Adenoviral Gene Delivery to Primary Human Cutaneous Cells and Burn Wounds

Tobias Hirsch,[1] Sebastian von Peter,[1] Grzegorz Dubin,[1,2] Dominik Mittler,[1] Frank Jacobsen,[1] Markus Lehnhardt,[1] Elof Eriksson,[3] Hans-Ulrich Steinau,[1] and Lars Steinstraesser[1]

[1]Department for Plastic Surgery, Burn Center, BG University Hospital Bergmannsheil, Ruhr University Bochum, Germany; [2]Faculty of Biotechnology, Jagiellonian University, Krakow, Poland; [3]Division of Plastic Surgery, Brigham and Women's Hospital and Harvard Medical School, Boston, MA, USA.

The adenoviral transfer of therapeutic genes into epidermal and dermal cells is an interesting approach to treat skin diseases and to promote wound healing. The aim of this study was to assess the in vitro and in vivo transfection efficacy in skin and burn wounds after adenoviral gene delivery. Primary keratinocytes (HKC), fibroblasts (HFB), and HaCaT cells were transfected using different concentrations of an adenoviral construct (eGFP). Transfection efficiency and cytotoxicity was determined up to 30 days. Expression was quantified by FACS analysis and fluorimeter. Cytotoxicity was measured using the trypan blue exclusion method. 45 male Sprague Dawley rats received 2×10^8 pfu of Ad5-CMV-LacZ or carrier control intradermally into either superficial partial thickness scald burn or unburned skin. Animals were euthanized after 48 h, 7 or 14 days posttreatment. Transgene expression was assessed using immunohistochemistry and bioluminescent assays. The highest transfection rate was observed 48 h posttransfection: 79% for HKC, 70% for HFB, and 48% for HaCaT. The eGFP expression was detectable in all groups over 30 days ($P > 0.05$). Cytotoxic effects of the adenoviral vector were observed for HFB after 10 days and HaCaT after 30 days. Reporter gene expression in vivo was significantly higher in burned skin compared with unburned skin ($P = 0{,}004$). Gene expression decreases from 2 to 7 days with no significant expression after 14 days. This study demonstrates that effective adenoviral-mediated gene transfer of epidermal primary cells and cell-lines is feasible. Ex vivo gene transfer in epithelial cells might have promise for the use in severely burned patients who receive autologous keratinocyte sheets. Transient cutaneous gene delivery in burn wounds using adenoviral vectors causes significant concentrations in the wound tissue for at least 1 week. Based on these findings, we hypothesize that transient cutaneous adenoviral gene delivery of wound healing promoting factors has potential for clinical application.

Online address: http://www.molmed.org
doi: 10.2119/2006-00031.Hirsch

INTRODUCTION

Gene therapy has been described as a promising approach for the treatment of skin diseases and impaired wound healing (1). Instead of an external application of therapeutic proteins, the genes of the therapeutic protein are brought into cells by vectors and are either integrated in the genome or remain episomal (2). Skin in particular has been described as an ideal target for in vivo and ex vivo gene therapy (3). Skin is easily attainable for an in vivo and ex vivo application and can be controlled and removed when side effects occur (4). The intense vascularization of the skin and the ability of dermal and epidermal cells to secrete proteins in the blood circulation enable skin therapy for the treatment of systemic diseases (5). The successful secretion of erythropoietin, transferrin, apolipoprotein E, and factor IX to treat metabolic and cardiovascular diseases after the genetic induction of keratinocytes and fibroblasts have been described (6-9). Moreover, skin therapy was beneficial in the local treatment of xeroderma pigmentosum (10), epidermolysis bullosa (11), and ichtthyoses (12). In addition to these applications in dermatological domains, gene therapy was of use in the treatment of wounds (13) caused by decubital, vascular, and diabetic pathologies (14,15). In particular the induction of the production of growth factors, such as PDGF-AA and IGF-1 (16), was shown to interact with and improve the molecular processes of wound healing.

Wound healing impairment and infection also remains one of the most evident problems in burn victims. More than 50,000 patients suffering from burn injuries are hospitalized each year in the United States and the mortality is about 5000 per year (17). The increasing incidence of wound infections with multidrug-resistant microbes on intensive care units leads to further increasing morbidity and mortality among these patients and is of considerable socioeconomic impact (18-21). The current clinical gold standard of topical treatment of chronic and burn wounds with synthetic agents is expensive and ineffective due to the high production costs and limited

Address correspondence and reprint requests to *Lars Steinstraesser, Department for Plastic Surgery, Burn Center, BG University Hospital Bergmannsheil, Ruhr University Bochum, Buerkle-de-la Camp Platz 1, 44789 Bochum/Germany. Phone: + 49 (0) 234/302-3442; fax: + 49 (0) 234/307-6379; e-mail: lars.steinstraesser@ruhr-uni-bochum.de. T. H and S. v. P. contributed equally to this work.*
Submitted April 11, 2006; accepted for publication July 25, 2006.

biological half-life (19,22-24). New concepts and therapeutic strategies to improve current wound care are needed. Instead of applying wound healing promoting factors topically, it is an interesting approach to deliver the gene of interest into the wound to utilize the cell machinery to overexpress wound healing–promoting factors for a limited time.

Aside from physical and chemical vector systems, viral vectors were used most frequently in the field of cutaneous gene therapy (25). The process of using viruses as vectors is based on their ability to transmit genes in host cells while infecting them.

Infections by adenoviruses are widespread in the population (26). Clinical symptoms vary from mild conjunctivitis and pharyngitis up to severe dysenteria (27). The genome of adenoviruses consists of double-stranded DNA of the length of 38 kb encapsulated by a hexon- and penton-based capsid. About 51 serotypes of the adenovirus were identified which can be divided into 6 subgroups (A-F) (28). Among them, mostly adenoviruses of the C-group, serotypes 2 and 5 were used for gene transfer, as they have the ability to transfect a wide range of cells, such as those of the lungs, liver, brain, blood, and skin (29). After the uptake via the ubiquitous CAR (coxsackie-adenovirus receptor), the adenovirus is transmitted into the nucleus of the host cell, where it remains episomal and, thus, leads to a transient expression of the transgenes (30). There are 2 phases in the expression of adenoviral genes. The "early genes" subdivide in 4 transcriptional parts (E1 to E4) and code for regulative proteins, which are responsible for the lytic growth of the virus in cell cultures, whereas the "late" transcripts code for structural proteins of the virus, such as the hexons of the capsid (29). In particular the E1-region is necessary for the production of the adenovirus and, thus, responsible for its cytotoxic effects, such that this part has to be removed to impair its replication in the setting of therapeutic gene transfer (31).

