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Adenovirus-mediated gene transfer of interleukin-4 into pancreatic stellate cells promotes interleukin-10 expression

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Abstract

Pancreatic stellate cells (PSC) are crucially involved in the development of fibrosis, a hallmark of chronic pancreatitis. Therefore, PSC represent an attractive target for the modulation of cellular functions providing the prerequisite for the establishment of novel therapeutic strategies like transfer of genetic material to the cells. Based on recent studies suggesting that the chronic course of pancreatitis is associated with immune deviation towards a Th1 cytokine profile, we have investigated the applicability of primary PSC to an adenovirus-mediated transfer of the cDNA encoding the Th2 cytokine interleukin (IL) 4 and the autocrine-acting effects of IL 4 on the cells *in vitro*. The transduction of primary PSC with a replication-incompetent adenovirus type 5 vector carrying the cDNA encoding rat IL-4 resulted in a distinct expression of the cytokine on mRNA and protein level for two weeks. Similar to recombinant IL 4, effects of the endogenously synthesized cytokine were mediated by the signal transducer and activator of transcription (STAT)6. Interestingly, beside the increase of PSC proliferation, IL 4 transduction was accompanied by an up-regulation in the endogenous expression of the anti-inflammatory cytokine IL 10. In summary, our data suggest that PSC are suitable targets for gene therapy modulating cellular interactions in the pancreas.

Keywords: adenoviral gene transfer • interleukin 4 • interleukin 10 • pancreatic stellate cells

Introduction

Chronic pancreatitis as well as pancreatic carcinoma are characterized by the development of an extensive fibrosis [1, 2]. Pancreatic stellate cells (PSC) have been identified as the pivotal target cell population for pro-fibrogenic mediators. Stimulating substances initiate an activation process in the specialized fibroblastic cells accompanied by an increased proliferation and the induction of the expression of extracellular matrix proteins (ECM) [3–5]. PSC activation can be mimicked *in vitro* by the cultivation of isolated stellate cells on plastic. Therefore, PSC cultures are an established model to analyze molecular mechanisms of biological cell functions [6]. In addition to response activity towards numerous substances, PSC themselves were shown to produce various mediators, especial-

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ly cytokines and therefore, the cells are thought to be involved in the regulation of inflammatory reactions [5, 7]. In conclusion, PSC represent an attractive target for modulating cellular functions that may intervene patho-physiological mechanisms potentially providing therapeutic options.

Gene therapy is a new therapeutic tool based on the transfer of genetic material to cells [8]. Recently, we could show that primary PSC were successfully transduced with a retroviral vector containing the SV40 large T antigen resulting in immortalized stellate cell lines [7]. In contrast to retroviral-based vectors, recombinant adenoviruses (Ad) are not integrated into the cellular genome. Replication-deficient Ad constructs represent versatile gene delivery systems that have been widely used for a variety of gene therapy applications *in vitro* and *in vivo* [9].

There is growing evidence that immunological mechanisms play a crucial role in the pathogenesis of chronic pancreatitis and the progressive fibrogenesis [10–13]. Using a rat pancreatitis model, we have recently shown that activated lymphocytes expressing Th1 cytokines are essentially involved in the perpetuation of inflammation associated with the deposition of components of extracellular matrix proteins (ECM) [13]. The Th2 cytokine interleukin-4 could not be detected both in the experimental pancreatitis model and human tissue of patients suffering from chronic pancreatitis.

It has been shown that IL-4 can down-regulate Th1 activity [14] and consequently, the cytokine was applied in various therapeutic approaches to influence diseases based on an inflammatory pathogenesis *e.g.* rheumatoid arthritis and diabetes [15–17].

The application of recombinant cytokines is limited by the short endurance of the proteins. In addition, the use of cytokines *in vivo* is hampered by low and frequently ineffective concentrations in the target tissue. These limitations can be avoided by over-expression of the respective protein using gene transfer techniques.

The purpose of the current study was the generation of IL-4-expressing pancreatic stellate cells using the adenoviral gene transfer technique. Subsequently, the effects of the genetic manipulation on cellular functions were characterized.

Materals and methods

Reagents

Reagents were purchased from the following sources: Nycodenz was obtained from Nycomed (Oslo, Norway); Hanks' Balanced Salt Solution (HBSS), non-essential amino acids, penicillin, streptomycin and trypsin from Invitrogen (Karlsruhe, Germany); Collagenase P, deoxyribonuclease I (DNase I), Cell Proliferation ELISA measuring 5-bromo-2'-deoxy-uridine (BrdU) incorporation into DNA from Roche (Mannheim, Germany); and Iscove's modified Dulbecco medium (IMDM) as well as fetal calf serum (FCS) from Biochrom (Berlin, Germany). Recombinant rat IL-4 (rrIL-4) and Quantikine® ELISA assays for measurement of rat specific IL-4 and IL-10, respectively, were purchased from R&D Systems (Wiesbaden, Germany); rabbit anti-phospho-STAT6 and anti-total STAT6 from New England Biolabs (Ipswich, MA, USA), rabbit anti-IL-4 from Biosource (Camarillo, CA, USA); anti-rabbit horseradish peroxidase (POD)-labelled Ig and the Enhanced Chemiluminescence (ECL) Plus kit from Amersham Biosciences (Freiburg, Germany); and pro-

