Primary research

Approaches to enhancing the retroviral transduction of human synoviocytes

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Abstract

This report concerns a clinical trial for rheumatoid arthritis (RA), approved by the US National Institutes of Health and the Food and Drug Administration. An amphotropic retrovirus (MFG-IRAP) was used *ex vivo* to transfer a cDNA encoding human interleukin-1 receptor antagonist (IL-1Ra) to synovium. The protocol required the transduced cells to secrete at least 30 ng IL-1Ra/10^6 cells per 48 h before reimplantation. Here we have evaluated various protocols for their efficiency in transducing cultures of human rheumatoid synoviocytes. The most reliably efficient methods used high titer retrovirus (approximately 10^8 infectious particles/ml). Transduction efficiency was increased further by exposing the cells to virus under flow-through conditions. The use of dioctadecylamidoglycylspermine (DOGS) as a polycation instead of Polybrene (hexadimethrine bromide) provided an additional small increment in efficiency. Under normal conditions of static transduction, standard titer, clinical grade retrovirus (approximately 5×10^5 infectious particles/ml) failed to achieve the expression levels required by the clinical trial. However, the shortfall could be remedied by increasing the time of transduction under static conditions, transducing under flow-through conditions, or transducing during centrifugation.

Keywords: arthritis, flow-through, high-titer retrovirus, interleukin-1 receptor antagonist

Introduction

Rheumatoid arthritis (RA) is a promising new target for gene therapy (reviewed in [1]). One approach to the genetic therapy of RA requires the transfer of anti-arthritis genes to the synovial linings of joints [2]. The first human trial of arthritis gene therapy approved by the Recombinant DNA Advisory Committee (RAC) of the National Institutes of Health and by the US Food and Drug Administration (FDA) involves the *ex vivo,* retroviral delivery to joints of a cDNA encoding the human interleukin-1 receptor antagonist (IL-1Ra) [3]. The procedure has been shown to be safe and effective in several animal models of RA [4–7].

When rabbit type B synoviocytes are transduced with the amphotropic retrovirus MFG-IRAP under standard, static conditions, they routinely secrete approximately 100 ng human IL-1Ra/10^6 cells per 48 h into their culture medium [7]. For the purposes of the clinical trial, transduced

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DOGS = dioctadecylamidoglycylspermine; IL-1Ra = interleukin-1 receptor antagonist [protein]; RA = rheumatoid arthritis; SD = standard deviation; SEM = standard error of the mean.

human synoviocytes were required to secrete at least 30 ng IL-1Ra/10^6 cells per 48 h [3]. We investigated several transduction strategies in order to identify conditions that result in the highest transduction efficiency for use in this clinical trial. This communication describes attempts to improve the transduction efficiency of human rheumatoid synoviocytes by MFG-IRAP.

Materials and Methods

Cell culture

Synovial tissue was recovered from joints of five patients undergoing surgery for the management of their RA. Cells were isolated by sequential digestion of synovium with trypsin and collagenase, counted with a hemocytometer, cultured in Ham's F-12 medium supplemented with 10% (v/v) fetal bovine serum and antibiotics, and seeded into 25 cm^2 plastic culture flasks with 4 ml medium at a density of 5×10^5 cells per flask (passage 0) [8]. Viabilty of >95% was confirmed by staining with trypan blue. Among cells of passage 0, type A and type B synovial fibroblasts and macrophages were the predominant adherent cells and lymphocytes were the predominant nonadherent cells. The latter were removed by medium changes every 3 days. Confluent cells were detached by trypsinizing, washed, and reseeded into 6-well culture plates at a density of $0.5–1.0 \times 10^5$ cells per well (first-passage cells) or into 25 cm^2 flasks. When the flasks reached confluence, they were trypsinized and seeded into six-well plates at a density of $0.5–1.0 \times 10^5$ cells per well (second-passage cells). Since type-A synoviocytes and macrophages are lost during trypsinization, passage 1 and passage 2 cells consist of type B synovial fibroblasts.

