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Abstract

OBJECTIVES: The viral gene transfer of Interleukin-1 receptor antagonist (IL-1ra) and Interleukin-10 (IL-10) into rheumatoid arthritis (RA) synovial fibroblasts (RASF) showed protective effects on cartilage destruction in the SCID mouse model for RA. Nevertheless, side effects of viral transduction are possible and a number of cytokines or cytokine inhibitors are not available encoded in viral vehicles. As the production of viruses coding for bioactive proteins is cost- and time-intensive, we established an in vivo long-term release model using osmotic minipumps in the SCID mouse model for RA.

METHODS: Isolated RASF were cultured for 4 passages and coimplanted together with human cartilage and an Alzet(R) Osmotic Miniature Pump Model 2004 containing 200 microl of IL-10 and IL-1ra for 40 days in SCID mice. Implants were removed after 40 days and evaluated histologically. The actual rates of IL-10 and IL-1ra in murine serum were measured by ELISA.

RESULTS: Release of IL-10 and IL-1ra by the pumps was effective as both could be measured in significant amounts in the serum of the mice. IL-10 and IL-1ra release showed protective effects towards the co-implanted cartilage, similar to the adenovirally IL-10-/IL-1ra-transduced RASF. The invasion scores for the implants with the osmotic pumps were: invasion 0.7+/-.5, degradation 0.5+/-.3 (all parameters significant vs. controls, p<0.05).

CONCLUSIONS: The results demonstrate that the combination of osmotic pumps with the SCID mouse model for RA can be used as approach for application and evaluation of cartilage-protective molecules. Furthermore, the effect of cartilage-protective cytokines is independent of the type of application.
The therapeutic use of osmotic minipumps in the SCID mouse model for rheumatoid arthritis

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ABSTRACT

Objectives: The viral gene transfer of Interleukin-1 receptor antagonist (IL-1ra) and Interleukin-10 (IL-10) into rheumatoid arthritis (RA) synovial fibroblasts (RASF) showed protective effects on cartilage destruction in the SCID mouse model for RA. Nevertheless, side effects of viral transduction are possible and a number of cytokines or cytokine inhibitors are not available encoded in viral vehicles. As the production of viruses coding for bioactive proteins is cost- and time-intensive, we established an in vivo long-term release model using osmotic minipumps in the SCID mouse model for RA.

Methods: Isolated RASF were cultured for 4 passages and coimplanted together with human cartilage and an Alzet® Osmotic Miniature Pump Model 2004 containing 200 µl of IL-10 and IL-1ra for 40 days in SCID mice. Implants were removed after 40 days and evaluated histologically. The actual rates of IL-10 and IL-1ra in murine serum were measured by ELISA.

Results: Release of IL-10 and IL-1ra by the pumps was effective as both could be measured in significant amounts in the serum of the mice. IL-10 and IL-1ra release showed protective effects towards the co-implanted cartilage, similar to the adenovirally IL-10-/IL-1ra-transduced RASF. The invasion scores for the implants with the osmotic pumps were: invasion 0.7±0.5, degradation 0.5±0.3 (all parameters significant vs. controls, p<0.05).

Conclusions: The results demonstrate that the combination of osmotic pumps with the SCID mouse model for RA can be used as approach for application and evaluation of cartilage-protective molecules. Furthermore, the effect of cartilage-protective cytokines is independent of the type of application.
INTRODUCTION

Predominant features of rheumatoid arthritis (RA) are synovial hyperplasia, synovial cell activation, and articular inflammation associated with subsequent destruction of cartilage and bone.[1] Molecular biology provides increasing evidence that metabolic activation of RASF is followed by an increased release of matrix degrading enzymes and inflammatory cytokines leading to progressive destruction of the affected joints.[2-7]

To understand the pathophysiology of RA various in vitro models are used but they provide only limited insight into the complex pathogenic mechanisms of the disease. Especially cell culture based models can not display all the pathogenic hallmarks in the joint during the initiation and further development of RA, including synovial hyperplasia, cartilage invasion and degradation. Thus, animal models are essential for detailed elucidation of the pathophysiology of RA and for the development of effective therapies.

Most of these models are rodent-based because of the possibility to investigate numerous animals at a time and their short generation interval. Of these, inbred rodent models such as transgenic- or knockout-models, in which the genetic background is known, are valuable to test the effects of a specific gene on the pathophysiology of a multifactorial disease such as RA. Nevertheless, to establish a rodent model for the overexpression or the knockout of a specific gene is time- and cost-intensive.