Table 1 List of rat specific primers used for PCR

HPRT	TCCCAGCGTC GTGATTAGTG	GGCTTTTCCA CTTTCGCTGA
IL-4	ATGCACCGAG ATGTTTGTACC	TTTCAGTGTT CTGAGCGTGGA
IL-10	TGCCTTCAGT CAAGTGAAGACT	AAACTCATTC ATGGCCTTGTA
TGF-β1	GCCTCCGCAT CCCACCTTTG	GCGGGTGACT TCTTTGGCGT
Collagen I	CAAGAATGGCGACCGTGGTGA	GTCCACCTCAAGGTCACGGTC
PPARγ	ATAAGGGACTCGAGGAGGTC	AACCATTGGGTCAGCTCTTG

tease IX as well as standard laboratory chemicals from Sigma (Deisenhofen, Germany). The RNeasy Mini RNA extraction kit and Taq polymerase were delivered by Qiagen (Hilden, Germany); and all reagents for reverse transcription [Superscript II RT, oligo(dT)12-18, dNTP] by Invitrogen (Karlsruhe, Germany). Rat-specific primers for PCR were generated using the NCBI gene bank as source for any sequences and synthesized by BioTez (Berlin, Germany). In detail, the following primer sequences were used (forward primer (5´-3´), reverse primer (5´-3´) (see Table 1). For quantitative PCR, cDNA was prepared with Reverse Transcription TaqMan™ reverse transcription reagents and random hexamer primers. For Real-time PCR, TaqMan™ universal PCR master mix and a rat IL-10-specific fluorescently labelled TaqMan™ MGB probe (Assay-on-Demand™) and TaqMan™ Rodent GAPDH Control Reagents as internal control were used (all Applied Biosystems, Foster City, CA, USA).

Isolation and culture of pancreatic stellate cells

Stellate cells were isolated from pancreas of male LEW.1W inbred rats (Karlsburg, Germany) as previously described [7]. Briefly, the pancreas was digested with a mixture of collagenase P (0.05 %), protease IX (0.02 %) and DNase I (0.1 %) in HBSS. After density gradient centrifugation (12 % Nycodenz; centrifugation at 1400 x g for 20 minutes) PSC were collected from the top of the gradient, washed and resuspended in IMDM supplemented with 10 % fetal calf serum, 1 % non-essential amino acids (dilution of a 100 x stock solution), 100 U/ml penicillin and 100 µg/ml streptomycin. Freshly isolated cells were cultured in 6-well culture plates at 37 °C in 5 % CO_2 humified atmosphere. With the first two medium changes (24 and 48 hrs after seeding), most of the contaminating cells were removed, and almost (>95 %) pure PSC cultures were received.

After reaching confluency, cells were harvested by trypsination and replated at equal seeding densities.

Adenovirus vectors and cell transduction

The generation of recombinant E1-deleted replication-incompetent adenovirus type 5 vectors carrying the genes encoding β–galactosidase (Adβ-Gal) and rat IL-4 (AdrIL-4), respectively, has been described elsewhere [18, 19]. Primary PSC activated by *in vitro* cultivation for 7 to 10 days after isolation from rat pancreas were used as target cells. Adenoviral transduction was performed by incubating PSC growing in 6-well plates with 0.05–500 *multiplicity of infection* (MOI) Adβ-Gal or AdrIL-4 for 2 hrs in serum-free culture medium. Then, FCS was added at a final concentration of 10 %, followed by incubation for additional 20 hrs. Transduced cells were washed with phosphate buffer saline (PBS, pH 7.4) and further treated according to the experimental requirements.

X-Gal staining

Cells pre-treated as indicated were washed twice with PBS and fixed by incubation in a solution of 2 % formaldehyde and 0.2 % glutaraldehyde (diluent: PBS) for 5 min at 4 °C. Afterwards, sections were washed two times with PBS, before staining solution composed of PBS containing 20 mM $K_3Fe(CN)_6$, 20 mM $K_4Fe(CN)_6 \bullet 3H_2O$, 2 mM $MgCl_2$ and 1 mg/ml X-Gal (5-bromo-4-chloro-3-indolyl β-D-galactopyranoside; dissolved at 50 mg/ml in dimethylsulfoxide) was added. Depending on the staining intensity, incubation (performed at 37 °C) was stopped after up to one h.

Cell proliferation assay

To assess cell proliferation, incorporation of 5-bromo-2`-deoxyuridine (BrdU) into newly synthesized DNA was quantified using the BrdU labelling and detection enzyme-linked immunosorbent assay kit. Cells were cultured for 24 hrs in 96-well plates in the presence of the indicated agents followed by a further incubation with BrdU for 4 hrs. BrdU uptake was measured according to the manufacturer's instructions.

Competitive RT-PCR

Total RNA was isolated from PSC as previously described [20]. 1 µg of total RNA was reversely transcribed (RT) into cDNA using $Oligo(dT)_{12-18}$ primer and reverse transcriptase Superscript II. Competitive polymerase chain reaction (PCR) was performed according to Brock *et al.* [21] using a synthetic DNA control fragment (CF) as internal standard. Sample cDNA and control DNA were amplified with the same

primers. The control and sample PCR products were distinguished by differences in length using agarose gel electrophoresis. To correct for variations in different preparations, the cDNA samples were adjusted to equal input cDNA concentrations, based on their content of the house-keeping gene HPRT, before determining the respective cDNA concentration. The PCR products were separated electrophoretically in an agarose gel containing 0.3 µg/ml ethidium bromide. The intensity of the ethidium bromide fluorescence, reflecting the amount of cDNA, was measured with an electronic camera. Data were analyzed with the EASY program (Herolab, Wiesloch, Germany). Expression was monitored for IL-4, IL-10, transforming growth factor (TGF)-β1, α–chain of collagen type I and PPARγ.

Real-time PCR

Total RNA of cells treated as indicated was isolated followed by cDNA synthesis as described above. IL-10 transcript levels were analyzed by quantitative PCR using ABI Prism 7000 sequence detection system. Standardisation of the system has been performed using the expression of the house-keeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH). PCR conditions were as follows: 95 °C for 10 min, 50 cycles of 15 s at 95 °C/ 1 min at 60 °C. PCR reactions were performed in quadruplicate. The relative expression of IL-10 mRNA normalized with GAPDH was calculated according to the equation $\Delta Ct = Ct_{target} - Ct_{GAPDH}$. IL-10 mRNA levels in cells cultured with rrIL-4 were expressed as $2^{-(\Delta\Delta Ct)}$, where $\Delta\Delta Ct_{rrIL\text{-}4} = \Delta Ct_{rrIL\text{-}4} - \Delta Ct_{\text{control (no rrIL-4)}}$.