Vectors

The construction of a ψ-CRIP [9] producer line for MFG-IRAP has been previously described [7]. The Human Gene Therapy Applications Laboratory of the University of Pittsburgh Medical Center produced clinical grade virus. The titer of this retroviral preparation was found to be 5×10^5 infectious particles/ml. This preparation is referred to as 'standard titer' retrovirus. High titer MFG-IRAP was produced by concentration using tangential flow filtration and size exclusion chromatography [10].

MFG-LacZ was also generated using the ψ-CRIP producer line. This vector contains the β-galactosidase gene, and was used as a marker of gene expression.

Transduction of synoviocytes

Transductions were carried out in six-well plates on passage 1 or passage 2 cells as noted below. On day 1, cells were plated at a density of $0.5–1.0 \times 10^5$ cells per well. When the cells were 50–60% confluent, the culture medium was removed and replaced with 3 ml of supernatant containing standard-titer MFG-IRAP, standard titer MFG-LacZ, high titer MFG-IRAP, or medium alone. All transductions were carried out in the presence of Polybrene (8 μg/ml) or dioctadecylamidoglycylspermine (DOGS) (5 μg/ml), as indicated.

For static transductions, viral supernatants were added to the cultures, which then were returned to the incubator for 2 h (one patient), 6 h (one patient) or 12 h (three patients), after which time the viral suspensions were replaced with 3 ml of fresh culture medium. For double static transduction, the media were removed 24 h from the time of the initial transduction and the process was repeated. In another series of experiments, the retroviral supernatants were allowed to remain on the cells for 24, 48, 72, or 96 h, with a change of retroviral supernatant every 24 h. At the end of the transduction period, all cells were washed with Gey's balanced salt solution and then 4 ml of culture medium was added to each well.

For transduction during centrifugation, the plates were centrifuged at 2000 ***g*** at 32°C during the 2 h transduction period. Centrifugation resulted in the development of a crescent-shaped 'dry' area in the center of each well. Because the cells in these areas were deprived of liquid during the transduction period, they did not survive. The dry areas were estimated to comprise approximately 10% of the area of the well.

For flow-through transduction, the method of [11] was followed. Briefly, synoviocytes were cultured on collagen-coated inserts (Transwell-COL™ from Costar), which were then placed into six-well plates. Retroviral supernatants (1 ml) were placed in the upper well and allowed to flow through the monolayer under gravity. After 8 h, the cells were washed and culture media were added to the wells.

Gene expression

After transduction, 4 ml of supplemented Ham's F-12 medium was added to each well, plates were returned to the incubator, and cells were allowed to reach confluence. Once the cells were confluent, the media were removed and the cells were washed with 4 ml Gey's balanced salt solution, which was then replaced with 4 ml culture medium. After 48 h, conditioned media were collected and stored at −20°C until they were assayed for IL-Ra expression. Cells were trypsinized and counted using a hemocytometer. Concentrations of IL-1Ra in the conditioned media were determined by ELISA (R&D Systems, Minneapolis, MN). Expression of LacZ was ascertained by histochemical staining with 5-bromo-4-chloro-3-indoyl-β-D-galactoside (X-gal) using standard techniques [7].

Data were analyzed with the statistical software Statview 4.5; unpaired *t*-tests were used to compare groups.

Results

Four different methods of retroviral transduction of rheumatoid synovium were compared: A) one static transduction at passage 1; B) one static transduction at passage 2; C) transduction at passage 1 during centrifugation; D) two static transductions made 24 h apart during passage 1 (Fig. 1).

Cells transduced once at passage 1 by static infection with standard-titer MFG-IRAP secreted 14.2 ± 2.8 ng IL-1Ra/10^6 cells per 48 h. None of the cultures secreted the $\geq$30 ng IL-1Ra/10^6 cells per 48 h required by the clinical protocol. Similar results were obtained with second passage cells. Static transduction twice within a passage resulted in an average secretion of 33.8 ± 8.7 ng IL-1Ra/10^6 cells per 48 h, but only two of the five cultures secreted more than 30 ng/10^6 cells per 48 h. First passage cells transduced once while being centrifuged secreted 110 ± 8.0 ng IL-1Ra/10^6 cells per 48 h ($P < 0.0001$ vs one or two static transductions) and all five cultures exceeded the cutoff threshold of 30 ng/10^6cells per 48 h. Routine static transductions with MFG-LacZ vector, carried out in parallel with these experiments, resulted in approximately 30% LacZ$^+$ cells, whereas transductions carried out during centrifugation resulted in nearly 100% transduction.