Another possibility to study the effects of specific gene products is viral or non-viral gene transfer. However, virus-based transduction of cells or application of viral vectors in rodent animal models in vivo often shows side effects by expressing viral proteins and the activation of unwanted signaling cascades. Another disadvantage can be a low transduction efficiency in the targeted cells or tissues.[8-10] Another problem is that numerous cytokines or their inhibitors encoded in viral vehicles are not readily available. Transduction of specific genes in cells with either lipofection or electroporation or other non-viral techniques is in most cases not stable over a longer time period and the time of expression of the transduced gene is too short to study its effects in vivo. In addition, using these methods several cell types are difficult to transduce. Moreover, the transduction efficiency of different cell types can vary when applied in vivo.

Furthermore, injection of a protein of interest in a rodent model to study the effects of the injected substance has the drawback that it is time-intensive. Animals have to be injected at regular intervals for a longer time period with high doses of protein. Constant protein levels in the animal can usually not be achieved because of the permanent catabolism and re-injection of high doses of protein.

To test the effects of specific proteins in the SCID mouse model of RA [5, 11] we searched for an experimental approach, which fulfils the above mentioned criteria, such as having a cost-saving method and a constant protein level over the complete trial period without the unwanted side effects. Therefore, we decided to use osmotic minipumps as a potential method for the continuous systemic treatment in the SCID mouse model with bioactive proteins. As underlying principle, the osmotic pressure of the pump ensures a controlled delivery of proteins to the SCID mice and avoids fluctuating protein concentrations in the experimental animal system.

Previously, we could show that the double gene-transfer of IL-10 and IL-1ra in the SCID mouse model of RA resulted in reduced cartilage invasion and
degradation.[11] Here, we administered IL-10 and IL-1ra with osmotic minipumps in the same mouse model to have a direct comparison of both methods. Taken together, in this study osmotic minipumps were used to establish a non-viral administration system in vivo over a defined period of time. The effects of the continuous administration of the cartilage-protective molecules IL-10 and IL-1ra by osmotic minipumps were compared with those previously achieved by the viral double gene-transfer method.
METHODS

Synovial Tissue and Cell Culture
Synovial tissues were obtained from synovial biopsies of 4 patients with RA undergoing joint surgery, who all met the criteria of the American College of Rheumatology. Approval of the local ethics committee and an informed written consent was obtained from all patients according to the declaration of Helsinki. Culture of synovial fibroblasts was performed as described recently. Following enzymatic digestion, fibroblasts were grown in Dulbecco’s MEM (Biochrom, Berlin, Germany) containing 10% heat inactivated fetal calf serum (Invitrogen, Karlsruhe, Germany), 100 U/ml penicillin and streptomycin (PAA Laboratories, Cölbe, Germany) and cultured for 4 passages at 37°C in 10% CO₂. Synovial fibroblasts were tested routinely for mycoplasmas and stained for fibroblast markers by immunohistochemistry.

Double Gene-Transfer of IL-10 and IL-1ra
Double gene-transfer of IL-10 and IL-1ra was performed according to established protocols. Briefly, RASF were transduced with adenoviral vectors, AdvIL-10 and AdIL-1ra, with equal MOI (multiplicity of infection) of 30. The expression of the transgene was confirmed by ELISA. As controls, RASF transduced with 30 MOI of AdEGFP or non-transduced RASF were used.

Mice and Alzet® osmotic pumps
Female Crl-scidBR mice, 4-5 weeks of age, and Alzet® osmotic pumps model 2004 were purchased from Charles River (Sulzfeld, Germany). Mice were housed under institutional Animal Care and Use Committee-approved germ-free conditions. SCID mouse experiments as described in the study were approved by the local Animal Care and Use Committee of the Regierungspräsidium Darmstadt.

Stability of proteins
Both proteins, IL-10 and IL-1ra, have to be kept stable in the osmotic pumps for the complete trial period of a minimum of 40 days at 37°C. To test whether the protein is stable during the experimental time period, osmotic minipumps were filled with a defined test concentration of IL-10 or IL-1ra, respectively, solubilized in 0.9% saline solution without or with 50% DMSO. The osmotic pumps were placed in 5 ml saline at 37°C as suggested by the manufacturer of the osmotic minipumps. Every other day 500 µl of the immersion saline of the osmotic pump was removed and analyzed by ELISA for the remaining concentration of IL-10 and IL-1ra. The residual volume of 4.5 ml was discarded and replaced with 5 ml fresh saline. The test concentration for the pump release was calculated as follows: a release of 0.25 µl in 1h corresponds to a daily release of 6 µl. This results in a release of 200 µl over a time period of approximately 33 days. For the measurement of the protein concentration by ELISA a concentration of 300 ng of protein is sufficient. Because the immersion solution was removed only every other day we postulated a daily protein release of 150 ng. Thus, to test the stability of the proteins in vitro, the osmotic minipumps were filled with 4.5 µg of protein (150 ng x 33 days).
Cytokine assays
Concentrations of IL-10 or IL-1ra in the immersion solution of the osmotic pump and in the murine sera were evaluated by ELISA. ELISA kits for human IL-1ra and human IL-10 were purchased from R&D Systems (Wiesbaden, Germany). Measurements were performed according to the manufacturer’s recommendations.