Immunoblotting

Protein extracts of PSC or culture supernatants (treated as indicated) were separated by SDS-polyacrylamide gel electrophoresis and blotted onto nitrocellulose filters. Next, membranes were blocked with 1 % bovine serum albumin (BSA) and incubated with the respective specific antibodies as indicated at 4 °C overnight. After a final incubation with a horseradish peroxidase-labelled anti-rabbit- or anti-mouse Ig antibody, blots were developed using the ECL Plus kit. For reprobing with additional antibodies, blots were stripped by incubation in stripping buffer (62.5 mM Tris HCl, pH 6.7, 2% SDS, 100 mM 2-mercaptoethanol) at 50 °C for 30 min.

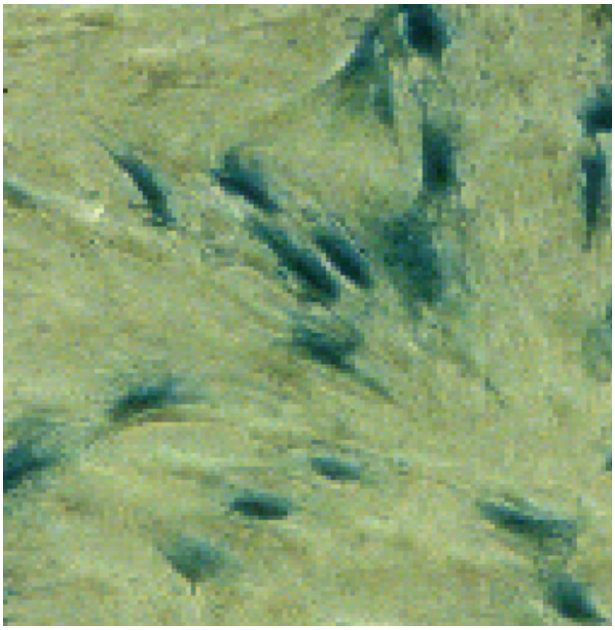

Fig. 1 Representative microphotograph of X gal-stained monolayer of cultured PSC one day after application of the adenovirus vector Adβ Gal (original magnification x 200).

Cytokine secretion

Cells were transduced with adenovirus vectors (Adβ-Gal and AdrIL-4, respectively) as decribed above. After further cultivation for periods as indicated, cell supernatants were collected and subjected to the measurement of IL-4 and IL-10 concentrations, respectively, using the ELISA technique according to the manufacturer's protocol.

Statistical analysis

Results are expressed as means ± SEM for the indicated number of separate cultures per experimental protocol. Statistical significance was checked using the t-test and SPSS software.

Results

Adenoviral gene transfer into pancreatic stellate cells

Initially, we investigated the applicability of adenovirus-mediated gene transfer into cultured primary PSC using a reporter construct containing the E.

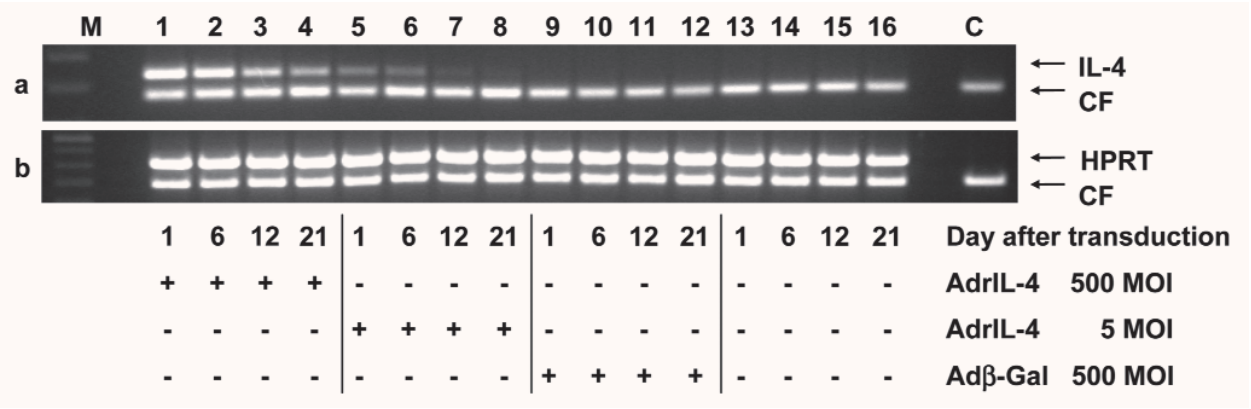

Fig. 2 IL-4 mRNA expression in adenovirus-transduced PSC. PSC were cultured in 75 cm^2 flasks and infected on day 0 (8 days after isolation) with adenovirus (AdrIL-4 and Ad -Gal, respectively) as indicated. On day 1 after transduction cells were harvested. Total RNA was isolated from 75% of the collected cells whereas 25% of the cells were re-cultured and underwent the same procedure on day 6 and 12. The last cells were harvested on day 21. Afterwards, RT reaction, competitive PCR and agarose gel electrophoresis were performed as described in the "Materials and methods" section. (**a**) The upper band represents the IL 4 amplicon at 275 bp, the lower band at 178 bp the control fragment (CF) as internal standard. M = 100 bp molecular weight marker, C = control without cDNA. (**b**) The bands are the amplicons of house-keeping gene HPRT at 608 bp and the CF at 499 bp. Adenovirus vectors were used in the following concentrations: 500 MOI AdrIL 4 (lanes 1-4), 5 MOI AdrIL 4 (lanes 5-8) and 500 MOI Adβ-Gal (lanes 9-12). Lanes 13-16 are controls without adenoviral infection. The results shown are representative of three independent experiments.

coli β-galactosidase gene (Adβ-Gal). As shown in Fig. 1, 24 hrs after infection with 500 MOI about 50% galactosidase-expressing cells could be detected by X-Gal staining.