Various studies demonstrated that the local application of adenoviral constructs improved wound healing, in particular in processes of reepithelialization and vascularization (32). Mainly growth factors, such as PDGF-BB, VEGF, IGF, and TGF, were used in these studies to transduce keratinocytes, fibroblasts, and adipocytes (14,33). All of these studies used adenoviral constructs, because only a transient expression of the transgene products was needed; as the transgene expression in acute and subacute processes, for example in impaired wound healing, is only desirable as long as the underlying pathology is eliminated and the wound closed (32,34).

The aim of this study was to investigate the in vitro transfection efficacy and cytotoxicity of a replication-deficient adenoviral reporter gene construct. In addition, the aim was to further investigate the feasibility, expression, duration, and kinetics after cutaneous adenoviral gene delivery in burn wounds and healthy skin.

MATERIAL AND METHODS

Isolation of Human Keratinocytes

Freshly received human skin was placed in sterile petri dishes and the hypodermis was cut off. The remaining layers were completely covered with freshly prepared 0.2% dispase-solution (4.7 units/mL, Gibco, 17105-041, Paisley, UK) and incubated overnight at 4°C. The epidermis was detached and placed in small pieces in a trypsin/EDTA solution (0.05%/0.02%, Gibco, 35400-027). The remaining dermis was used for the isolation of fibroblasts. After incubating the epidermis for 5 min at 37°C in a gently shaking water bath (100 to 150 rpm), the cell suspension was vortexed and finally stopped by adding FBS (10%, Hyclone, Logan, USA). The suspension was filtered through a 100-μm cell strainer (Becton Dickinson, Heidelberg, Germany) and centrifuged at 400*g* and 4°C for 5 min. The cells were resuspended in 5 mL keratinocyte medium (3:1 DMEM [Gibco, 21969-035], Ham´s F12 [Gibco, 21765-029], 10% FBS [Hyclone], 1% penicillin/streptomycin [ICN, Aurora, USA], 4 mM L-glutamine [ICN], 24.3 μg/mL adenine [Calbiochem, Darmstadt, Germany], 5 μg/mL insulin [Sigma, St. Louis, USA], 0.4 μg/mL hydrocortisone [Calbiochem], 1.36 ng/mL triiodothyronine [Sigma], 10/–10 M cholera toxin [Sigma], 10 ng/mL EGF [Sigma]) and counted by CASY-1 (Schärfe-System, Reutlingen, Germany). They were seeded out into collagen type 1–precoated culture flasks (Becton Dickinson Falcon, 354236, Heidelberg, Germany) and cultivated at 37°C and 5% CO_2.

Isolation of Human Fibroblasts

The dermis was placed in sterile petri dishes. A sterile collagenase-type 2 solution (Gibco, 17101-015) was prepared in PBS at 3000 units/mL (3 mL collagenase-solution/g tissue). The tube was placed in a rotor oven at 37°C overnight (Becton Dickinson). After digestion of the tissue, the cell suspension was filtered through a 100 μm cell strainer and centrifuged at 400*g* for 10 min (Heraeus Suprafuge, Hanau, Germany), and the pellet was resuspended in fibroblast culture medium (DMEM [Gibco 21696-035], 10% FBS [Hyclone], 1% penicillin/streptomycin [50 units and 50 μg/mL; ICN]). Cells were counted by CASY-1, seeded, and cultured at 37°C and 5% CO_2. The medium was changed every second day.

HaCaT Cell Culture

HaCaT cells were cultured in sterile petri dishes using Dulbecco's modified Eagle's medium (Gibco 21696-035), 10% FBS (Hyclone), 1% penicillin/streptomycin (50 units and 50 μg/mL; ICN). Cells were counted by CASY-1, seeded, and cultured at 37°C and 5% CO_2. The medium was changed every second day.

Adenovirus Propagation

HEK-293 cells were brought to confluence overnight in a culture of DMEM, 10% FCS, and 1% penicillin/streptomycin. After changing the medium to DMEM, 2% FCS, 1% penicillin/streptomycin, the culture was transfected with the virus and incubated at 37°C for 48 h. After harvesting, cells were pelleted and resuspended

(20 mM Tris/HCl, pH 8.0, containing 2 mM $MgCl_2$). Three freeze-and-thaw cycles were performed in liquid nitrogen and in a waterbath at 37°C. After centrifugation of the lysate at 4°C and 3300*g* for 10 min, the supernatant was used and the debris was discarded. Virus was titered by measuring the optical density of the supernatant at 260 nm (double-stranded DNA) by a spectrophotometer (BioPhotometer, Eppendorf, Hamburg, Germany). The titration was controlled by plaque assays with a dilution series of 10^{-1} to 10^{-9} of the supernatant. HEK-293 cells were covered with agar, containing medium and the dilutions, and cultures were controlled for plaques after 48 h and 5 days. Each plaque was thereby assigned to 1 infection-competent virus particle. Cell cultures were transfected with the sterile but unpurified virus stock at MOIs of about 10 to 20. The cells were incubated for 45 h at 37°C and 5% CO_2, harvested, and centrifuged at 250*g*, 4°C for 5 min. The pellet was resuspended in 20 mM Tris/HCL, pH 8.0, and cells were lysed by 3 freeze-and-thaw cycles, as described above. After centrifugation at 3300*g* and 4°C for 10 min, the supernatant, containing the virus, was collected and purified of proteins and cell debris by a cesium chloride-gradient (Sigma). The gradient was centrifuged in a swinging-bucket-rotor of an ultracentrifuge (Haereus Suprafuge, Hanau, Germany) for 4 h at 105,000*g* and 20°C. The purified virus was harvested with a 5-mL syringe and collected in a Falcon tube. The preparation was desalted and fractioned by a gel filtration with sepharose CL-4B (Amersham Pharmacia, Buckinghamshire, UK). The fractions were measured by photometer, and those fractions which contained the virus protein were kept. After concentrating the volume to 1 mL in a Millipore Centricon concentrator (Amicon, Billerica, USA), 10% glycerol was admixed to the virus solution and frozen at –80°C.

Rat burn model. All procedures adhered to all regulations related to animal use and other federal statutes. Experiments were performed to the principles in the "Guide for the Care and Use of Laboratory Animals" from German Animal Welfare Act.

