Adenoviral transduction of human osteoblastic cell cultures

A new perspective for gene therapy of bone diseases

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This article confirms the susceptibility of osteoblastic cells to adenoviral transduction. Osteoblasts were harvested from human cancellous bone. Cells were transduced, using various amounts of adenoviral vectors carrying the cDNA encoding interleukin-1 receptor antagonist (IL-1Ra), or the marker genes ß-galactosidase and luciferase. Expression of the transgenes and the biological activity of IL-1Ra produced by gene transfer were measured quantitatively in a time-course by ELISA. The rate of transduction was 100% after exposure to 1×10^7 infective particles of adeno-LacZ. No expression of IL-1Ra was seen after transduction with adeno-IL-1Ra at titers of 1×10^4 and less. However, after transduction at titers of 1×10^7, infective particles cells expressed IL-1Ra consistently for 72 days, with levels up to 1 μg IL-1Ra/1×10^6 cells/48 hours. None of the control samples expressed detectable levels of IL-1Ra. The biological activity of the transgenic IL-1Ra was demonstrated by its ability to suppress successfully IL-1-induced nitric oxide synthesis by rabbit articular chondrocytes. After transduction with 1×10^7 infective particles of the adeno-luciferase vector, up to 81,000 Units transgenic luciferase/$\times 10^6$ osteoblastic cells were measured 2 days after gene transfer. Our results show that adenovirus transduces osteoblastic cells at a high rate in vitro.

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Cytokines are soluble factors that play a critical role in mediating cell to cell interactions within skeletal tissues and are known to be involved in the pathogenesis of bone loss in inflammatory conditions such as rheumathoid arthritis (Ralston and Grabowski 1996) as well as in systemic bone diseases such as osteoporosis (Kimble et al. 1995, Zheng et al. 1997). Among those cytokines known to promote bone resorption, interleukin-6 (IL-6), tumor necrosis factor-alpha (TNF-α) and, especially, interleukin-1 (IL-1) appear to be of major importance (Thompson et al. 1986). Certain naturally occurring proteins, such as interleukin-1 receptor antagonist (IL-1Ra), can oppose the IL-1 induced effects on osteoclasts and, thus, could be used in the treatment of osteoporosis (Hung et al. 1994, Kimble et al. 1995). On the other hand, bone morphogenetic proteins (BMPs) may be used to improve bone healing, especially in bone defects or delayed bone union. Unfortunately, the clinical use of these therapeutic proteins is limited by their short physiological half-lives, which range from minutes to hours, due to their fast clearance in vivo. This means that such agents must be used at frequent intervals, or even continuously to achieve therapeutic effects. Moreover, since there is no good way to target these factors locally to specific sites of disease, non-target tissues become exposed to them, leading to side-effects.

Gene therapy represents a new approach to delivering these therapeutic agents at relatively large concentrations for a longer time in discrete locations (Evans and Robbins 1995). Being locally produced after cell transduction, cytokine inhibitors, such as IL-1Ra and soluble tumor necrosis

factor-α receptor (sTNF-αR), may prevent bone loss, while avoiding the side-effects of conventional therapeutic regimens.

This study was designed to evaluate the ability to transduce human osteoblastic cells derived from cancellous bone, and to determine whether we could induce high level expression of biologically active transgenic cytokine inhibitors.

Material and methods

Cell culture

Human osteoblastic cell cultures were established by obtaining cancellous bone of the ulna and radius intraoperatively during osteotomy of informed healthy donors with posttraumatic forearm deformities and from the patella, following patellectomy (4 patients, mean age 45 years). After removing the cortical and fibrous tissue completely, the cancellous bone was treated with collagenase 0.1% (Seromed, Berlin, Germany) overnight and, after removing the collagenase, the cells were plated out the next morning. The human osteoblastic cells reached confluence after 3 weeks, at the earliest. After the second passage, the cells exhibited a uniform morphology.

Alkaline phosphatase activity

Alkaline phosphatase (ALP) activity was measured in second-passage human osteoblastic cells, using a commercial kit from Sigma (Sigma Chemical, St. Louis, USA) according to specifications of the manufacturer. Immortalized synovial fibroblasts (HIG-82) (Georgescu et al. 1988) served as negative controls; immortalized bone marrow stem cells from osteogenesis imperfecta mice (OIM) (Balk et al. 1997) served as positive controls. OIM-cells are known to produce ALP, after treatment with BMP-2.