Then, experiments were performed using the AdrIL-4 vector. Monolayers of *in vitro* activated PSC were incubated with different vector concentrations for 20 h. At indicated time points cells were subjected to extraction of RNA or protein as well as to proliferation assays, respectively.

Fig. 2 shows the expression of IL-4 mRNA in PSC detected by RT-PCR. The transfer of 500 MOI AdrIL-4 resulted in high IL-4 mRNA transcript levels during 6 days after the transduction. Subsequently, the expression declined but remained detectable for up to 3 weeks. An AdrIL-4 concentration of 5 MOI resulted only in low expression levels during the first 3 days after gene transfer. Untreated PSC (data not shown) as well as cells transduced with the reporter gene construct Adβ-Gal did not express IL-4 mRNA.

Protein synthesis and secretion of IL-4 were assessed by investigation of the cell culture supernatant using the immunoblot technique. Similar to the mRNA expression studies, a vector concentration of 5 MOI resulted only in a weak signal whereas the IL-4 production strongly increased after the treatment with 500 MOI (Fig. 3). Regarding the time course there was a remarkable difference between RNA levels and the secreted protein. The transcript levels persisted at least 14 days (Fig. 2), whereas the IL-4 protein decreased dramatically between day 2 and day 4 after gene transfer. On day 5 only a faint IL-4 - specific band was visible (Fig. 3). This discrepancy may be due to the much more sensitive PCR technique compared with immunoblotting.

Influence of IL-4 transduction on the expression of selected genes in PSC

To address the issue of molecular effects mediated by IL-4, we investigated the transcript levels of selected genes of interest in IL-4-expressing PSC (Fig. 4). Interestingly, we found a strong up-regulation of IL-10 mRNA levels simultaneously to the expression of IL-4 (Fig. 4a).

The transcript levels of the pro-fibrogenic molecules TGF-β1 and collagen type I did not change (Fig. 4b, c). Interestingly, the expression of PPARγ (Fig. 4d), which has been shown to inhibit

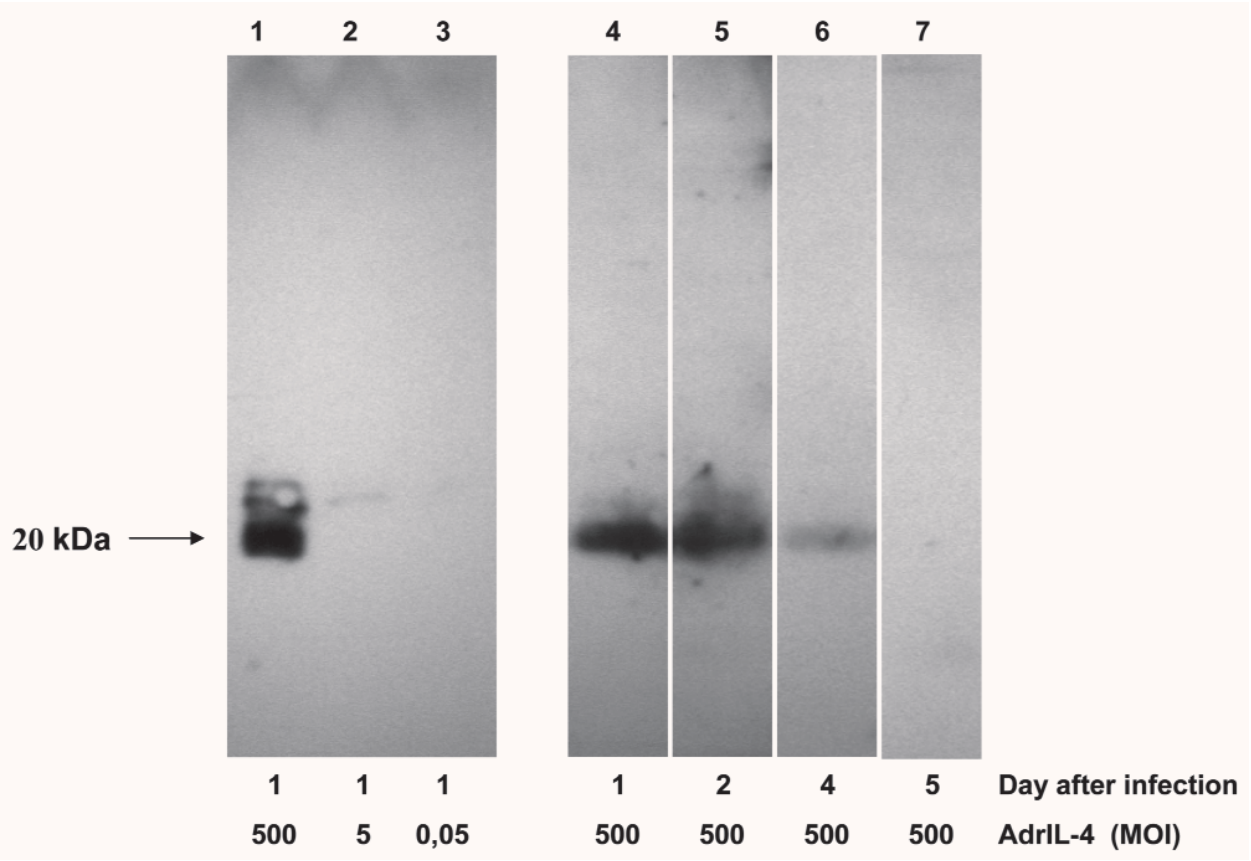

Fig. 3 Adenoviral gene transfer-dependent of IL 4 expression. PSC were cultured in 6-well plates and infected with the indicated MOI of AdrIL 4 (lane 1-3). Samples of supernatants (75 µl) were collected at the indicated time points and subjected to immunoblot analysis. Note that the medium was replaced every day, so that the expression of protein shown by immunoblots is just the amount of one day. Expression of IL 4 was indicated by a 20 kDa band.

stellate cell activation [22], also corresponded with the up-regulation of IL-4, albeit to a lower extent compared to IL-10.