Male Sprague-Dawley Rats were obtained from Charles River. Animals arrived 2 weeks prior to investigation and were maintained in a 12-h night/day rhythm in groups of 5 animals per cage under constant access to water and food. Rats were weighed (220 to 260 g per rat was presumed). Three groups of 15 animals were randomized and investigated: group 1 served as unburned skin group (positive control); group 2 received burn injuries on both flanks as described below; and group 3 served as unburned/burned negative control. Animals were clipped and depilated on the back and both flanks under intraperitoneally delivered anesthesia with 100 mg/kg ketamine (Ratiopharm, Ulm, Germany), xylazine 20 mg/kg (Bayer, Leverkusen, Germany), and were single-caged at this time. On day 2, 1 area per flank (which corresponds to 20% of the total body surface area) was marked and fitted into an specially prepared insulation mold. Rats were immersed for 20 s in 60°C hot water bath under anesthesia named above plus 0.04 mL Temgesic (buprenorphinhydrochloride, Essex Pharma, Munich, Germany) 30 min prior to operation. Burned areas were dried and disinfected using Softasept (Braun, Melsungen, Germany). Then wounds were dressed by Tegaderm (3M Healthcare, Borken, Germany) and received occlusive dressing with Peha-Haft (Hartmann, Heidenheim, Germany), fixed with 2 clips (Visistat, Weck Closure Systems, USA) on both sides. Animals were treated with postburn pain control of 0.04 mL Temgesic every 12 h. Rats were inspected daily to ensure adequate feeding and mobility and to repair dressing as needed.

Immunohistochemistry. Skin sections were stained using immunohistochemistry to detect β-galactosidase. Samples were fixed in 4% paraformaldehyde and embedded in paraffin. Immunohistochemistry was performed on 4 µm sections with a polyclonal anti–β-galactosidase antibody (DPC Biermann, Bad Nauheim, Germany) at a dilution of 1:800 and incubation for 30 min at room temperature. Slides were then incubated for 30 min with a corresponding biotinylated antibody (anti-rabbit in goat, 10 µL in 1 mL PBST and 15 µL normal horse serum), followed by 30 min of incubation with ABC *Elite* reagent (Vector, Burlingame, USA) and 2 to 15 min in DAB peroxidase solution (Vector SK 4100) and counterstained with hematoxylin. Slides were dehydrated and covered with Entellan mounting medium (Zeiss, Jena, Germany). Results were analyzed by light microscopy (Axioskop 2 plus, Zeiss). Pictures were taken with a connected AxioCam HRC (Zeiss) at a 200× magnification.

In vitro transfection. Overnight, the cells were brought to confluence in 12-well plates. The corresponding seeding density of cells was about 90,000 cells/cm^2 for fibroblasts, 130,000/well for keratinocytes, 150,000 cells/well for HaCaT-cells, and 175,000/well for HEK-293-cells. The adenovirus was diluted in a medium with 2% FBS and cells were transfected with MOIs of 0.1, 1, 10, and 100. The plates were incubated in a humidified atmosphere at 37°C and 5% CO_2 and gently shaken every 30 min during 4 h to guarantee the homogeneous distribution of the virus in the culture. Then the virus-containing medium was replaced by fresh medium, the plates were further incubated and medium was changed every second day.

In vivo transfection. At 48 h postburn, a square of 1.5 by 1.5 cm per area was transfected with 2×10^8 infectious units (IU) Ad5-CMV-LacZ solved in 300 µL PBS (PAA Laboratories, Linz, Austria). The vector was applied by a single injection partial subcutaneously (150 µL) and partial intradermally (150 µL). After transfection, the wound was dressed as mentioned above. Animals were euthanized 2, 7, and 14 days after administration. Skin was excised, and samples for quantitative analysis of β-galactosidase activity were weighed and immediately stored in liquid nitrogen. Tissue was homogenized in lysis buffer (23 mM KH_2PO_4, 0.2% Triton X-100, 1 mM dithiothreitol, pH 7.8), using a rotor-stator

homogenizer (Polytron PT3100, Kinematika, Luzern, Switzerland).

Quantification of the Reporter-Gene Expression and Measuring of Cytotoxicity

The transfection efficacy was determined at 12, 24, and 48 h and 3, 10, 20, and 30 days after transfection for in vitro experiments. The expression of eGFP was quantified in a microplate fluorescence reader (Bio-Teck, Flx-800, Winooski, USA) and, after trypsination of cell cultures, by a FACS cell counting system (Becton Dickinson, Heidelberg, Germany). The cytotoxicity was determined by trypan blue method. Total protein amount of homogenated and lysed skin samples were measured using a BCA Protein Assay Reagent Kit (Pierce, Rockford, USA) in a microplate reader (Bio-Teck, ELx-808) at 562 nm. β-Galactosidase activity was determined in a luminometer using Galacto-Light-Plus (Tropix, Lincoln, USA) with a microplate luminometer (Berthold Detection Systems, Orion, Pforzheim, Germany). All samples were measured in triplicate. Luminometric results have been adjusted to BCA-analysis by determining relative light units per mg total protein as well as pg β-galactosidase per mg total protein.

Statistical Analysis

Data was analyzed using analysis of variance (ANOVA) and *t* test (StatView, Abacus Concepts/SAS Institute, Cary, USA). Results are presented as means ± standard error of the mean. Outcomes were considered significant with a value of $P < 0.05$. Statistical analysis was performed using SPSS 1.0 (SPSS, Chicago, USA). Results were considered to be significant at $P < 0.05$.

RESULTS

Quantification of Gene Expression by FACS Cell Counting System

Measured by FACS, the transduction rate was given as a percentage of the transfected cells of the total cell count.