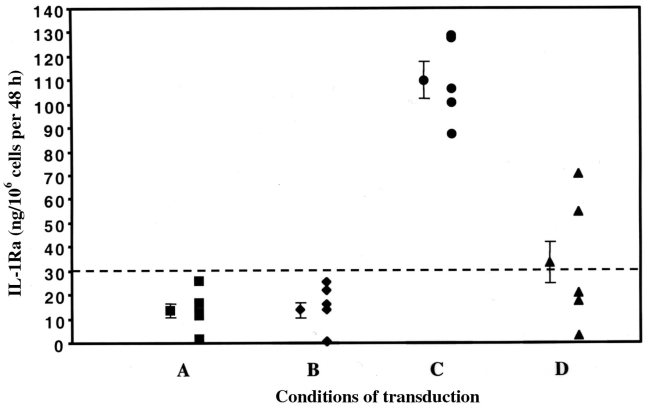
Lowering the temperature from 37 to 32°C or increasing the volume of the retroviral supernatant from 1 to 3 ml per well produced small (25% or less; statistically insignificant) improvements in transgene expression (data not shown). Increasing the time of transduction beyond 24 h raised the transduction efficiency considerably (Fig. 2). With transduction periods of 48 and 72 h, all cultures produced at least 30 ng IL-1Ra/10^6 cells per 48 h.

In another series of experiments, high titer MFG-IRAP, flow-through transduction [11], and DOGS [12] instead of Polybrene were evaluated for their effects on transduction efficiency and gene expression (Table 1). Qualitatively equivalent results were obtained with two different patients in triplicate experiments. Transduction was significantly more efficient with high titer than with low titer virus, regardless of the polycation used ($P < 0.05$) or the transduction method (static or flow-through). The flow-through transduction method was significantly better ($P < 0.05$) than static transduction when Polybrene was used. In the presence of DOGS, flow-through transduction was significantly better than static transduction if low titer virus was used ($P < 0.05$). Overall, DOGS did not consistently provide significantly better transduction efficiency than Polybrene.

Discussion

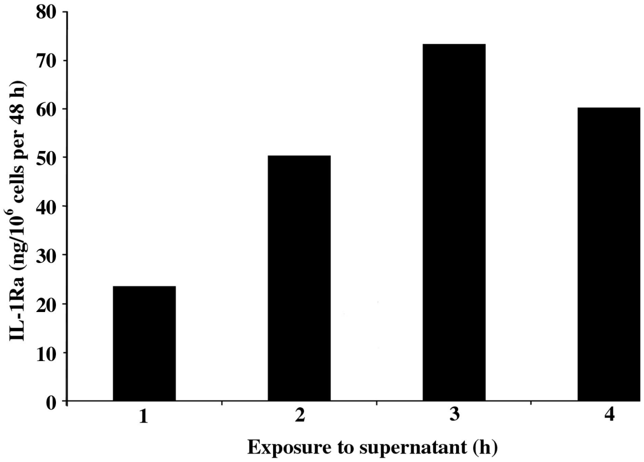
These data support a growing body of evidence that centrifuging enhances the efficiency of retroviral transduction [13]. Similar experiments using MFG-LacZ confirmed that

Figure 1



IL-1Ra production by human synoviocytes after transduction by MFG-IRAP under various conditions. Monolayer cultures of synoviocytes from five different patients with RA were transduced with standard-titer MFG-IRAP under the following conditions: A) first passage cells; static conditions; single transduction; B) second passage cells; static conditions; single transduction; C) first passage cells; with centrifuging; single transduction; D) first passage cells; static conditions; double transduction. Each point represents the average of two determinations for each patient under the indicated conditions. To the left of each column are the overall means for each method ± SD. The dashed line indicates the minimum level of 30 ng IL-1Ra/10^6 cells per 48 h required by the clinical protocol. IL-1Ra production after transduction with centrifuging (column C) exceeds all other methods, with $P < 0.0001$.

