×10^6 cfu/ml for the amphotropic and 5×10^6 cfu/ml for the ecotropic producer cells.

Infection of rodent cells and skin citrullinemic fibroblasts: Patient fibroblasts and rodent cell lines (3T3, XC) were cultured in the presence of 5 ml of filtered (0.45 μm) supernatant from GP + envAm 12 cells producing the AS virus, for 48 h in DMEM supplemented with 10% FCS. Polybrene was added to the medium at a concentration of 4 μg/ml.

Infection and culture of hematopoietic cells: Human bone marrow cells were obtained from healthy volunteers. 3×10^6 producer cells were plated and the same number of bone marrow cells were added to the dish. Cells were co-cultivated for 48 h in the presence of 20% FCS, 10% bovine serum albumin, 4 μg/ml polybrene, transferrin (0.3 μg/ml) and hematopoietic growth factors as provided by conditioned media (5637 (ATCCMTB9) conditioned medium, 10%; WEHI3b (ATCCTIB68) conditioned medium, 10%. The use of these conditioned media were shown to improve gene transfer into hematopoietic cells). After co-cultivation, hematopoietic cells were separated from producer cells on a Percoll gradient (d = 1.055). Mock infections were carried out by co-cultivating bone marrow cells with GP + envAm12 cells. Long-term cultures were performed as described by Dexter et al.[16]. The medium was changed every week and non-adherent cells were collected. Those cells were either assayed for the presence of retroviral sequences and gene expression or subcultured in semi-solid medium to generate colonies (cfu-c) derivated from hematopoietic progenitors. Clonogenic cultures were performed in methyl cellulose as described[17]. 2×10^5 cells were plated onto 35 mm dishes in the presence of 5% lymphocyte conditioned medium, 50 μM β2-mercaptoethanol and 2 U of erythropoietin (Terry Fox Lab., Vancouver, Canada) and allowed to grow for 14 d. Individual colonies were harvested and used to determine retroviral insertion and expression.

DNA analysis and enzymatic assays: Retroviral sequences were detected in cultured cells using the polymerase chain reaction (PCR). The sequences of the primers and the PCR conditions have been previously described[18]. The primer sequences were derived from a sequence within the LTR and from the human AS cDNA. Other general techniques were performed as described by Maniatis et al.[19]. AS activity in colonies and bone marrow cells was determined as described by Demarquoy et al.[18]. In some cases, the citrulline incorporation assay was used as an in vivo test to measure AS activity. This assay measures citrulline conversion into arginine in cultured cells. The assay was performed in MEM medium without arginine and leucine supplemented with ^{14}C-citrulline and ^{3}H-leucine. After 48 h of incubation, ^{14}C-arginine and ^{3}H-leucine contents were analyzed in the total cellular protein. The use of ^{3}H-leucine allows normalization, which was done as ^{14}C cpm per 10^5 cpm of [^{3}H]-leucine incorporated.

Results

Amphotropic infection of skin fibroblasts from citrullinemic patients. Under normal conditions, patients' cells (AC and HJ) showed no significant activity, while normal cells presented an AS activity of 0.30 using the citrulline incorporation assay. After infection with an amphotropic virus the level of activity measured in patient cells was dramatically increased (table) and those levels were found to be in the same range as for cells from normal individuals. Ecotropic virus was not capable of transducing the AS cDNA into human cells. Both ecotropic and amphotropic viruses allowed gene transfer into rodent cell lines and AS expression.

Long term detection and expression of the AS retroviral sequences in bone marrow derived cells. Bone marrow cells from normal donors were infected by co-cultivation for 48 h with amphotropic virus-producing cells. Freshly infected cells were assayed for the presence of retroviral sequences. Cells were then kept in long-term culture, and every week, the AS activity in the non-adherent cells of the culture and the percentage of infected cfu-c derived from the culture were monitored. Just after infection the percentage of cfu-c carrying the AS retroviral vector was found to be between 80–95%. An example of amplification is given in figure 1. This percentage remained stable for two weeks and then

Table. Rodent cells or human fibroblasts were infected by incubation with supernatant from virus-producing cells for 48 h in the presence of polybrene. AS activity was determined by the citrulline incorporation assay. This assay monitors the conversion of radiolabelled citrulline into arginine and its incorporation into proteins in a selection medium in which [^{14}C]-citrulline has been substituted for arginine and [^{3}H]-leucine for leucine. The determination of ^{14}C in proteins was expressed in cpm and normalized with [^{3}H]-leucine incorporation. Mock infections were carried out with medium obtained from GP + E-86 (ecotropic infection) or GP + envAm 12 cells (amphotropic infection).