Recombinant adenoviral vectors

3 different E1/E3-deleted adenoviral vectors were used for different purposes, the adeno-LacZ, the adeno-luciferase, and the adeno-IL-1Ra vector. In each case, the transgene was cloned into the E1 region of the viral genome and its expression was driven by a human CMV early promoter. The viruses were stored until use at a concentration of 7×10^{12} particles/mL at –80 °C. The infective potential of the particles was determined at a rate of 1:100, stating that 1×10^7 of the 1×10^9 particles are infective and ready for transduction (platelet forming units (pfu)).

Transduction

Third-passage cells were transferred to either T 25 cm flasks at a density of 1×10^5 cells per flask or 24-well plates at a density of 5×10^4 cells per well. After they reached 80% confluence, cells were transduced, using an adenoviral vector carrying the human recombinant IL-1Ra gene or the marker genes encoding firefly luciferase or bacterial ß-galactosidase (LacZ). Cells in the flasks were transduced with either 1×10^7, 1×10^4, or 1×10^1 pfu to evaluate the rate of transduction, and with 1×10^7 infective particles (pfu) to test for the time of expression.

Non-transduced cells and cells transduced with adenovirus encoding the LacZ gene were used as controls for IL-1Ra expression. The efficiency of transduction was estimated by the X-gal staining of LacZ-transduced cells. Conditioned media was collected daily from wells or every other day from flasks and stored at –80 °C until testing for IL-1Ra expression by ELISA (Biosource, US).

IL-1 bioassay

IL-1 is known to upregulate the expression of the inducible form of nitric oxide synthetase (iNOS) which produces nitric oxide in rabbit articular chondrocytes (Stadler et al. 1991, Palmer et al. 1993). This effect was used as the basis of a bioassay to determine the biologic activity of the transgenic IL-1Ra. Chondrocytes were received from articular cartilage of New Zealand white rabbits and cultured in 24-well plates (Dulbecco's Modified Eagle Medium (DMEM), supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin). After reaching 80% confluence, cells were transferred to serum-less medium, and 20 Units recombinant IL-1 protein were added to induce nitric oxide production (GibcoBRL, Grand Island, NY, USA). To the individual wells were added 100, 50, 25, 5, 1, or 0 µL of medium conditioned by transduced cells producing transgenic IL-1Ra or LacZ. Following 48 hours incubation, the activity of iNOS was measured by assaying

Table 1. Expression of alkaline phosphatase (ALP; units/ 1×10^6 cells) by human osteoblastic cells . Immortalized synovial fibroblasts (HIG-82) served as negative controls, immortalized bone marrow stem cells (OIM) after treatment with BMP-2 served as positive controls

Type of cells	Mean ALP-expression	SE
OIM	30.0	7.0
Osteoblastic	9.5	0.5
HIG-82	0.0	0
None	0.0	0

the nitric oxide concentration in the medium using a spectrophotometric method based on the Griess reaction (Green et al. 1982).

Results

Cell cultures

Human osteoblastic cells were able to generate large healthy colonies and grew finally to confluence. The cells multiplied continually and were subcultured every other week. After the second trypsinization, cells developed a uniform appearance.

Alkaline phosphatase activity

Alkaline phosphatase (ALP) is known to be one of the earliest markers expressed by an immature osteoblast, and its expression persists throughout osteoblastic maturation. The human osteoblastic cells spontaneously expressed ALP. In contrast, the immortalized synovial fibroblasts (HIG-82) that served as negative controls did not. Immortalized murine osteoprogenitor cells, known to express ALP after stimulation with BMP-2, served as a positive control (Table 1).

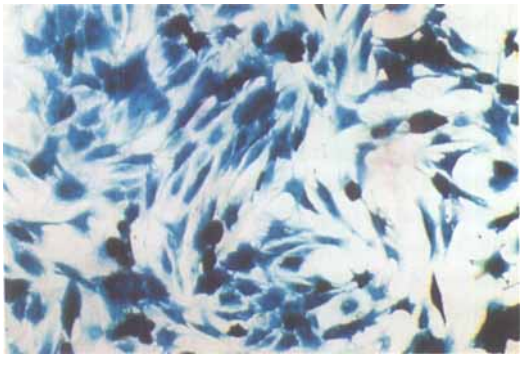

Figure 1. Human osteoblastic cells stained with X-gal after transduction with adeno-LacZ. The blue staining of the osteoblastic cells following the X-gal procedure shows that the LacZ-gene was expressed at a rate of 100% after transduction with 1×10^7 infective particles (pfu) per 1×10^6 cells (× 200).

