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"Inverse wrap": an improved implantation technique for virus-transduced synovial fibroblasts in the SCID mouse model for rheumatoid arthritis

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Abstract The SCID mouse model for rheumatoid arthritis (RA) is an established and reliable approach to examining the distinct mechanisms operative in RA synovium, and evaluating novel gene therapy strategies. However, serum concentrations of circulating gene therapy products following gene transfer are frequently too low to allow detection. This problem stimulated us to develop a novel implantation technique to improve the yield of these soluble gene products. Synovial fibroblasts from patients with RA were cultured, passaged, and transduced with Ad5 sTNFRp55:Ig. sTNFRp55:Ig production was confirmed by ELISA, and then cells were implanted into SCID mice using a novel implantation strategy in which pieces of human cartilage were engrafted into a fibroblast-saturated inert sponge. Thereafter, the sponges were implanted under the skin of the mice instead of under the kidney capsule, as in the original approach, allowing co-implantation of larger pieces of cartilage together with higher numbers of adenovirus-transduced RA synovial fibroblasts. The improved implantation technique not only resulted in a reduction in the number of mice needed in each experiment by approximately 60%, and a reduction of the time taken for surgery by about 50%, but also considerably enhanced the serum concentrations of the gene product sTNFRp55-Ig, allowing detection of the soluble TNF receptor p55 by standard ELISA. In summary, the improved implantation technique for the SCID mouse model for RA results in more economic animal treatment, and facilitates the detection and quantification of circulating gene products following adenovirus-based gene transfer into synovial fibroblasts.

Key words Gene transfer · Inverse wrap · Rheumatoid arthritis · SCID mouse · TNF receptor

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Introduction

Rheumatoid arthritis (RA) is characterized by a chronic, progressive destruction of the affected joints, accompanied by inflammation and altered cellular and humoral immune responses. There is strong evidence that various T-cell-independent pathways may contribute significantly to the destructive process.[1–3] In this situation, synovial fibroblasts at the site of invasion into articular cartilage and bone appear to be one of the key actors.

In recent years, it has been shown that synovial cells form nodulary structures when implanted in nude mice.[4] This finding stimulated the development of the SCID (severe combined immunodeficiency) mouse model,[5] which was used to examine the interaction between human synovium and cartilage in the absence of human blood components.[6] Subsequently, this model was used to assess whether cultured synovial fibroblasts derived from RA patients maintain their agressive–invasive phenotype towards normal human cartilage in the absence of other synovial cells and human matrix components.[7] In these experiments, it was shown that the RA fibroblast phenotype persisted for several passages in cell culture, and also during a 60-day implantation period when co-implanted with normal human cartilage under the renal capsule of SCID mice.[7]

The alteration of key cellular pathways by gene therapy has been proposed in order to modulate the molecular mechanisms that contribute to joint destruction in RA, and the first human studies have already been initiated.[8] In addition, several studies have shown the beneficial effects of anti-TNFα therapy in rheumatoid arthritis models such as

murine collagen-induced arthritis,[9–11] and in various clinical trials using monoclonal anti-TNF-α antibodies as well as soluble TNF receptor constructs.[12,13] This therapeutic advance led to the clinical use of anti-TNF therapy.[14] However, basic research data are urgently needed to evaluate the effect of anti-TNF-α gene transfer approaches prior to application in RA patients.

The SCID mouse model has proved to be very useful in the evaluation of the effects of gene transfer into synovial fibroblasts.[15,16] A potential shortcoming of this approach is the fact that owing to the small sample size of the implants, serum concentrations of circulating gene therapy products (such as soluble TNF receptor) following virus-based gene transfer are frequently too low to allow detection using commercially available ELISA and Western blot systems. This problem results in difficulties in monitoring whether the molecules of interest are synthesized continuously for the complete duration of the experiment. Therefore, we developed a novel implantation strategy named the "inverse wrap technique," in which the synovial fibroblasts and the normal cartilage are enclosed in an inert sponge and are then co-implanted under the skin, in contrast to the previous technique in which much smaller pieces of cartilage and fewer RA fibroblasts are co-implanted under the renal capsule of SCID mice.[15,16]

Materials and methods

Synovial tissue and cell culture

Synovial tissues were obtained from five patients with RA who were undergoing routine joint surgery (synovectomy or joint replacement by prosthesis implantation) at the Department of Orthopedics, University of Regensburg. All patients met the criteria of the American College of Rheumatology.[17] The culture of synovial fibroblasts was performed as previously described.[15,16] In brief, following enzymatic digestion, fibroblasts were grown in Dulbecco's MEM (Biochrom, Berlin, Germany) containing 10% heat-inactivated fetal calf serum (Gibco Life Technologies, Grand Island, NE, USA), 100 U/ml penicillin, and streptomycin (PAA Laboratories GmbH, Linz, Austria), and cultured for four passages at 37°C in 10% CO_2. The synovial fibroblasts were stained for fibroblast markers by immunohistochemistry. More than 95% could be stained positively for the fibroblast enzyme prolyl 4-hydroxylase, and none were positive for the macrophage marker CD68 or the neutrophil marker cathepsin G (data not shown). In addition, testing for mycoplasma contamination was routinely performed.