Moreover, mRNA expression analysis of other cytokines (IL-6, IL-15) showed no changes in IL-4 over-expressing PSC (data not shown).

In order to confirm on protein level the IL-4-induced up-regulation of IL-10, we analyzed the secretion capacity of adenovirus-transduced PSC evaluating the concentration of both cytokines in the cell supernatant using the ELISA technique. In agreement with the RNA data, IL-4 as well as IL-10 were still highly expressed 6 days after the AdrIL-4 transfer (Fig. 5). Subsequently, the concentration decreased but it was clearly detectable at least up to 2 weeks. The traces of IL-4 measured in the supernatant of Adβ-Gal-infected PSC may be attributed to an unspecific reaction or/and a moiety of serum in the culture medium.

To definitely show that the induction of IL-10 expression was indeed mediated by IL-4 and did not represent a consequence of the adenovirus construct incorporated in the cells, we analyzed the impact of recombinant rIL-4 on IL-10 transcript levels in PSC using the Real-time PCR-technique. Total RNA of PSC was extracted at different time points after the application of 10 ng/ml rrIL-4 and reversely transcribed into cDNA which was subjected to the quantitative PCR. As shown in figure 6, IL-10 mRNA levels in rrIL-4 - treated PSC were increased 5-fold after 2 h and 11-fold after 12 h with respect to untreated controls. Longer incubation times had no significant enhancing effects (data not shown). These results suggest that the induction of IL-10 in AdrIL-4-infected PSC was attributed to the endogenously synthesized IL-4 in a paracrine and / or autocrine manner.

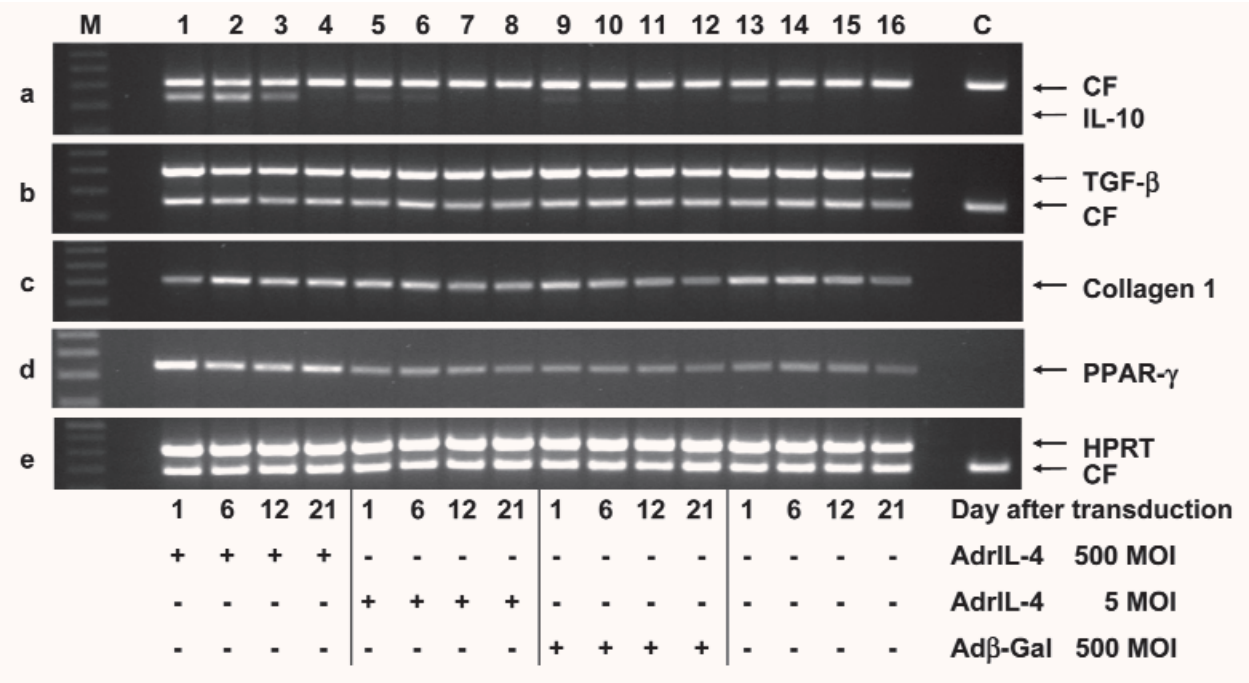

Fig. 4 Changes of IL-10-, TGF-β1-, collagen I- and PPARγ mRNA expression in adenovirus-transduced PSC. Transduction of the cells with different concentrations of adenovirus (AdrIL-4 and Ad -Gal, respectively), cell harvest and RT-PCR were performed as described in the legend to Fig. 2 and the "Materials and methods" section. (**a**) The lower band represents the IL 10 amplicon at 346 bp, the upper band at 421 bp the control fragment (CF) as internal standard. (**b**) TGF-β amplicon at 396 bp, CF=257 bp. (**c**) Collagen I amplicon at 513 bp. (**d**) PPARγ amplicon at 347 bp. (**e**) house-keeping gene HPRT at 608 bp, CF at 499 bp. M = 100 bp molecular weight marker, C = control without cDNA. Lanes 13-16 represent controls without adenoviral infection. The results shown are representative of three independent experiments.