Testing of mouse sera for IL-6
To exclude T- or B-cell activity of the SCID mice, ELISAs for murine IL-6 and murine TNF (R&D Systems, Wiesbaden, Germany) were performed with the sera of SCID mice. All SCID mice sera were negative for murine IL-6 and TNF (data not shown).

Modified ‘Inverse Wrap’ technique for tissue and pump implantation into the SCID mouse
Preparation of fibroblasts and cartilage
Synovial fibroblasts were cultured as described above. Immediately before implantation, fibroblasts were trypsinized, spun down and resuspended in saline solution in a final volume of 50 µl for each sponge.

Preparation of the osmotic minipumps
An Alzet® osmotic miniature pump model 2004 with constant delivery rates of 0.25 µl/h for about 33 days was filled with the corresponding solution (200 µl IL-10 and IL-1ra in NaCl/50% DMSO or with NaCl/50% DMSO as control) (figs 1A,B). As the osmotic agent is outside of the pump reservoir (separated by an impermeable but flexible wall), the content within the pump is independent from the pump delivery rate. The pump has a delayed starting time of approximately two days after filling. When immediately implanted after filling the protein solution into the pump (fig. 1C), the protein delivery starts very early in the experimental time, in which no cartilage destruction is detectable (day 0 – 4). As the proteins still circulate for a few days after the pump is empty, an experimental setting of 40 days was selected.

Implantation of fibroblasts and cartilage together with osmotic minipumps
At the day of implantation, normal human cartilage was obtained from non-arthritis knee joints of patients undergoing routine surgery at the Department of Orthopedics at the University of Regensburg. The cartilage was cut in 1-5 mm³ pieces. One piece was immediately snap frozen, stored at -70°C and served as control for scoring after explantation.

A cube of inert sponge (≈ 80 mm³) was incised and a piece of cartilage (1 - 5 mm³) was inserted. The sponge was soaked with 5 x 10⁵ fibroblasts suspended in 50 µl sterile saline as described previously.[11, 13] Implantation of the osmotic minipump and the sponge under the skin of the mice was performed under sterile conditions. The left flank of the skin of an anesthesized mouse was opened surgically and the osmotic minipump was implanted subcutaneously. Afterwards, 2 implants containing fibroblasts were inserted under the skin of the SCID mouse adjacent to the osmotic pumps (fig 1C). As additional control a SCID mouse received a sponge-containing cartilage and RASF but no osmotic pump. In parallel, transduced RASF were co-implanted with vital human cartilage but without osmotic minipumps as control.

In an additional experiment, RASF were coimplanted together with human cartilage subcutaneously under the skin of two SCID mouse. An osmotic minipump was implanted at the contralateral flank of the same animals: one animal received a pump filled with NaCl/50% DMSO as control, the minipump of the other animal was
filled with IL-1ra and IL-10 in 50% DMSO. This experiment was done to analyze whether it was necessary to implant the osmotic pump adjacent to the cartilage.

Sacrifice
After 40 days, mice were sacrificed and examined for macro- and histopathologic abnormalities. The implants and the osmotic pumps were removed. Immediately after removal of the implants (including a part of attached murine skin), they were embedded in TissueTek embedding medium (Miles, Elkhart, IN), snap-frozen and stored at −70°C until further use. At the day of explantation, murine serum was collected for the analysis of human IL-10- and human IL-1ra concentrations by ELISA and the residing volume of the osmotic minipump was determined.

Histological evaluation of implants
Fixed sections of the implants (5 µm) were stained with hematoxylin-eosin and each specimen was evaluated for the grade of invasion of fibroblasts into cartilage and perichondrocytic cartilage degradation as published recently.[11, 14, 15] Statistical analysis was performed using the t-test, p values < 0.05 were regarded significant. Scoring was performed by a minimum of three different researchers for all patients. Scores were evaluated for all implants.