Transduction

The synovial fibroblasts were transduced with Ad5sTNFRp55:Ig. This vector originates from replication-deficient type 5 adenovirus lacking the E1 loci. It encodes a divalent human p55 sTNFR/murine IgG1 fusion protein consisting of the extracellular domain of human 55-kDa TNFα receptor, and the CH2–CH3 domains of a mouse IgG1 heavy chain under the control of the CMV promoter.[18]

For adenoviral transduction in vitro, five fibroblast populations of patients with RA were grown to 80% confluence in six-well plates (5×10^5 cells per well). The cells were washed with saline solution (sterile physiologic saline, 0.9% NaCl), and 50 MOI (multiplicity of infection) of Ad5-sTNFRp55:Ig adenovirus, suspended in 500 µl saline, was added. After incubation at 37°C for 2 h, cells were washed three times and 1 ml DMEM was added. sTNFRp55 production was confirmed by ELISA (R&D Systems, Wiesbaden, Germany) prior to implantation (i.e., 48 h after transduction).

Implantation

Four-week-old SCID mice were obtained from a germ-free breeding colony (Charles River, Sulzfeld, Germany) and examined for macroscopic anomalies before and during surgery, as well as for macro- and histopathologic abnormalities after they had been killed. On the day of implantation, normal human cartilage was obtained from nonarthritic knee joints of patients undergoing routine surgery at the Department of Orthopedics at the University of Regensburg. Implantation of fibroblasts and cartilage was performed under sterile conditions using the novel "inverse wrap" (IW) technique: a cube-like piece of inert sponge (on average 80–100 mm^3) was incised and a piece of cartilage (approximately 3–5 mm^3) was inserted into its center. Thereafter, the sponge was soaked in fibroblasts (about 5×10^5 cells) suspended in sterile saline. Then the skin of the anesthesized mouse was opened surgically and up to three pieces of sponge containing cartilage and fibroblasts were inserted (Figs. 1 and 2). As a control, smaller pieces of cartilage (about 1 mm^3) were implanted under the renal capsule (RC) of the mouse, together with a smaller number of the same transduced fibroblasts (about 0.5×10^5 cells), as previously published.[15,16] Briefly, the mice were anesthetized, a small incision was made in the left flank, and the

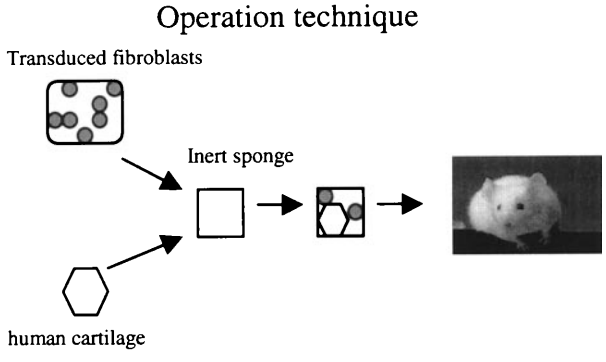

Fig. 1. Inverse wrap (IW) implantation technique. Following embedding of the normal cartilage into the inert sponge and soaking the sponge in transduced fibroblasts, up to three implants were inserted under the skin of a SCID mouse

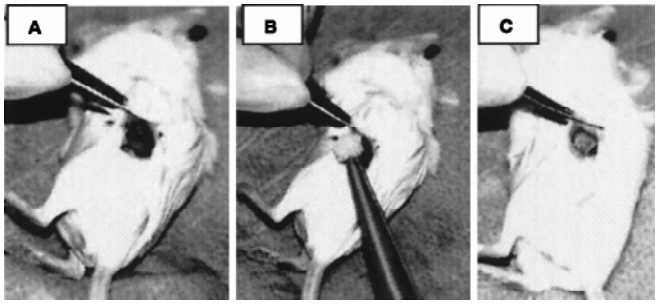

Fig. 2. A Surgical implantation procedure. **B** Sponge containing fibroblasts and cartilage. **C** sponge implanted under the skin of the mouse

kidney was partly exteriorized. Then the renal capsule was incised and the cartilage and fibroblast-containing sponge were engrafted side by side under the renal capsule. After 60 days, the mice were killed, and the implants were removed, embedded immediately in TissueTek embedding medium (Miles, Elkhart, IN, USA), snap-frozen, and stored at −70°C until needed. On the day of explantation, mouse serum was collected and tested for sTNFRp55 production by ELISA (R&D Systems). In both approaches, the duration of the operation (the injection of anesthesia to the end of skin suture and the return of the mouse to the cage) was measured.