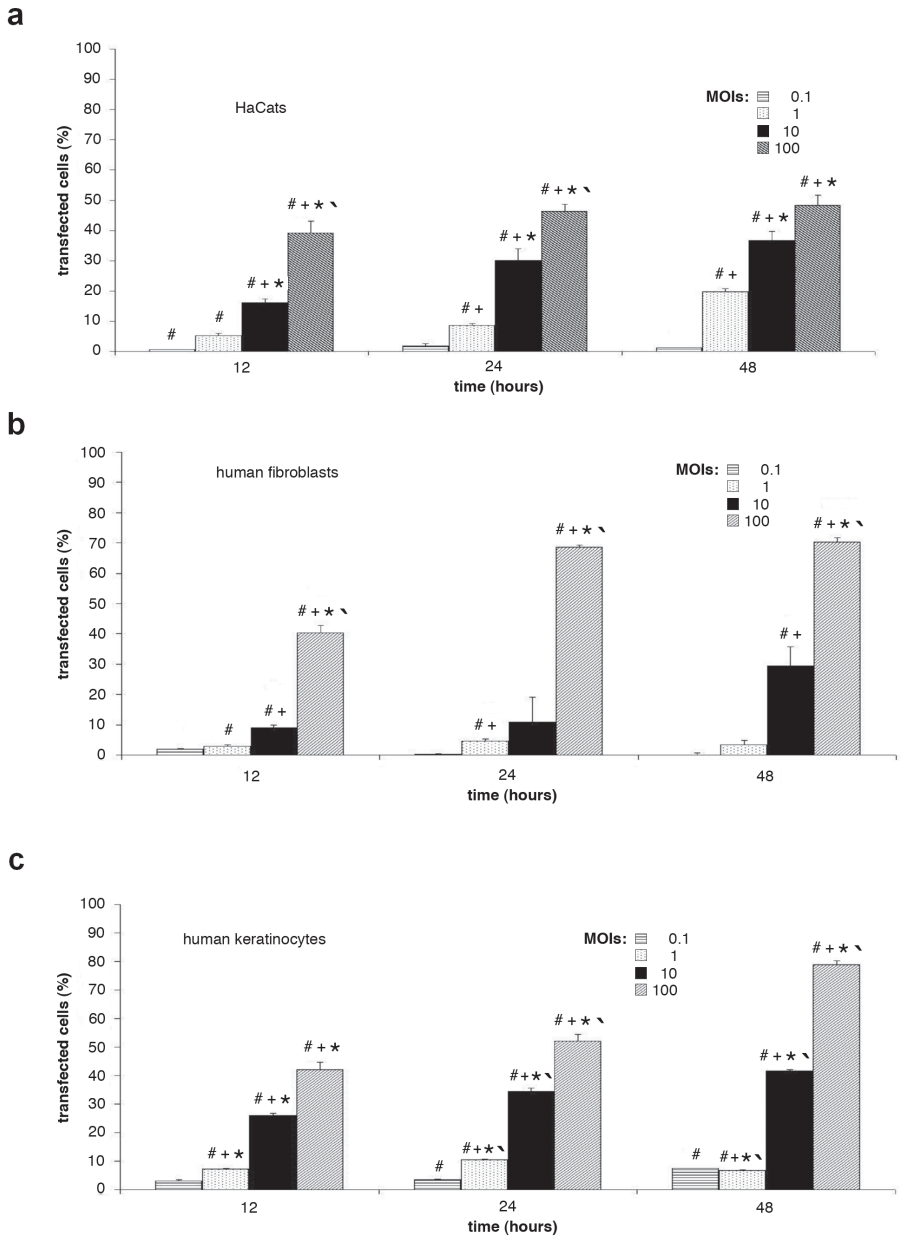

Figure 1. Transgene expression at 12, 24, and 48 h and MOIs of 0.1 to 100 in HaCaT cells (A), human fibroblasts (B), and human keratinocytes (C), measured by FACS. Significance (P = 0.05): #, MOI compared with negative control; +, MOI compared with 0.1; *, MOI compared with 1; `, MOI compared with 10.

For HFB, HKC, and HaCaT-cells, the increasing MOI resulted in an increase in the EGFP expression (Figure 1). The maximal transfection rate at an MOI of 100 after 48 h was 79% ± 1.55 for HKC, 70% ± 1.59 for HFB, and 48% ± 3.42 for HaCaT cells. Thus the transfection efficacies of both the primary cell types were comparable, whereas the transfection efficacy of the immortalized cell line was significantly ($P = 0.05$) lower.

Quantification of Gene Expression by Fluorimeter

The quantitative determination of gene expression by fluorimeter was

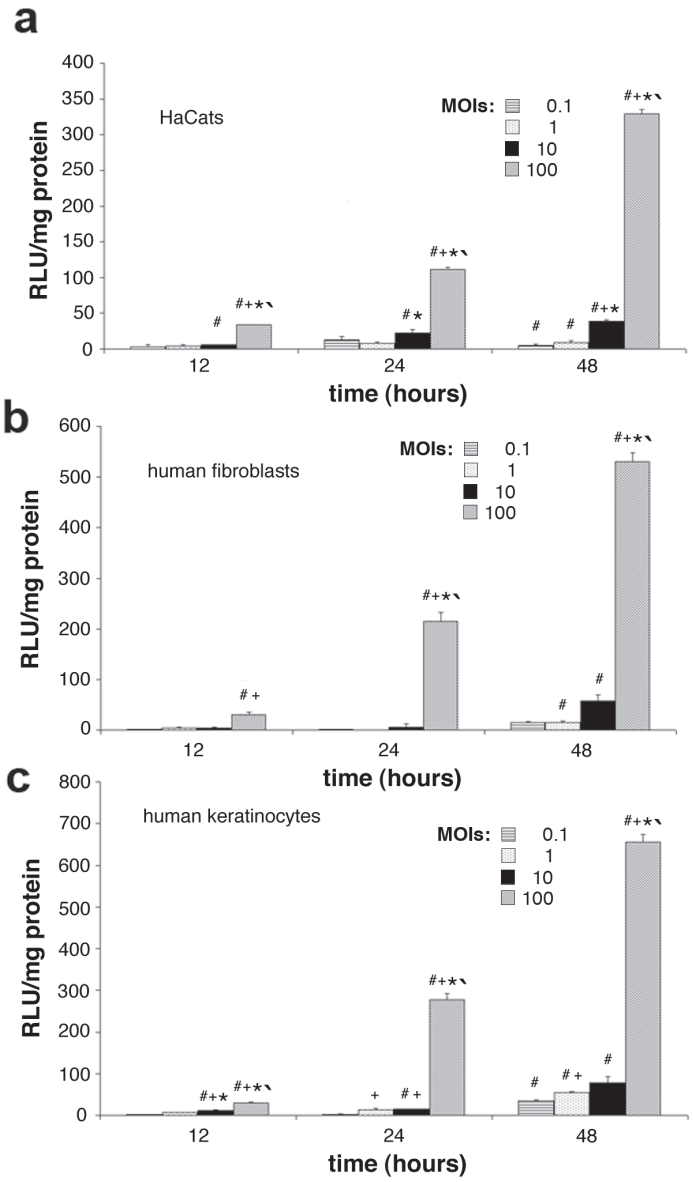

Figure 2. EGFP expression at 12, 24, and 48 h and MOIs of 0.1 to 100 in HaCaT cells (A), human fibroblasts (B), and human keratinocytes (C), measured by fluorimeter. Significance (P = 0.05): #, MOI compared with negative control; +, MOI compared with 0.1; *, MOI compared with 1; `, MOI compared with 10.

given as relative light units (35) per mg total protein. As in the measurements by FACS, the expression rates increased according to raising MOIs (Figure 2). The maximal rates at an MOI of 100 after 48 h was 655 ± 19.54 RLU/mg for HFB, 530 ± 17.83 RLU/mg for HKC, and 330 ± 5.25 for HaCaT cells. Comparable to the results received by the FACS cell counting system, the transfection rates of the primary cell types were again in the same range, whereas that of the HaCaT cell line was significantly lower. The transfection of HEK-293 cells served as positive control and increased to a maximum of 4052 ± 849 RLU/mg, followed by a rapid decrease to 27 ± 880 RLU/ mg after 48 h.