Influence of IL-4 on proliferation of PSC

Next, we studied the influence of IL-4 on the S phase of the cell cycle measured by BrdU incorporation into the DNA of PSC. Fig. 7 summarizes the effects of rrIL-4, transfer of AdrIL-4 and Adβ-Gal, respectively, on PSC proliferation. AdrIL-4 as well as rrIL-4 enhanced significantly the growth of *in vitro* cultivated PSC. In contrast, the proliferation rate of Adβ-Gal transduced cells did not differ from untreated controls.

STAT6 activation

Activation of *signal transducer and activator of transcription* (STAT) 6 represents an essential step of IL-4-induced signal transfer [23]. Therefore, we have investigated the activation status of STAT6 in adenovirus-transduced PSC. Western blots revealed that untreated control cells and Adβ-Gal-transduced PSC contained no detectable phospho-STAT6 (Fig. 8, lanes 2 and 3). As expected, cell treatment with recombinant IL-4 strongly stimulated tyrosine phosphorylation of STAT6. Interestingly, efficient activation of STAT6 was also observed in PSC transduced with AdrIL-4 at 5-500 MOI (lanes 4–6). Together, these results support the hypothesis that the biological effects initiated in adenovirus-transduced PSC are mediated by the IL-4 gene product.

Discussion

The current study provides a proof of principle that regulatory mediators can be expressed in pancreatic stellate cells *in vitro via* adenoviral gene transfer. The major requirement for a successful gene transfer is the efficient delivery of an exogenous

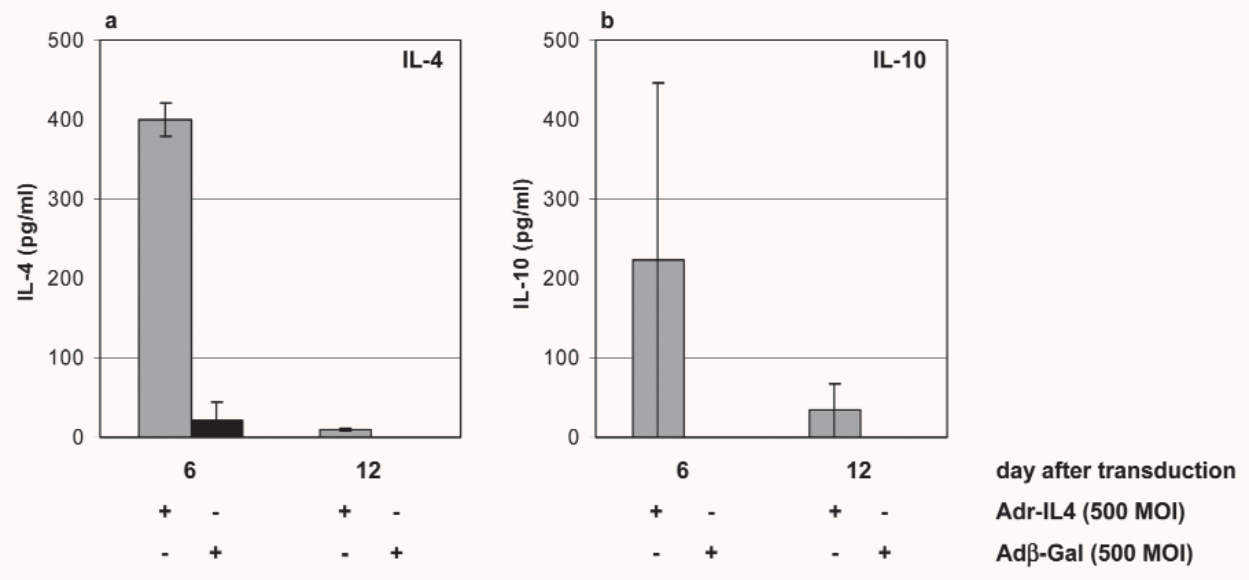

Fig. 5 Expression of IL 4 and IL 10 after AdrIL 4 and Adβ Gal administration. Cultured PSC were infected by adenovirus encoding for IL 4 or β-Gal (500 MOI each). One day after infection media were taken off and cells were splitted 1/4. 5 days later (day 6 after transfection) supernatants were collected and cells were splitted again. The same procedure was done on day 12 after transduction. Samples were applied to Quantikine® ELISA. Results for IL 4 are shown in part a, IL 10 in part b. Data are indicated as mean values of concentrations of 3 experiments ± SEM.