Histologic evaluation

To evaluate the outcome parameters established from the retrovirus gene transfer experiments,[15,16] the implants were cut, sectioned, and stained by hematoxylin–eosin staining. The sections from the standard procedure (RC) and from our new method (IW) were evaluated by two independent and blindfolded examiners according to the criteria described below. Invasion: 0, no or minimal invasion; 1, visible invasion (about 2 cell depths, 20–30 μm); 2, invasion (about 5 cell depths); 3, deep invasion (about 10 cell depths).[15,16] Perichondrocytic cartilage degradation: 0, no degradation, sharp halo; 1, visible degradation (less than 1 diameter of the chondron); 2, degradation (1–2 diameters of the chondron); 3, intensive degradation (more than 2 diameters of the chondron).

Immunohistochemistry

To verify the presence of human cells in the implants at the time of explantation, immunohistochemistry for the detection of human β2-microglobulin and MHC class I was performed using the alkaline phosphatase antialkaline phosphatase (APAAP) method with primary monoclonal antibodies against β2-microglobulin and MHC class I (Pharmingen, Erembodegem-Aalst, Belgium). After a trypsin digestion step, the sections were covered with a 4% nonfat dry milk, 2% normal goat serum buffer to block nonspecific binding, rinsed in Tris-NaCl, and incubated with the primary antibody diluted 1:50–1:100. The slides were rinsed and incubated with a secondary goat antimouse IgG antibody (Jackson Immunoresearch Laboratories, West Grove, USA) in a 1:400 dilution. Color development was performed as previously published.[7] The slides were mounted immediately using Faramount aeqous mounting medium (Dako, Copenhagen, Denmark).

Immunogold–silver immunohistochemistry was used for the detection of matrix metalloproteinase 13 (MMP 13). Snap-frozen sections were cut (4–6 μm), fixed in acetone, and blocked with milk and normal goat serum. The slides were then washed and incubated with the MMP13 antibody (RDI, Flanders, USA), diluted according to the manufacturer's instructions. The slides were rinsed in Tris buffer and incubated with a biotinylated goat antimouse antibody (Jackson) diluted 1:600 in Tris buffer. After washing, the sections were incubated with peroxidase-conjugated streptavidin (Jackson) diluted 1:600 in Tris buffer. After rinsing, 6 nm gold-labeled goat antihorseradish peroxidase (Jackson), diluted 1:40 in Tris buffer, was applied. For photochemical silver amplification, the sections were incubated in a mixture of 50 ml double-distilled deionized water (Fisher Scientific) and 50 ml 0.05 M citrate buffer (pH 3.8), containing 250 mg hydroquinone (Fluka, Buchs, Switzerland), for 5 min at room temperature. The sections were immediately transferred into a mixed solution of 0.05 M citrate buffer (pH 3.8) containing hydroquinone (5 mg/ml, Fluka) and double-distilled deionized water containing silver acetate (2 mg/ml, Fluka). After 18 min, the sections were quickly rinsed with distilled water and placed in 10% photographic fixative (Kodafix solution, Kodak, Rochester, NJ, USA) for 2 min, rinsed thoroughly with distilled water, and mounted (Gel Mount, Biomeda, Foster City, CA, USA).

Results

Gene transfer outcome parameters

When compared with the results obtained using retrovirus-based gene transfer,[16] the use of the Ad5 adenovirus resulted in similar invasion and degradation scores (data not shown), confirming that sTNFR overexpression alone is not effective in inhibiting cartilage destruction or fibroblast invasion in the SCID mouse model for RA.[16] Furthermore, it could be shown that the cells invading the cartilage are of human origin, as shown by immunohistochemistry for human β2-microglobulin and MHC class I. Mouse cells were only found in the area of the sponge surrounding the cartilage distant from the area of invasion. In addition, it could be shown that MMP-13 (collagenase-3) was expressed by human fibroblasts adjacent to the co-implanted cartilage (Fig. 3).