Duration of In Vitro Transgene Expression

Long-term measurements after 3, 10, 20, and 30 days were performed with HaCaT cells and HFB cells at an MOI of 100. Determined by FACS cell counting system, results demonstrated an increase in expression with a peak for the HaCaT cell line of 76% ± 1.093% at day 10, followed by a decrease up to the day 30 (Figure 3a). These results were confirmed by measurements in the fluorimeter, also showing a peak of eGFP expression on the day 10 of 1577 ± 236 RLU/mg (Figure 3b) and a subsequent decrease up to day 30. For HFB a peak of 79% ± 0.88% was observable at day 10, as measured by FACS, and of 2700 ± 162 RLU/mg in the measurements by fluorimeter at day 20 (Figure 3).

Cytotoxicity of Adenoviral Vector

Quantification of the viral cytotoxicity was determined using the trypan blue method. Figure 4 shows dead cells as a percentage of the absolute cell count, as well as the results of the untransfected negative control. For HaCaT cells, no significant difference between transfected cells and negative control was visible up to day 30 (Figure 4a). However, significantly ($P = 0.05$) more cytopathic effects were detected for primary cells at days 10, 20, and 30 (Figure 4b). HEK-293 cells served as positive control, showing a continuous increase in cytopathic effects, which was significantly different from negative control as of the 24-h mark (data not shown).

Rat Burn Model

No macroscopic differences between transfected and untransfected areas could be detected. No signs of inflammation and bacterial contamination occurred.

Immunohistochemistry. Immunohistochemical analysis showed β-galactosidase–expressing cells in the unburned area mainly within the epidermis and the hair bulbs. Expressing cells are detectable in the basal layer of the epidermis (Figure 5A) as well as

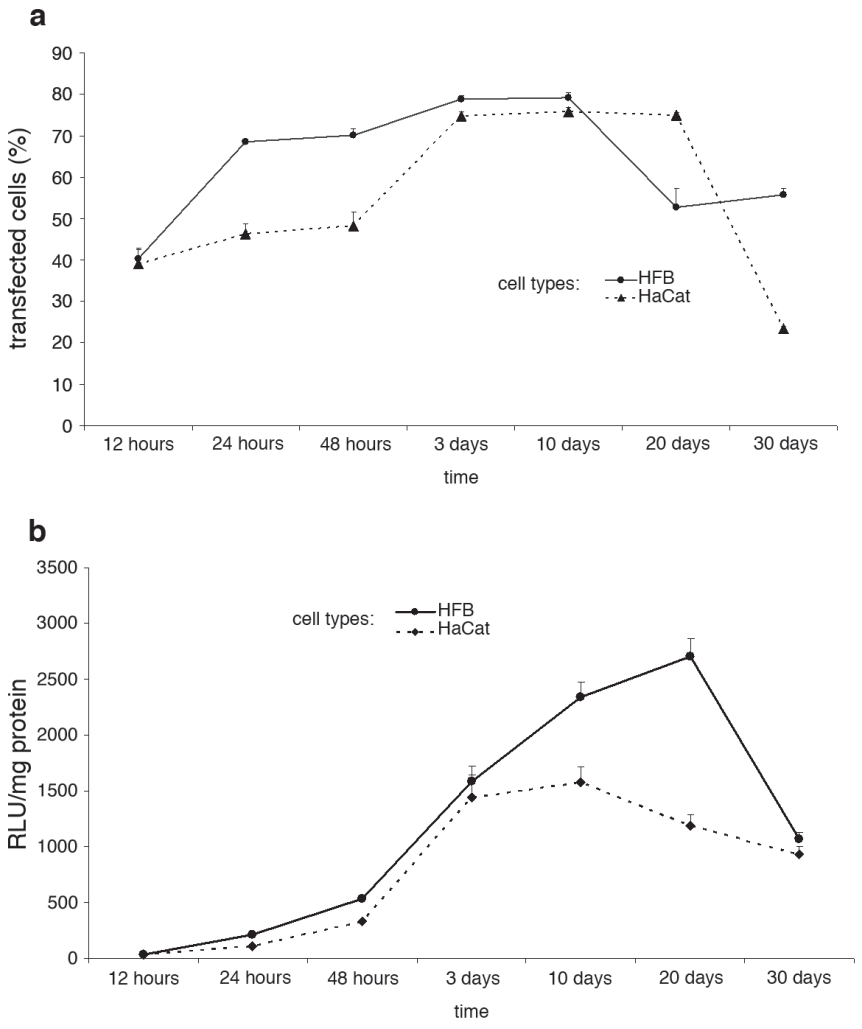

Figure 3. EGFP expression at 12, 24, and 48 h and 3, 10, 20, and 30 days and MOI 100 in HFB and HaCaT cells, measured by FACS (A) and fluorimeter (B).

in the dermis and deeper skin appendages and are focused on dermal fibroblasts, keratinocytes, and hair follicles (Figure 5B). In the burned sections, transgene expression is localized more diffusely and only few expressing cells are detectable in the superficial epidermis, which is visibly affected by the trauma. However, high expressing cells were detected in the dermal reticular layer where expressing fibroblasts are reorganized in typical islands to rebuild a functional epidermis (Figure 5C and D).

In vivo gene transfer in burned and unburned rat skin. Unburned and burned areas were transfected with 2×10^8 IU Ad5 CMV LacZ and were followed for 48 h and 7 and 14 days. The results after 48 h determined that an efficient expression was established in both groups: Quantitative analysis showed 482 pg β-galactosidase/mg of total protein for unburned and 2563 pg β-galactosidase/mg of total protein amount for burned areas. Thus, the expression in the burned group was 5 times higher than in the unburned group ($P = 0.018$) and both groups differ significantly from negative control (Figure 6). After 7 days, a similar ratio between the groups was detected. Burned skin showed again higher β-galactosidase protein content compared with the unburned skin, and both groups were significantly higher compared with negative control (Figure 6). According to the transient effect of adenoviral gene transfer, the β-galactosidase activity decreased in both groups, unburned as well as burned, from 48 h to 7 days in its expression. Fourteen days after gene transfer, no significant expression could be detected (Figure 6).