Figure 2



IL-1Ra production by human synoviocytes after static transduction for extended periods. Monolayer cultures of synoviocytes from five different patients with RA were transduced statically in duplicate with standard titer MFG-IRAP for the indicated lengths of time. Supernatants were changed every 24 h, until transductions were completed for all cultures. The cells were then washed, given complete media, and incubated for a further 48 h. At this time, media were harvested and IL-1Ra content was determined. Values are means ± SD. IL-1Ra production increased significantly ($P < 0.05$) when the time of transduction was increased from 24 to 48 h and from 48 to 72 h.

Table 1

Effect of flow-through, high retroviral titer and DOGS on the transduction of human synoviocytes.

	IL-1Ra production (ng/10^6 cells per 48 h)			
	High titer		Standard titer	
	Patient 1	Patient 2	Patient 1	Patient 2
Stationary PB	235 ± 6	177 ± 9	9 ± 1	6 ± 1
Flow PB	721 ± 17	249 ± 4	171 ± 6	17 ± 2
Stationary DOGS	299 ± 12	210 ± 21	8 ± 1	5 ± 0
Flow DOGS	908 ± 105	258 ± 5	260 ± 8	39 ± 3

Values are means ± SEM. Each result is the average of three determinations for each patient. PB = Polybrene.

centrifuging increased the proportion of transduced cells, suggesting that the main effect is on transduction efficiency. The disadvantage of centrifuging is loss of cells due to the formation of lunate dry areas. Increasing the volume of supernatant minimizes these dry areas, but increases the consumption of supernatant.

Length of exposure to retroviral supernatant also affected transduction. The MFG retroviral vector used in this study is based on the Moloney murine leukemia virus, which provides all the elements necessary for infection of target cells. The half-life of Moloney-based retroviruses at 37°C is 6–9 h [14], so they would survive long enough to transduce cells as they entered mitosis during prolonged incubations. The plateau in efficiency between 72 and 96 h of transduction probably reflects the fact that the cells became confluent during that time.

In general, our data using flow-through transduction support those of Chuck and Palsson [11]. The disadvantage of the flow-through method was the need for regular attention to ensure that wells had not run dry and to recycle the viral supernatant. However, the method was economical, because it was possible to cycle 1 ml of supernatant over the cells. Flow-through improved IL-1Ra expression using the lower titer virus, but the degree of enhancement differed between the two patients; in one, there was an approximately 3- to 8-fold increase, depending on whether Polybrene or DOGS was used (Table 1). In the other patient, increases of approximately 20- and 30-fold were obtained. The improving effects of the flow-through method were less dramatic with high titer virus, where transduction was already higher under static conditions, but increases of up to 3-fold were obtained.

Increasing the titer of the retrovirus improved transduction efficiencies 25- to 30-fold when Polybrene was used and approximately 40-fold when DOGS was used. Although the results were not as dramatic as those reported by Themis and colleagues [12], the use of DOGS instead of Polybrene gave a small to moderate improvement in IL-1Ra production. Overall, the highest levels of transgene expression were obtained using high titer retrovirus in combination with flow-through transduction conditions. These values far exceeded those obtained by centrifuging with standard titer virus. The effects of centrifuging with high titer virus were not evaluated.

Conclusions

Collectively, these data suggest that, of the parameters evaluated here, the single biggest improvement in retroviral transduction of human synoviocytes is obtained with high titer retrovirus. Further increases in transgene expression were achieved by using high titer retrovirus with flow-through transduction or centrifuging. When these factors were combined, IL-1Ra production was increased 50- to 100-fold relative to static transductions performed with standard titer virus. Improvements of this magnitude will be particularly important when performing gene therapy with transgenes such as IL-1Ra, which need to be produced in large molar excess over the molecules whose activities they antagonize [15].

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