Advantages of the inverse wrap implantation method

The experiments were performed using the conventional implantation method (implantation under the renal cap-

sule[15,16]) and our new inverse wrap method in parallel using different sets of mice. Both methods gave comparable results concerning the invasion and degradation of the co-implanted cartilage (Fig. 4). IW was as safe as RC with regard to mouse survival, as all mice stayed alive and physically active until the end of the implant period. Comparison of both methods revealed three main advantages of the IW technique.

1. Owing to the fact that in the IW method only a skin incision is made and no retroperitoneal (renal) surgery needs to be performed, the time needed for the operation was reduced significantly, resulting in an average operation time of 15 min (IW) rather than 30 min (RC). This also resulted in a shorter anesthesia time and less anesthetic (Table 1). In addition, as the IW surgery was less strain on the animals, their recovery was much faster (normal activity, such as running around the cage, after about 1 h (IW) rather than 1–3 h (RC).

2. For each fibroblast population examined, it is necessary to have at least two or three implants in order to perform different immunohistochemistry and in situ hybridization experiments. The possibility of implanting two or three pieces of cartilage in one mouse using the IW method (Fig. 1), as compared with only one implant per mouse using the RC method, therefore reduces the number of mice necessary for the complete experiment by about 60%.

In addition, much larger implants can be placed under the skin than under the renal capsule, further enhancing the number of transduced cells per SCID mouse. The nourishment of the implants was the same in both methods (Fig. 5).

3. The main benefit of the novel IW method with regard to monitoring gene transfer experiments was that the gene product sTNFR-Ig could be detected by ELISA in mouse serum after explantation in all mice (6/6), as compared with no (0/6) mice using the RC method. TNFR-Ig levels in circulating mouse blood in the IW experiments ranged from 40 to 100 pg/ml mouse serum, whereas sTNFR-Ig was below detection level in serum obtained from experiments performed with the original RC implantation method (Table 2).

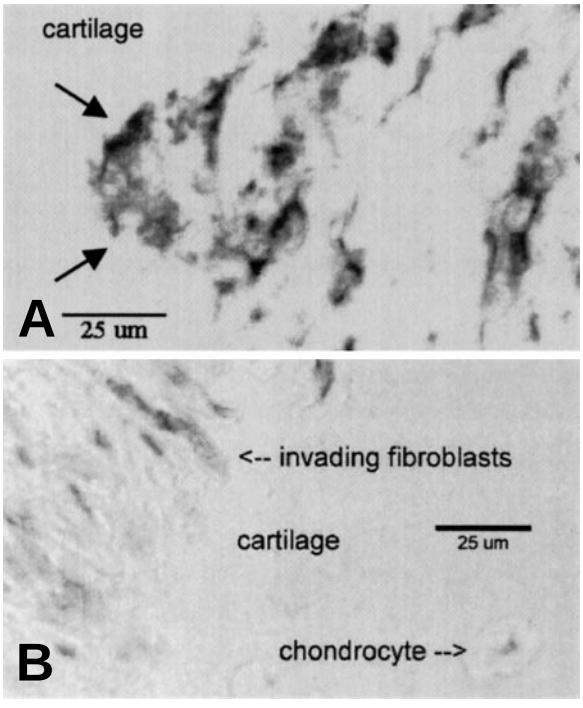

Fig. 3. **A** Immunohistochemistry for human MMP-13 (collagenase-3), showing intensive expression of this matrix-degrading enzyme in transduced fibroblasts at sites of invasion into the co-implanted cartilage (*arrows*: invasion site). **B** Negative control (primary antibody omitted)

Discussion

The pathophysiology of RA is reflected by a progressive inflammatory destruction of joints, and despite the use of numerous anti-inflammatory and disease-modifying drugs, new approaches are urgently required to inhibit the progression of joint destruction. A promising way to develop novel therapeutic agents is to study synovial cell-mediated

Table 1. Comparison of the duration of anesthesia and amount of anesthetic between the inverse wrap (IW) and renal capsule (RC) methods

	IW	RC
Duration (average, min)	30	45
Amount of anesthetic (mg/kg body weight)	Ketamine: 75	100
	Xylazine: 11.25	15