DISCUSSION

In this study we demonstrate that adenoviral gene delivery is highly efficient in primary human epithelial cells and particularly in burn wounds. However, significant differences between primary cells and the HaCaT cell line have been observed. This indicates that transfection efficacy after adenoviral gene delivery is cell dependent. Previous studies confirmed these differences in adenoviral cell tropism, which is consistent with results in the literature (36,37). Other authors describe a generally decreased transfection ratio of cell lines, when compared with primary cells (33,38).

Measured by FACS, the transfection efficacy was dose dependent but did not display any logarithmic progression. Thus, the expression of eGFP did not increase in proportion with the added amount of virus. This corresponds to the results of the study of Gilbert et al. (35), who measured the eGFP production in HEK-293 cells that were previously transduced with an adenoviral construct. They found a direct correlation between the measured fluorescence and the concentration of eGFP, but no correlation between eGFP concentration and the amount of added virus. Thus, the fluorescence was not related to the employed viral titer, but only to the amount of produced reporter-gene. In this context, our results would mean that the lack of logarithmic progression of transduction ratio was not based on a systemic loss of infectious units, but on a limitation of synthetic capacities of transduced cells. This may be caused by a saturation of the cells' capacities to produce proteins, the precise mechanisms of which, how-

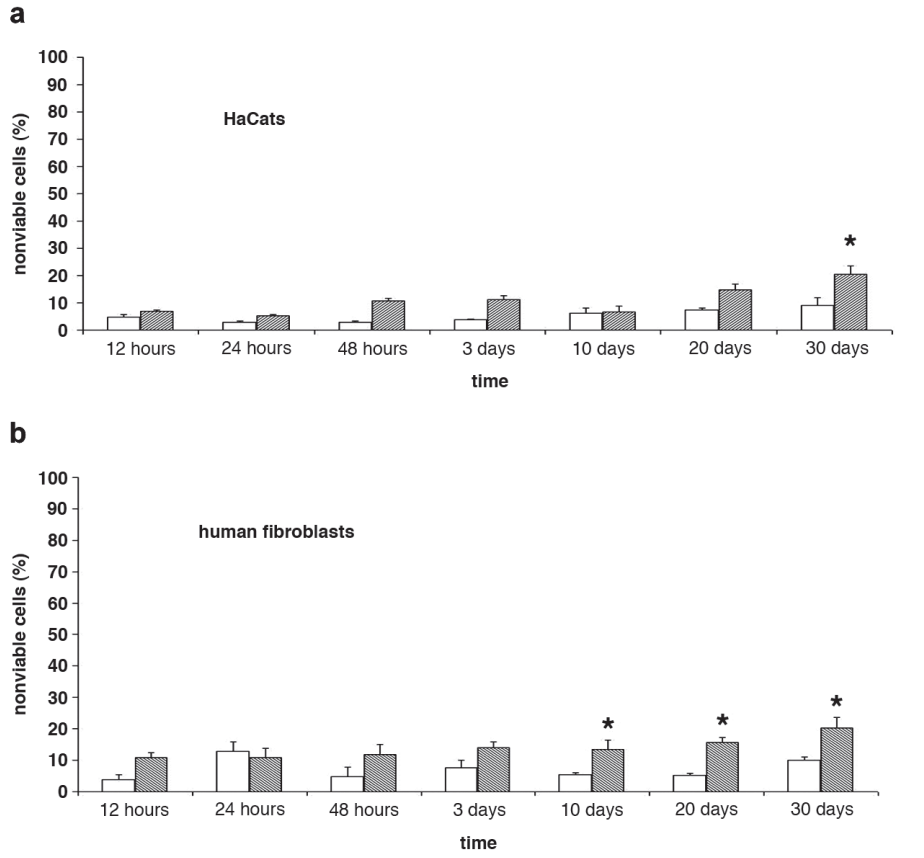

Figure 4. Cytotoxicity at 12, 24, and 48 h and 3, 10, 20, and 30 days of MOI 100 and negative control in HaCaT cells (A) and human fibroblasts (B). Significant differences of cytopathic effects after 30 days, marked with * ($P < 0.05$).

ever, are certainly not yet thoroughly understood.

Fluorimeter analysis showed a considerable increase of expression between the MOIs of 10 and 100. However, this result was inconsistent with the continuous increase of expression measured by FACS analysis. This observation may be explained by the results of Wahlfors et al. (39), who describe that the detection sensitivity of eGFP by fluorimeter analysis is dependent on a threshold of 10^5 eGFP molecules within the sample. The sensitivity of measuring eGFP by fluorimeter is, thus, limited, and the restricted sensitivity of the fluorimeter may explain the "gap" of expression between the MOIs of 10 and 100. These results highlight the importance of applying several analytic methods by quantifying transgene eGFP expression in cells and tissue samples.

In vivo, burned skin areas as well as unburned skin showed high expressions over at least 7 days. The β-galactosidase expression is clearly detectable at both time points (48 h and 7 days) by quantitative analysis and immunohistochemistry. We showed that the transfection efficacy in burned wounds was higher than in the unburned areas. Strieter et al. (40) found higher expressions in inflamed compared with uninflamed tissue. Furthermore, our results are in accordance with previous studies in burned wounds using the gene gun technique in the same animal model. In this study, the authors showed higher expression rates in burned skin compared with unburned skin tissue but only achieved lesser transfection results which is presumably due to the inferior transfer amount of the gene gun technique compared with adenoviral gene transfer (13). Presumably, higher expression is caused by increased vascular permeability, inflammatory cell diapedesis, extravasation, and higher metabolic activity in the harmed tissue (41). Denham et al. (42) also showed increased transgene expression of liposome:plasmid constructs in a model of acute experimental pancreatitis in the injured pancreas.

Our results showed a low cytotoxic potential of the adenovirus in vitro. Significant cytopathic effects in comparison to negative control occurred for HaCaT cells at day 30. HEK-293 cells served as positive control and showed increasing cytotoxicity over total time course. These results are consistent with the overall opinion in the literature that describes adenoviruses as vectors with a high safety profile. Crystal et al. (43) presented a detailed analysis of the safety profile and toxicity of adenoviruses. Further, adenoviral constructs have been widely used in clinical trails and have been shown to have only low oncogenic potential and rarely provoke serious clinical symptoms (16). In a period of 10 years of research, involving more than 3500 patients, only a few undesirable effects were shown and only when unusually high dosages were used (2,44). The death of a patient in 1999, following the systemic application of an ornythintranscarbamylase encoding adenovirus, was caused by the highest dose of adenovirus that has been applied so far (3.6×10^{13} virus particles), such that, in the context of the literature and our findings, the cytotoxic potential of adenoviral vectors in the topic application can be described as being low.