Immunohistochemistry
Immunohistochemistry was performed as published previously.[11] Fixed sections of the implants were stained for mouse anti-human vimentin (DAKO, Hamburg, Germany), mouse anti-human follistatin (R&D Systems, Wiesbaden, Germany), rabbit anti-mouse IL-1R (R&D Systems, Wiesbaden, Germany) or mouse anti-mouse H2-Dd (BD Biosciences, Heidelberg, Germany).
RESULTS

Stable protein release by osmotic minipumps of IL-10 and IL-1ra over 40 days
The stability of the proteins with or without 50% DMSO in saline was tested at 37°C prior to the animal experiments. The concentration of human IL-10 and human IL-1ra in the immersion solution of the osmotic minipump was measured by ELISA. The protein solution had to be stabilized by the use of 50% DMSO. After 40 days, the residual pump volume in the pumps containing 50% DMSO as well as the circumsolution saline still contained sufficient concentrations of the protein. Minipumps filled with protein solution without DMSO did not contain appropriate amounts of protein after the trial period. Between days 6 and 30 a constant flow was visible (fig 2A, regression line). The decrease is most likely due to degrading protein over time even though 50% DMSO were used.

Protein concentrations in sera of SCID mice
Concentrations of human IL-10 and human IL-1ra in mouse sera collected at the day of explantation were evaluated by ELISA. The concentration of human IL-1ra was 351.17±143.49 pg/ml and the human IL-10 concentration was 232.75±128.93 pg/ml (n = 12) (fig 2B).

Residual volumes in the osmotic minipumps
After 40 days, osmotic minipumps were removed and the residual volume in the osmotic minipumps was measured. The volumes ranged between 0 and 27 µl (fig 2C).

Effects of IL-10 and IL-1ra release on cartilage degradation in the SCID mouse model
The implants of the controls, mice without osmotic pumps and with osmotic pumps filled with NaCl/50% DMSO, showed an intensive perichondrocytic cartilage degradation similar to previous experiments (figs 3A, B, 4).[11, 14, 15] At sites of invasion into the cartilage, human synovial fibroblasts could be detected. There was no difference in the degradation and invasion of the cartilage when the the osmotic minipump was implanted at the contralateral flank, i.e. not adjacent to cartilage and RASF. In order to minimize the number of lesions and the duration of surgery we coimplanted cartilage/RASF together with the osmotic pump at one flank of the SCID mouse. To confirm that the cells invading into the cartilage were human RA synovial fibroblasts, immunohistochemistry using species specific antibodies was performed. Invading cells were positive for human but not for murine markers, such as anti-mouse IL-1R (data not shown).

Constant release of IL-10 and IL-1ra by osmotic minipumps containing both proteins in saline with 50% DMSO resulted in a significant reduction of cartilage invasion and a similar decrease of cartilage degradation (figs 3C, 4). This confirms previous results [11] regarding the beneficial effect of IL-10 /IL-1ra on RASF invasion and degradation into coimplanted cartilage in the SCID mouse model (figs 3D,E, 4). The average degradation scores of all implants are shown in figure 4.
DISCUSSION

IL-10 and IL-1ra play central roles in the pathophysiology of RA but neither one of them is able to inhibit the progression of cartilage destruction completely. We have previously shown, that the double gene transfer of IL-10 and IL-1ra has beneficial results on cartilage protection in the SCID mouse model for RA. Although the retro- or adenoviral transfer of IL-10 and IL-1ra achieved long-term stable serum levels of both proteins, a disadvantage is the time- and cost-intensity in the development of the viral vectors and the production of the viral particles. In addition, the transduction with viruses can cause side-effects by the expression of viral proteins and their activation of signaling cascades and numerous cytokines or their inhibitors are not available encoded in viral vehicles. Therefore, the intention of the present study was to establish a reliable method for the systemic treatment with bioactive proteins in the SCID mouse model of RA. We used osmotic minipumps in this mouse model for the application of IL-10 and IL-1ra and compared the effects of this method with the results achieved with the viral transfer. We could demonstrate that the application of proteins via osmotic minipumps is an effective tool to evaluate the effects of cytokines and inhibitors in vitro. The delivery rate of IL-10 and IL-1ra remained stable over 40 days and was still detectable at day 40 (fig 2B). The systemic treatment of our SCID mouse model with IL-10 and IL1-ra reduced the invasion and the degradation of the implanted cartilage (fig 3C). The effect was similar to those results achieved with viral overexpression. In summary, the results of this study show that the application of IL-10 and IL-1ra with osmotic minipumps is an effective experimental approach and comparable to virus-based double gene-transfer of joint-protective genes into rheumatoid synovial fibroblasts. Furthermore, compared with the virus-based double gene-transfer method the use of osmotic minipumps for protein-release is time- and cost-saving and facilitates a constant protein level over an extended period of time, which exceeds even the usual time period for standard murine arthritis models such as collagen- (CIA) or antigen- (AIA) induced arthritis. Thus, the use of osmotic minipumps is not linked solely to the SCID mouse model, but could also be applied to other animal models such as CIA or AIA. Indeed, Kurosaka and coworkers used this device for the administration of endostatin in CIA induced arthritis in mice. In this study, the continously administration of endostatin using osmotic pumps over 14 days and a daily therapeutic administration resulted in inhibition of CIA in mice. Moreover, administration of osteogenic protein using osmotic minipumps in a rabbit OA model with anterior cruciate ligement transection (ACLT) showed a protection of articular cartilage. Thus, the delivery of proteins using osmotic minipumps is an effective tool and the obtained results are comparable to the devices of therapeutic application or gene transfer.