Table 2. Successful transduction using 1×10^7 infective particles (pfu) of the adeno-luciferase vector. The luciferase expression (in thousand units/1×10^6 cells) from osteoblastic cells is high 2 days after transduction. Non-transduced and adeno-LacZ-transduced osteoblastic cells failed to show luciferase activity

Type of cells	Mean (thousands)	SE
Adeno-luciferase-transduced osteoblastic	66.25	7.92
Adeno-LacZ-transduced osteoblastic	0.025	0.005
Non-transduced osteoblastic	0.010	0.0

Transduction efficiency and marker gene expression (LacZ, luciferase)

1, 3, 5, 10, 15, and 20 days after transduction, cells were stained with X-gal and were microscopically examined for LacZ-positive cells. Virtually every osteoblastic cell was transduced after transduction with 1×10^7 pfu and expressed the LacZ gene, which suggests an efficiency of the adenoviral gene transfer approaching 100% (Figure 1). Thus LacZ gene expression lasted for at least 20 days after transduction.

Adenoviral transduction with the luciferase gene led to high levels of intracellular luciferase activity. 2 days after transduction, the cells expressed luciferase up to 81,000 Units/1×10^6 cells. Non-transduced cells and cells transduced with the adeno-LacZ vector failed to do so (Table 2).

Expression of the IL-1Ra transgene

The IL-1Ra content of media conditioned by osteoblastic cell cultures transduced with adeno-IL-1Ra was measured every other day. After transduction with 1×10^7 pfu adeno-IL-1Ra, the osteoblastic cells expressed up to 1.107 μg IL-1Ra/1×10^6 cells/48 h. The peak of expression was seen between 2 and 4 weeks after transduction. Expression of the transgene was detectable for 72 days

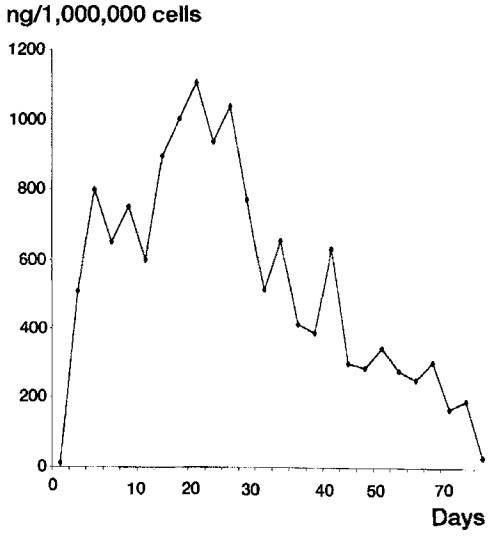

Figure 2. IL-1Ra production by 1 x 10^6 osteoblastic cells within 48 hours, following transduction with 1 x 10^7 infective particles (pfu) of the adeno-IL-1Ra vector. The highest levels of expression were found 12–30 days after transduction, whereas detectable levels of the transgenic IL-1Ra were seen up to 72 days.

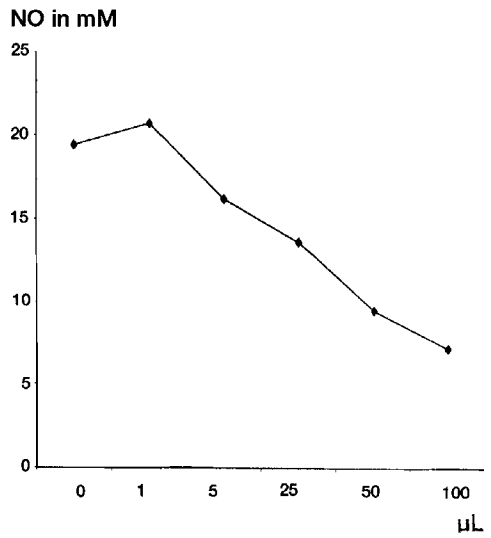

Figure 3. Bioactivity of the transgenic IL-1Ra. The medium conditioned by adeno-IL-1Ra-transduced osteoblastic cells successfully inhibits the IL-1-induced nitric oxide (NO) synthesis in rabbit articular chondrocyte cell cultures in a dose-dependent manner. Control media conditioned by non-transduced cell cultures of osteoblastic cells failed to inhibit the IL-1-induced NO production.

(Figure 2). Transduction with 1 x 10^4 pfu and less did not result in measurable gene expression nor did the control cells transduced with adeno-LacZ or the non-transduced cells express human IL-1Ra (data not shown). The medium was changed every other day in the period of IL-1Ra expression. After 5 weeks, the cells first stopped dividing and then died 73 days after transduction.

Bioactivity of the transgenic IL-1 Ra

Nitric oxide (NO) is synthesized in large amounts by chondrocytes activated by inflammatory mediators such as IL-1. Addition of medium conditioned by osteoblastic cells transduced with adeno-IL-1Ra to in vitro cultures of rabbit articular chondrocytes suppressed the IL-1-induced NO synthesis in a dose-dependent manner (Figure 3). These results show that the expressed IL-1Ra was biologically active. Conditioned media of non-transduced cells and cells transduced with the adeno-LacZ vector failed to do this (data not shown).