In our study, transgene expression of eGFP was detected in vitro over a time course of 30 days. This correlates to studies where the medium gene expression of reporter genes in epithelial cells was 2 to 4 weeks (45). The decline of transgene expression toward day 30 has been explained in various studies: Genes transmitted by adenoviral constructs remain episomally in the nucleus of the host cells, thus producing only a transient expres-

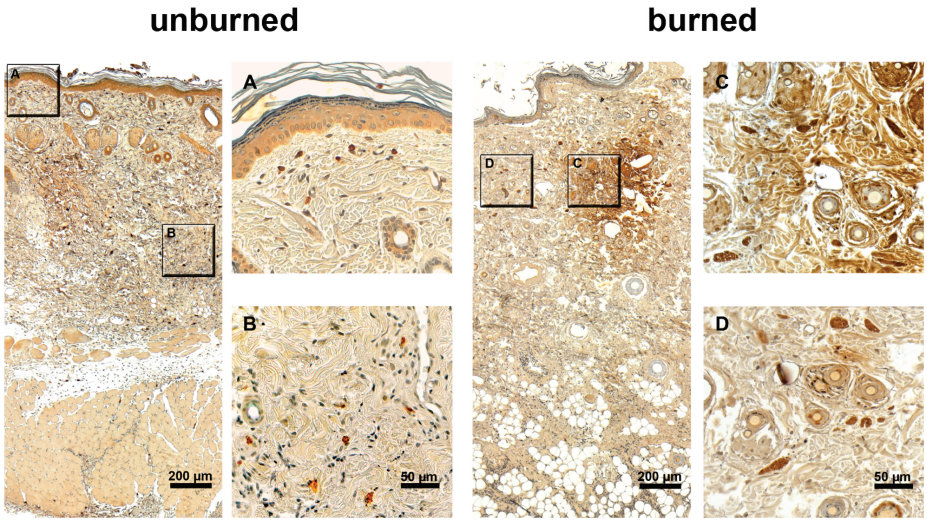

Figure 5. Localization of transgene expression in burned and unburned skin. Viral particles were introduced intradermally and subcutaneously into rat skin; 48 h after gene transfer, unburned and burned tissue samples were harvested and prepared for imunohistochemistry. β-Galactosidase was detected and visualized using a polyclonal rabbit antibody. A and B show higher magnifications of the corresponding area in unburned skin. C and D show burned tissue specimen.

sion (46). In contrast to that, for example, retroviral-mediated transgenes are incorporated into the chromosomes of the host cell and cause long-term expression (36). Although adenoviral vectors only cause transient gene transfer, our results showed high but decreasing gene expression for at least 1 week. This transient effect might be important and of advantage to treat wounds after a single application. Therefore, a transient gene expression is by all means eligible to avoid persistent gene expression after the wound healing process is completed.

To conclude, the results of this study demonstrated an efficient time- and dose-dependent adenoviral transfection in vitro and in burn wounds. As described in the literature, the virus was produced easily in high titers and showed few cytotoxic effects. These results, as well as the ability of adenoviruses to transduce a wide range of proliferating and postmitotic cells and the comparably huge insertion capacity, make adenoviral constructs suitable vectors for inducing transient expression of transgenes.

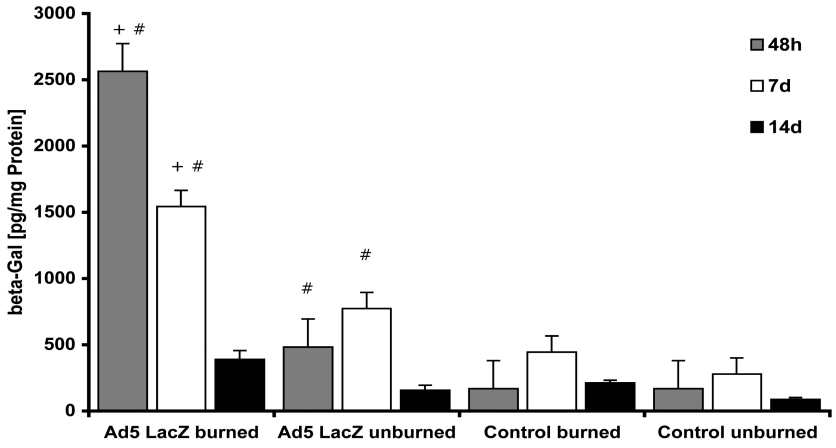

Figure 6. Transgene expression 48 h and 7 and 14 days posttransfection in the rat burn model. Significance (P = 0.05): +, burned versus unburned group; #, transfected versus control group.

It becomes evident that this technique is feasible for further therapeutic approaches to introduce therapeutic genes in burn wounds. This could become a path-breaking perspective concerning the evident and increasing obstacles in the therapy of burn victims in intensive care and therapy. Further in vivo studies concerning immune response and clinical effect of delivered therapeutic genes have to be conducted to investigate the feasibility of therapeutic genes in a burn wound environment to get further insights in this high-potential technique of gene therapy in burned patients.

ACKNOWLEDGMENTS

We sincerely thank Janine Mertens-Rill and Andrea Gerhardts for expert technical assistance. This work was supported by the Deutsche Forschungsgemeinschaft (DFG; Ste1099).

REFERENCES

1. Meng X et al. (2002) Keratinocyte gene therapy: cytokine gene expression in local keratinocytes and in circulation by introducing cytokine genes into skin. *Exp. Dermatol.* 11:456-61.
2. Slama J et al. (1997) Gene transfer. *Ann. Plast. Surg.* 35:429-39.
3. Trainer AH, Alexander MY. (1997) Gene delivery to the epidermis. *Hum. Mol. Genet.* 6:1761-7.
4. Christensen R, Jensen UB, Jensen TG. (2001) Cutaneous gene therapy-an update. *Histochem. Cell. Biol.* 115:73-82.
5. Braun-Falco M, Hallek M. (1998) Cutaneous gene therapy-perspectives of gene transfer in keratinocytes. *Hautarzt* 49:536-44.
6. Siprashvili Z, Khavari PA. (2003) Lentivectors for regulated and reversible cutaneous gene therapy. *Mol. Ther.* 9:93-100.
7. Petersen MJ et al. (1995) Sustained production of human transferrin by transduced fibroblasts implanted into athymic mice: a model for somatic gene therapy. *J. Invest. Dermatol.* 104:171-6.
8. Fenjves E et al. (1989) Systemic distribution of apolipoprotein E secreted by grafts of epidermal keratinocytes: implications for epidermal function and gene therapy. *Proc. Natl. Acad. Sci. U. S. A.* 86:8803-8807.
9. Gerrard AJ, Hudson DL, Watt FM. (1993) Toward gene therapy for hemophilia B using primary human keratinocytes. *Nat. Genet.* 3:180-3.