AS activity Cell	Non-infected	Mock-infected	Infected
Ecotropic infection			
XC	0.012	0.015	0.851
3T3	0.018	0.014	0.986
HC (patient)	0.017	0.021	0.009
		0.014	0.013
Amphotropic infection			
XC	0.011	0.008	1.020
3T3	0.014	0.016	0.880
HJ (patient)	0.017	0.021	0.462
	0.021	0.024	0.364
			0.197
			0.332
AC (patient)	0.016	0.015	0.298
	0.014	0.015	0.265
			0.328
Normal individuals			
	0.291		
	0.246		
	0.324		

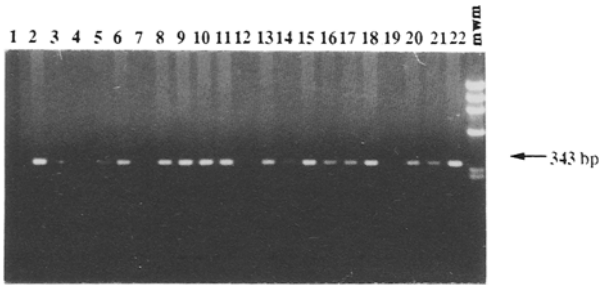

Figure 1. Detection of AS retroviral sequences in cfu-c by polymerase chain reaction. Individual colonies were picked from methyl cellulose, washed in PBS twice and lysed using Triton in a final volume of 50 µl. 10 µl portions were used for 30 cycles of amplification. The product was analyzed on a 1.3% agarose gel containing ethidium bromide. Lane 1, negative control; lane 2; positive control; lanes 3–22, cfu-c from infected human bone marrow-derived cells in culture; mwm, molecular weight marker: *Hae*III digest of φX 174.

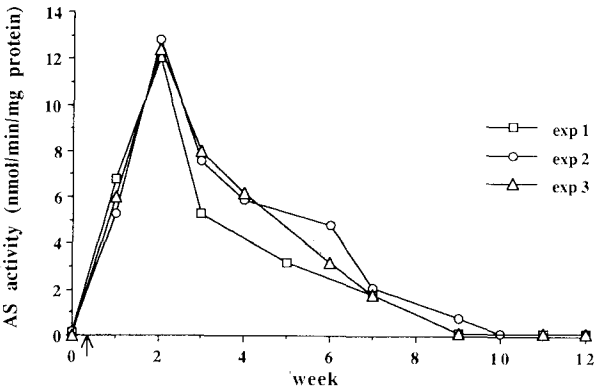

Fig. 3. As activity was determined in pooled non-adherent bone marrow-derived cells maintained in long term culture. AS activity was expressed in mU/mg protein. Three independent experiments are represented, the symbols used refer to the same individuals as in figure 2.

decreased to reach less than 30% at week 4. The percentage decreased further after this period, and no infected cfu-c were detected after 12 weeks in culture (fig. 2).

The AS activity was determined in non-adherent cells. The activity followed the same pattern as the percentage of infected cfu-c. AS activity in bone marrow-derived cells before infection was virtually undetectable (<0.1 mU/mg protein). This activity increased during the first 2 weeks following the infection. After 2 weeks in culture, AS activity was averaging 12.4 mU/mg protein. After this date, enzyme activity decreased and the average activity was found to be 6.2 mU/mg protein after 4 weeks in culture and no significant activity was measured after 10 weeks (fig. 3). No infection of human hematopoietic cells was observed when the ecotropic virus was used.