Discussion

We found that first-generation adenoviruses can transduce human osteoblastic cells in vitro efficiently, leading to persistent expression of the transgene product. This result was obtained with adenoviral vectors encoding the LacZ gene, the luciferase gene and the human IL-1Ra gene. Our results encourage the pursuit of further animal studies, investigating the possible use of gene transfer to osteoblasts for the treatment of bone diseases or bone-healing disorders.

IL-1Ra is a specific competitive inhibitor of IL-1 that has no agonistic properties (Evans and Robbins 1994, Hung et al. 1994). It has been found that blocking the actions of IL-1 reduced the bone resorption following ovariectomy in mice (Boyce et al. 1989). Cytokine inhibitors such as IL-1Ra or soluble TNF-α receptors also prevent early postmenopausal osteoporosis in rats (Kimble et al. 1995). Moreover, IL-1 is known to be one of the pathologic agents causing inflammation in rheumatoid arthritis (Chole et al. 1994, Ralston and Grabowski 1996, Kang et al. 1997). This makes IL-1Ra, a physiological antagonist of IL-1, a potentially important drug for the downregulation of the effects of IL-1 on the musculoskeletal system including bone. The administration of cytokines, cytokine inhibitors, or growth factors to bone and

bone marrow might be clinically beneficial in various local and systemic bone diseases, but their clinical use is limited by the short half-lives of these proteins. Gene delivery offers an alternative administration system, avoiding daily injections or continuous, systemic delivery by, for example, pumps. In addition, gene therapy offers the possibility of localized delivery, restricted to the tissue of interest, where therapeutic effects may occur without systemic side-effects (Evans and Robbins 1995).

Our results after X-gal staining show that the adenoviral vector encoding ß-galactosidase (LacZ) was able to transduce human osteoblastic cells efficiently. In vitro transduction with the adeno-IL-1Ra and adeno-LacZ led to extended expression of both genes. In the case of IL-1Ra, high expression was seen for at least four weeks. In addition, we have proven the transgenic IL-1Ra to be biologically active by demonstrating that the expressed cytokine inhibitor could suppress IL-1-induced NO synthesis by rabbit articular chondrocytes in a dose-dependent manner. The mechanisms that limit in vitro transgene expression of cultured cells are not completely understood, but they may be related to the episomal nature of adenoviral gene delivery (Dai et al. 1995, Yang et al. 1995, Foley et al. 1997).

Gerich et al. (1996) found LacZ-positive cells in bone after accidental injection of the adeno-LacZ vector into the insertion site of the patella tendon, suggesting the ability of adenoviral vectors to transduce bone cells in vivo. We are currently investigating a growth factor-based gene transfer of bone, to develop new strategies in the treatment of fractures with segmental defects and fractures resulting in non-unions (Baltzer et al. 1999). The length of transgene expression noted in the present in vitro study may be considered to be ideal for the purpose of fracture-healing (Niyibizi et al. 1998).

For therapy of chronic bone diseases, such as osteoporosis, the transient effects of adenovirally-mediated gene transfer may limit the use of adenoviral vectors, unless a convenient method of repeated application can be devised. Moreover, further investigation on the effect of immunization by the administration of viral vectors should be done. But in case of local, non-chronic bone diseases, a limited but high expression as shown in this study may be considered ideal. If transgene expression in vivo has a duration like that of adenoviral vectors, encoding growth factors may be used as part of a new approach to accelerate fracture healing and treat non-unions (Niyibizi et al. 1998, Baltzer et al. 1999). Due to the ability of the adenovirus to transduce human bone cells at a high rate, such that the transduced cells express high levels of the biologically active gene product, the adenovirus may be appropriate for use as an in vivo vector to treat local and systemic bone diseases, where long-term gene expression is not required. We are currently evaluating the use of gene therapy to improve the ossification in a critical size defect of long bones in the rabbit, and to prevent postovariectomy-induced bone loss in mice by transduction with the adeno-IL-1Ra and adeno-sTNF-αR vectors.

Introduction of exogenous therapeutic genes into osteoblasts, as described in our study, may open new avenues for the delivery of potentially therapeutic substances that normally undergo fast metabolism in vivo to treat bone diseases. The high levels of expressed gene products offer not only the opportunity for gene-based treatments of local bone disorders but, depending on the amount of administered vectors, gene therapy might also become an appropriate approach for the treatment of systemic bone diseases, such as osteoporosis.

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