10. Qielliet X, Chevallier-Lagente O, Zeng L. (1997) Retroviral-mediated correction of DNA repair defect in xeroderma pigmentosum cells is associated with recovery of catalase activity. *Mutat. Res.* 385:235-242.
11. Dellambra E et al. (1998) Corrective transduction of human epidermal stem cells in laminin-5-dependent junctional epidermolysis bullosa. *Hum. Gene Ther.* 9:1359-70.
12. Freiberg R et al. (1997) A model for corrective gene transfer in X- linked ichthyosis. *Hum. Mol. Genet.* 6:927-933.
13. Steinstraesser L et al. (2001) Feasibility of biolistic gene therapy in burns. *Shock* 15:272-7.
14. Yao F, Eriksson E. **(2000)**Gene therapy in wound repair and regeneration. *Wound Repair Regen.* 8:443-51.
15. Eming SA, Morgan JR, Berger A. (1997) Gene therapy for tissue repair: approaches and prospects. *Br. J. Plast. Surg.* 50:491-500.
16. Crombleholme TM. (2000) Adenoviral-mediated gene transfer in wound healing. *Wound Repair Regen.* 8:460-72.
17. Hansbrough JF, Franco ES. (1998) Skin replacements. *Clin. Plast. Surg.* 25:407-23.
18. Lindberg RB et al. (1965) The successful control of burn wound sepsis. *J. Trauma* 5:601-16.
19. Gorecki P et al. (1999) Antibiotic administration in patients undergoing common surgical procedures in a community teaching hospital: the chaos continues. *World J. Surg.* 23:429-32; discussion 433.
20. Estahbanati HK, Kashani PP, Ghanaatpisheh F. (2002) Frequency of Pseudomonas aeruginosa serotypes in burn wound infections and their resistance to antibiotics. *Burns* 28:340-8.
21. Jacobsen F et al. (2005) Activity of histone H1.2 in infected burn wounds. *J. Antimicrob. Chemother.* 55:735-41.
22. Bacchetta CA et al. (1975) Biology of infections of split thickness skin grafts. *Am. J. Surg.* 130:63-7.
23. Steinstraesser L et al. (2002) Activity of novispirin G10 against Pseudomonas aeruginosa in vitro and in infected burns. *J. Antimicrob. Agents Chemother.* 46:1837-44.
24. Jacobsen F et al. (2005) Transient cutaneous adenoviral gene therapy with human host defense peptide hCAP-18/LL-37 is effective for the treatment of burn wound infections. *Gene Ther.* 12: 1494-502.
25. Spirito F et al. (2001) Cutaneous gene transfer and therapy: the present and the future. *J. Gene Med.* 3:21-31.
26. Nadeau I, Kamen A. (2003) Production of adenovirus vector for gene therapy. *Biotechnol. Adv.* 20:475-89.
27. Monto AS, Arden NH. (1992) Implications of viral resistance to amantadine in control of influenza A. *Clin. Infect. Dis.* 15:362-9.
28. John R, Burnett RM. (2004) Adenovirus structure. *Hum. Gene Ther.* 15:1167-1176.
29. Gerard R, Meidell S. (1995) Adenovirus Vectors. *DNA Cloning: A Practical Approach*, BD Hames, Oxford University Press, Oxford, p. 285-307.
30. Philipson L, Pettersson RF. (2004) The coxsackie-adenovirus receptor – a new receptor in the immunoglobulin family involved in cell adhesion. *Curr. Top. Microbiol. Immunol.* 273:87-111.
31. Ali M, Lemoine NR, Ring CJ. (1994) The use of DNA viruses as vectors for gene therapy. *Gene Ther.* 1:367-84.
32. Setoguchi Y et al. (1994) Ex vivo and in vivo gene transfer to the skin using replication-deficient recombinant adenovirus vectors. *J. Invest. Dermatol.* 102:415-21.
33. Doebis C et al. (2002) Efficient in vitro transduction of epithelial cells and keratinocytes with improved adenoviral gene transfer for the application in skin tissue engineering. *Transpl. Immunol.* 9:323-9.
34. Huber M et al. (2000) Efficient in vitro transfection of human keratinocytes with an adenovirus-enhanced receptor-mediated system. *J. Invest. Dermatol.* 114:661-6.
35. Gilbert PA et al. (2000) On line measurement of green fluorescent protein (GFP) fluorescence for the monitoring of recombinant adenovirus production. *Biotechnol. Lett.* 22:561-567.
36. Chen M et al. (2003) An efficient gene transduction system for studying gene function in primary human dermal fibroblasts and epidermal keratinocytes. *Clin. Exp. Dermatol.* 28:193-199.
37. Ghazizadeh S, Doumeng C, Taichman LB. (2002) Durable and stratum-specific gene expression in epidermis. *Gene Ther.* 9:1278-85.
38. Christensen R, Jensen UB, Jensen TG. (2001) Cutaneous gene therapy: an update. *Histochem. Cell Biol.* 115:73-82.
39. Wahlfors J et al. (2001) Green fluorescent protein (GFP) fusion constructs in gene therapy research. *Histochem. Cell Biol.* 115:59-65.
40. Strieter RM et al. (1990) Host responses in mediating sepsis and adult respiratory distress syndrome. *Semin. Respir. Infect.* 5:233-47.
41. Moldawer LL et al. (1999) Application of gene therapy to acute inflammatory diseases. *Shock* 12:83-101.
42. Denham W et al. (1998) Cationic liposome-mediated gene transfer during acute pancreatitis: tissue specificity, duration, and effects of acute inflammation. *J. Gastrointest. Surg.* 2:95-101.
43. Crystal RG et al. (2002) Analysis of risk factors for local delivery of low- and intermediate-dose adenovirus gene transfer vectors to individuals with a spectrum of comorbid conditions. *Hum. Gene Ther.* 13:65-100.
44. Crystal RG. (1995) Transfer of genes to humans: early lessons and obstacles to success. *Science* 270:404-10.
45. Lotze MT, Kost TA. (2002) Viruses as gene delivery vectors: application to gene function, target validation, and assay development. *Cancer Gene Ther.* 9:692-9.
46. Hermens WT, Verhaagen J. (1998) Viral vectors, tools for gene transfer in the nervous system. *Prog. Neurobiol.* 55:399-432.