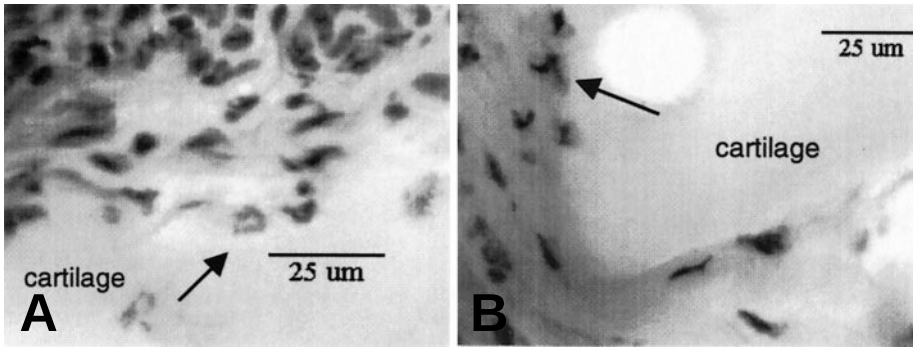

Fig. 4. HE-stain of fibroblasts invading the co-implanted cartilage. **A** Implant under the renal capsule (RC). **B** Implant under the skin (IW). *Arrows*, invading fibroblasts

Table 2. TNFR-Ig concentrations (pg/ml) in mouse serum using IW vs. implantation under the RC

Cells (patient)	Not transduced	LacZ control	TNFR-Ig/IW	TNFR-Ig/RC
OA45R	n.d.	n.d.	55.52	n.d.
RA70R	n.d.	n.d.	102.13	n.d.
OA39R	n.d.	n.d.	40	n.d.
RA79R	n.d.	n.d.	31	n.d.

n.d., not detectable; ELISA detection limit as stated by the manufacturer, 3 pg/ml

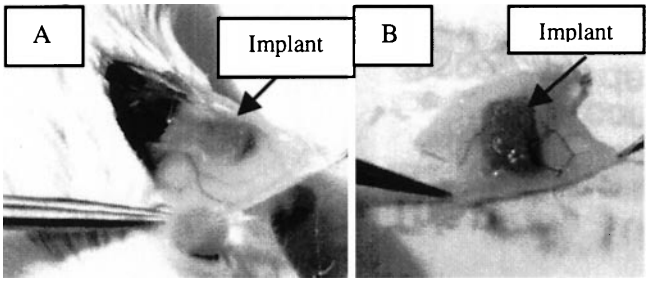

Fig. 5. Implant performed using the inverse wrap technique at the time of explantation. Note the adequate nourishment of the implant, and the minimal scar formation and low inflammation around the implant. **A** Implant in situ under mouse skin. **B** Close-up of implant

cartilage destruction using the SCID mouse model for RA. This does not reflect the complete course of the disease, but it allows us to study some distinct pathophysiological mechanisms, i.e., the interaction of human synovium or synovial fibroblasts with human cartilage under in vivo conditions, including gene therapy approaches.[15,16] In this model, in which RA synovium or RA synovial fibroblasts are co-implanted together with normal human cartilage under the renal capsule of the SCID mouse, a similar, invasive growth of the RA synovium or of the RA synovial fibroblasts into the cartilage over an extended period of time can be shown.

The maintenance of therapeutic levels of molecules which protect the cartilage in the arthritic joint is a difficult goal not only to achieve, but also to monitor. When using the original SCID mouse model to study the effects of retrovirus-based gene transfer of potentially inhibitory molecules into human RA synovial fibroblasts, sophisticated and labor-intensive molecular biology techniques such as in situ hybridization and in situ reverse transcriptase polymerase chain reaction (RT-PCR) are generally required to detect the gene products and validate the effectiveness of the gene transfer in the implants.[15,16] Although commercially available detection systems such as ELISA (e.g., for the measurement of sTNFRp55 protein) have sufficient sensitivity to measure gene transfer products in the supernatants of cell cultures,[15,16] in the original SCID mouse model they fail to detect the minute amounts of soluble gene product released by the relatively few fibroblasts located under the renal capsule of the SCID mice.

As was demonstrated by the experiments in our study, the novel implantation strategy named the "inverse wrap technique" is capable of improving the yield of gene transfer products such as sTNFRp55 synthesized by the transduced synovial fibroblasts as outlined above, and will therefore facilitate a much easier way to monitor and evaluate the gene products of choice in future gene therapy experiments, including double gene transfer.[19,20] In addition, the possibility of inserting more than one implant per SCID mouse using the IW method not only considerably reduces the number of mice needed for an experiment, as well as costs and space in animal housing, but also facilitates the broader use of the SCID mouse model due to the lower requirements for surgery and surgical skills.

Conclusions

This improved implantation technique for the SCID mouse model for RA results in more economic animal treatment, as the surgical procedure is less strenuous and considerably shorter. In addition, it is possible to place two to three implants in one mouse, therefore reducing the total number of mice needed. Owing to the fact that this method results in a higher number of transduced human cells in one mouse, the novel inverse wrap technique also facilitates the detection and quantification of circulating gene products following virus-based gene transfer into synovial fibroblasts, including most recent double gene transfer approaches.[19,20]

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