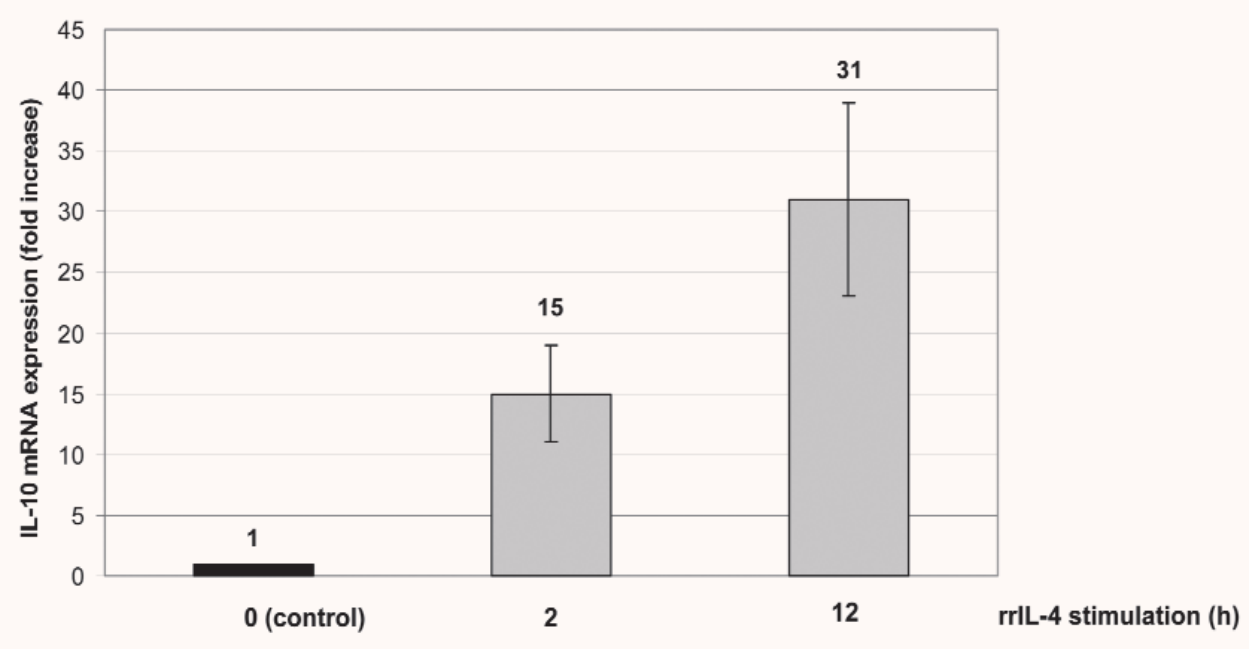

Fig. 6 Quantitative RT-PCR (qRT-PCR) of IL 10 after application of recombinant rat IL 4. Recombinant rat IL 4 (rrIL 4; 10 ng/ml) was added to cultured PSC 12 h and 2 h before harvesting, respectively. Total RNA were isolated and 1 µg were reversely transcribed. cDNA of each sample were used to perform Real-time PCR. Each time point represents the mean value of quadruplicate measurements (see also chapter "Materials and methods"). Demonstrated are the resulting ratios of IL 10 expression versus non- stimulated controls (± SEM). Data shown are representative of three independent experiments.

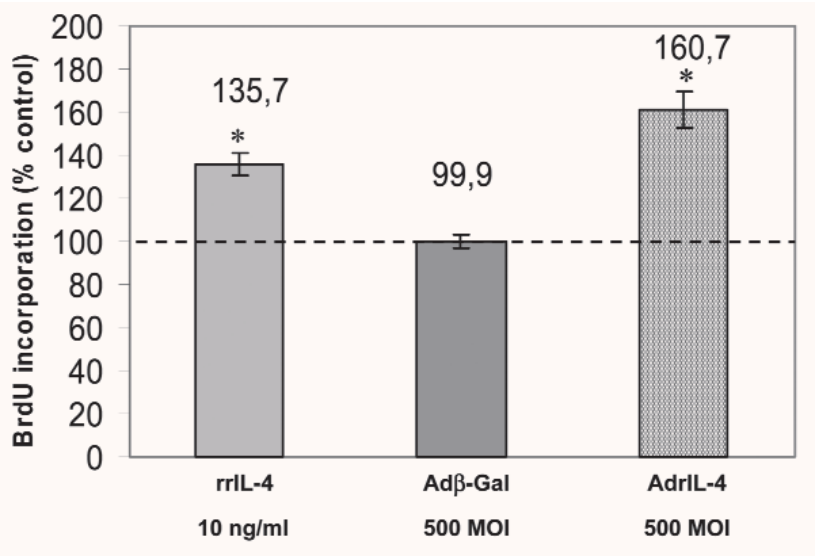

Fig. 7 Proliferation assays of PSC after adenoviral gene transfer of IL 4 using BrdU incorporation technique. Cultured PSC were infected with AdrIL 4 and Adβ-Gal, or recombinant rat IL 4 was applied as indicated. 24 h later, BrdU was added and incubation continued for 4 h. Results are demonstrated versus the untreated control (=100 %). Values are the means (± SEM) of six experiments. (* $p < 0.01$; n=6)

therapeutic gene to the appropriate cell type with subsequent synthesis of high and controllable protein levels. In this context, viral systems are superior to non-viral approaches. In comparison to retroviral vectors, adenovirus constructs generally have the advantage of high transduction rates, of capability introducing genes into dividing as well as quiescent cells with equal efficiency, of accommodation of large DNA cassettes, and that huge amounts of vector constructs can be produced and purified [24, 25]. The adenovirus DNA is not integrated into the cellular genome, an important aspect with regard to the bio-safety of any virus technology. Disadvantages of recombinant Ad-virus systems are the relatively short duration of the transgene expression and the immunogenicity of the virus particles [9].

Most gene therapy trials have used adenovirus-derived vectors for gene delivery in different tissues. More than 50 known serotypes, especially type 2 (Ad2) and type 5 (Ad5) have been used to generate vectors [9]. The incorporation efficiency depends - beyond the vector construction - on the expression of the coxsackie/adenovirus receptor (CAR) in the targeted host cell. Thus, generally primary lymphocytes are not susceptible to adenovirus infection, in part because of the absence of CAR [26]. Fibroblastic cells have been shown to express only low amounts of CAR [27] and consequently, the necessary adenovirus concentration of 500 MOI for an efficient IL-4 gene transfer into PSC was an order of magnitude higher compared with data reported for the transduction of epithelial cells [28].

To our knowledge we could show for the first time a successful adenovirus-mediated gene transfer into pancreatic stellate cells *in vitro*. Based on previous data showing Th1-driven immune reactions in an experimental model of chronic pancreatitis we used IL-4 as a relevant therapeutic gene [13].

After the AdrIL-4 transfer into primary PSC cultivated for 8 days the IL-4 expression remained nearly constant during one week. Subsequently, mRNA and protein levels gradually declined but were detectable for at least three weeks. This persisting expression is noteworthy insofar as cultivated primary stellate cells undergo a degeneration process once having achieved the activated myofibroblast-like phenotype during 14 days after isolation [7].