In summary, the combination of the established SCID mouse model for RA and the long-term osmotic pump-based delivery system for bioactive molecules adds a novel tool for the detailed analysis of RA pathophysiology.
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COMPETING INTERESTS

The authors declare that they have no competing interests.
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FIGURE LEGENDS

Figure 1: Preparation of osmotic minipumps.
A) Schematic structure of an osmotic minipump showing the technical features. The reservoir tank is isolated from the outer chamber containing high concentrations of salt by an impermeable but flexible layer. The outer surface of the pump is a semipermeable but solid layer. Water enters the osmotic minipump by this semipermeable layer and thereby increases the volume of the chamber with the osmotic agent. This causes a compression of the flexible inner reservoir and thereby the delivery of the protein solution into the animal.
B) Filling the reservoir tank of an osmotic pump by using a blunt syringe. After covering the reservoir tank with the flow moderator the osmotic pump is ready to use.
C) Subcutaneous implantation of an osmotic minipump into the left flank of an anesthesized SCID mouse.

Figure 2: IL-10 and IL-1ra protein concentration in murine sera and residual volumes in osmotic minipumps.
A) Analysis of protein stability in the osmotic minipumps using filling concentrations of 4500 ng in 200 µl (pump volume). An example of the protein measurements over time for 4500 ng IL-1ra in NaCl/50% DMSO is presented. The protein release starts approximately at day 3-4. A constant release is visible between days 6 and 30 with a slight decrease in the protein amount, most likely due to protein degradation.
B) Protein levels in murine sera after 40 days of implantation measured by ELISA show significant amounts of circulating bioactive proteins.
C) The residual pump volumes after 40 days of implantation show a nearly complete emptying of the reservoir tank.

Figure 3: SCID mouse implants.
A) In control implants (mice without osmotic pumps) RASF showed strong invasiveness into the cartilage (red arrows) together with perichondrocytic cartilage degradation (green arrows).
B) Control implants of mice implanted with osmotic minipumps containing NaCl/50% DMSO revealed an intensive perichondrocytic cartilage degradation (green arrows) and strong invasion (red arrows) similar to that without osmotic minipumps.
C) IL-1ra and IL-10 release by osmotic minipumps resulted in a decreased invasion into the cartilage (red arrow) and a reduced cartilage degradation (green arrow).
D) AdEGFP-transduced control mice showing a strong invasiveness into the cartilage (red arrows) together with perichondrocytic cartilage degradation (green arrows).

E) AdvIL-10/AdIL-1ra transduced RASF showing a decreased cartilage degradation and invasion.

**Figure 4: Scoring of the SCID mice implants**

Invasion and Degradation scores of the SCID mouse experiments showing a significant decrease of cartilage destruction in the implants of IL-10/IL-1ra treated mice. Scoring was performed by a minimum of 3 different researchers. Mean of all implants and standard deviation was calculated. *Significant reduction versus respective controls without IL-10/IL-1ra (p < 0.05) is presented.*
REFERENCE LIST

A delivery portal is shown with a semipermeable membrane. The process involves filling a reservoir carefully without contact to the reservoir wall. An osmotic agent is inserted into the flow moderator, and the setup is demonstrated on a live animal.
Protein | — | — | — | IL10/IL-1ra | IL10/IL-1ra
Osmotic pumps | — | — | + | — | +
Viral transduction | — | + (EGFP) | — | + | —
Invasion | 2.4 ± 0.5 | 2.1 ± 0.5 | 1.9 ± 0.4 | 0.5 ± 0.3* | 0.7 ± 0.5*
Degradation | 2.0 ± 0.5 | 1.9 ± 0.5 | 1.7 ± 0.5 | 0.3 ± 0.2* | 0.5 ± 0.3*