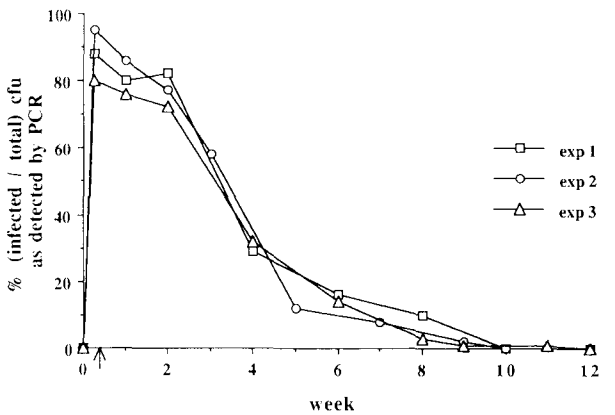

Figure 2. After infection, cells were plated for long term culture. Cfu-c derived from non-adherent cells were assayed by PCR every week, or as indicated, for the presence of AS viral sequences. Data shown represent the percentage of infected cfu-c among the total number of tested cfu-c. The arrow represents the end of the period of co-cultivation. The results of 3 independent experiments from 3 different individuals are presented.

Discussion

Citrullinemia and the AS locus provide some technical advantages such as cell selection and rapid analysis of infection[20]. Furthermore, they represent a feasible model for gene therapy. Patients with citrullinemia can now survive for several years with a strict dietary regimen and pharmacological therapy. However, some patients are still dying from hyperammonemia which occurs episodically. Most first-affected citrullinemic children born into a family suffer permanent brain damage prior to diagnosis, but dozens of citrullinemic patients are still living, some with brain damage and some with normal neurological functions. Citrullinemic patients maintain a high level of citrulline in the blood ($\times 200$), which provides a detection system for any therapeutic approach. Citrulline can enter any cells, and expression of high levels of AS activity in bone marrow-derived cells and skin fibroblasts could be expected to be beneficial. Furthermore, no special co-factors are required for this enzyme and AL, the subsequent enzyme in ureagenesis, is expressed in most cell types and would allow for the conversion of argininosuccinate in arginine.

Using the packaging cell lines GP + envAm12 and GP + E-86 we were were able to obtain a high viral titer ($>10^6$ cfu/ml) from a previously-described recombinant retrovirus containing the human argininosuccinate synthetase gene[6]. The presence of helper virus was monitored using a rescue assay. Throughout these experiments, no such virus was found. Comparable titers, in the absence of replication-competent viruses, have been previously described using this packaging cell line[21] or the ecotropic equivalent (GP + E-86[18]).

The amphotropic viruses carrying the AS cDNA were able to transduce to AS gene into fibroblasts from citrullinemic patients. The level of activity in infected cells was in the same range as in fibroblasts from normal individuals, when measured by the conversion

of radiolabelled citrulline to arginine in infected fibroblasts. This expression was monitored for up to 2 weeks, in cultured cells, and no major change in the level of activity was observed during this period and no transformant cells were ever observed.

We were able to demonstrate infection and expression of the virus in cultured human bone marrow-derived cells for up to 10 weeks. When hematopoietic cells were infected, 80–90% of the progenitor cells were found to be carrying the retroviral sequences just after the infection. However, when those cells were maintained in culture for several weeks the percentage of infected cells slowly decreased and no viral sequences were found after 10 weeks in culture. The level of activity measured in non-adherent bone marrow cells maintained in culture was found to be closely correlated to the percentage of infected cfu-c. This pattern has been described in the past[15] for other constructs. This loss of activity may be due to the inability to infect a sufficient number of primordial stem cells or to the decay of the culture of human hematopoietic cells. Our experience with murine cells using both ecotropic and amphotropic vectors[18] suggested that with equivalent titers an amphotropic vector was not capable of infecting murine hematopoietic cells as well as an ecotropic virus. One hypothesis is that an ecotropic vector is not able to infect primordial hematopoietic stem cells efficiently, or that the titer is too low.

Bone marrow transplantation has never been used for the treatment of citrullinemia. However, most of the damage due to the defect is related to increased levels of ammonia and citrulline in the blood. We think than an increased concentration of AS in the blood might allow degradation of citrulline in the blood, and would reduce the concentration of circulating ammonia and mitigate the disorder. Furthermore, it is possible that bone marrow-derived macrophages that have been infected with the AS cDNA virus would migrate throughout the organism, and participate in the degradation of citrulline in several organs such as the liver or the brain, as 'microglial cells'[22]. Because of the decay of AS expression in cultured bone marrow cells, repeated transfusion of infected bone marrow cells might be an approach that would allow elimination of citrulline and ammonia from the blood and correction of the disease.

Abbreviations. cfu, colony forming unit; DMEM, Dulbecco's modification of Eagle's medium; MEM, Eagle's minimum essential medium; PCR, polymerase chain reaction; PBS, Phosphate buffer saline.

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