To investigate IL-4-mediated biological impact, we analyzed the expression profile of genetically modified PSC. Interestingly, IL-4 enhanced the transcription and synthesis of IL-10 in PSC. IL-10 represents a crucial immunosuppressive Th2 cytokine [29]. It has been shown to prevent monocyte differentiation towards dendritic cells, which are the most effective professional antigen presenting cells, especially for primary immune responses [30]. Furthermore, IL-10 inhibits the proliferation as well as the cytokine synthesis of T lymphocytes [31, 32]. Therefore, this cytokine has been applied in different therapeutic concepts [17]. Because of similar biological mechanisms targeted by therapy strategies with

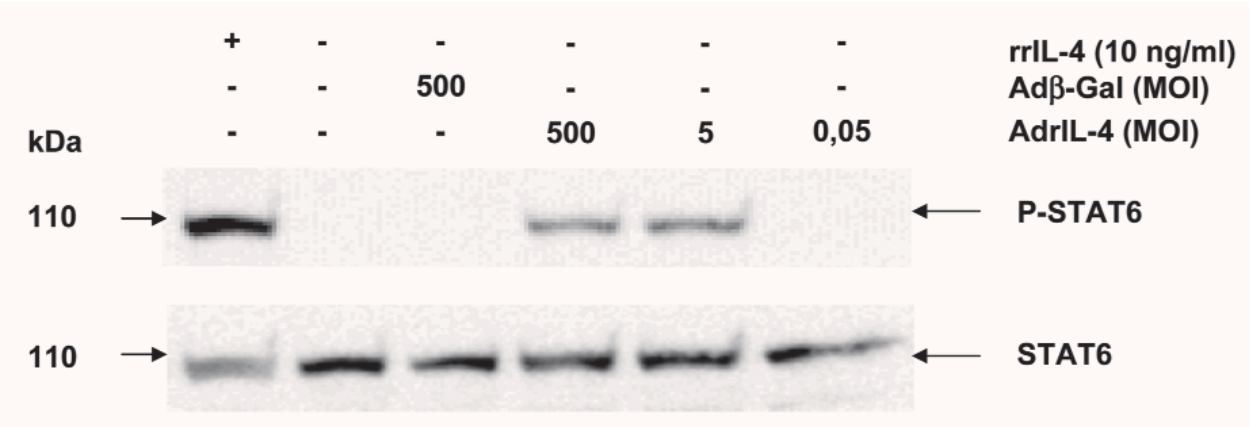

Fig. 7 Representative Western Blot of Phospho-STAT6. PSC monolayer were harvested 22 h after infection with adenovirus (AdrIL 4 and Adβ–GAL; MOI as indicated). Control cell were either left untreated, or incubated with rrIL-4 for 20 min. Tyrosine phosphorylation of STAT6 was assayed by immunoblotting using an antibody specific for the tyrosine-phosphorylated protein (P-STAT6; upper panel). To control loading, the blot was stripped and reprobed with an anti-STAT6 protein-specific antibody (lower panel).

IL-4 or IL-10, the combined application of both cytokines has been used in several studies [33, 34].

Recently, we have shown in a rat model of chronic pancreatitis induced by dibutyltin dichloride (DBTC) that adenoviral gene transfer of IL-4 modulates the immune response in terms of cytokine expression as well as of immunocompetent cell infiltration [21]. Thus, in the scenario of chronic pancreatitis, the additional up-regulation of IL-10 in IL-4-expressing PSC could amplify the anti-inflammatory effect which was aimed by the cytokine treatment strategy.

The production of IL-4 in the manipulated PSC had no effect on the expression of the pro-fibrogenic substances TGF-β1 and collagen. Kuroda *et al.* have reported a collagen synthesis-stimulating effect of IL-4 on human dermal fibroblasts [35]. This discrepancy to our results obtained for PSC might be attributed to the different cell populations used in the experiments.

Furthermore, we investigated in AdrIL-4-infected PSC the expression of PPARγ. Similar to IL-10, PPARã transcript levels correlated with the IL-4 mRNA level. This receptor has been suggested to be relevant in the inhibition of the active PSC phenotype [22].

Interestingly, the proliferation of *in vitro* cultivated PSC was slightly elevated by IL-4. The relevance of this effect in the physiological microenvironment of PSC has to be investigated.

IL-4 expression resulted in the phosphorylation of STAT6 in PSC. In contrast, IL-4 gene transfer had no effect on tyrosine phosphorylation and activation of STAT1 as well as STAT3 (data not shown), suggesting that activation of STAT6 was a specific effect. The activation of the transcription factor STAT6 as an essential member of the IL-4-initiated intracellular signal cascade has been reported for various cell types [23]. STAT6 is thought to be substantially involved in the IL-4-mediated differentiation of type 2 T-helper lymphocytes [36].

The results of the experiments performed in the course of this study were consistent insofar as the exogenous application of recombinant IL-4 has induced functional and molecular effects similar to the endogenous cytokine expression in PSC. These results indicate an autocrine or/and paracrine mechanism of IL-4 produced by the genetically engineered PSC themselves.

Taken together, we could show that the adenovirus-mediated gene transfer of IL-4 into primary pancreatic stellate cells resulted in an expression of the functional active protein. The regulatory Th2 cytokine induced the synthesis of the anti-inflammatory mediator IL-10. In comparison with the short time effects of the recombinant protein in cell culture, production of biologically active IL-4 by AdrIL-4-infected PSC persisted about two weeks.

In summary, this study suggests adenovirus-mediated gene transfer as an efficient approach to modulate PSC functions *in vitro*. It will be interesting to analyze in follow-up studies if such genetically engineered PSC are applicable *in vivo,* possibly as therapeutic tools in the context of pancreatic disorders.

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