Gene Therapy for Pyoderma Gangrenosum: Optimal Transfection Conditions and Effect of Drugs on Gene Delivery in the HaCaT Cell Line Using Cationic Liposomes

Alexandra R. Teagle a, James C. Birchall b, Rachel Hargest a

a Department of Surgery, School of Medicine, and b School of Pharmacy and Pharmaceutical Sciences, Cardiff University, Cardiff, UK

Key Words
Pyoderma gangrenosum · Gene therapy · Liposome · Lipofectamine ®

Abstract
Background/Aims: Pyoderma gangrenosum (PG) is a rare ulcerative skin disease, currently treated empirically with immunosuppression. PG is a good target for gene therapy since the skin is easily accessible. This study used the FDA-approved vector Lipofectamine ® 2000 to investigate in vitro transfection of skin keratinocytes. The aim was to determine an optimum transfection protocol, including the effect of drugs currently used to treat PG on the efficiency of gene transfer, since gene therapy is unlikely to be used as monotherapy. Methods: Cells of the HaCaT line were transfected with the lacZ reporter gene, and transgene expression was measured after a given time period. Conditions tested were: relative concentrations of DNA and Lipofectamine ®, time from transfection to measurement of expression, pH, and exposure to clinically relevant drugs (hydrocortisone, methotrexate, infliximab). Results: The greatest levels of β-galactosidase expression were observed using a DNA:Lipofectamine ® ratio of 1:5 (μg/μl) on day 3 after transfection, using culture medium at pH 7, and in the presence of hydrocortisone. Transfection efficiency was reduced by the presence of methotrexate and not significantly affected by infliximab. Conclusion: Gene therapy is a potential future strategy for the management of PG; this study is a step towards the development of a topical gene-based agent.

Introduction
Pyoderma gangrenosum (PG) is a rare neutrophilic dermatosis, characterised by painful cutaneous ulcers [1], with an annual incidence of 3–10 per million [1]. Although relatively uncommon, the ulcers of PG are exquisitely painful, and, like many skin disorders, the disease has a significant negative impact on quality of life. Patients with PG are often systemically unwell, and extracutaneous manifestations have been described [1]. Around 50% of cases are associated with a systemic disease, most commonly inflammatory bowel disease, rheumatoid arthritis, and haematological malignancy [2]. The pathogenesis is not known, but inflammatory and immunological processes are thought to be the underlying mechanisms [3]. Current treatment is mostly empirical, with corticosteroids and other immunosuppressants as first...
line [4, 5]. Several therapies may need to be trialled to achieve remission of the disease, all of which have potentially severe toxicities. The development of novel therapeutics is therefore an interesting and important area for study, and gene therapy is a promising therapeutic strategy. Although gene therapy has not turned out to be the game-changing new treatment for many intractable conditions, it may still prove to be useful in combination with other modalities of treatment.

The accessibility of the skin makes diseases such as PG an attractive target for gene therapy. Liposomes are an ideal vector in this setting. Liposomes are spherical vesicles characterised by lipid bilayers and are formed upon hydration of a dry lipid film. Liposomes resemble small cellular organelles [6] and have high potential as low-toxicity drug carriers. Successful transfection in vitro with liposomes was first described in 1987 [7], and their use in gene transfer has been investigated in vitro in a wide range of cell lines [8–13], as well as in vivo [14]. Being non-viral vectors, liposomes have a superior safety profile and acceptability compared to their viral counterparts [15, 16], as well as the ability to carry larger nucleic acid sequences [16, 17].

Whilst many groups have investigated lipofection as a method of gene transfer in vitro, there is no optimised transfection protocol for its use in epidermal cell lines. Furthermore, in relation to skin disease, and specifically PG, no studies have investigated the effect of the drugs used currently in clinical management on the efficiency of gene transfer. This is particularly noteworthy, given that any future gene therapy strategy would likely be used alongside at least one of these drugs.

This study therefore aimed to determine the optimum conditions for in vitro transfection of the HaCaT (keratinocyte) cell line using the liposomal vector Lipofectamine® 2000 with a lacZ reporter gene, and, subsequently, to determine the effect on transfection of the presence, during cell culture and transfection, of drugs commonly used in the treatment of PG, i.e. hydrocortisone, infliximab and methotrexate.

Materials and Methods

Cell Lines and Culture Conditions
All media and supplements were obtained from: PAA Laboratories Ltd., Yeovil, UK; Invitrogen Life Technologies, Paisley, UK; Sigma-Aldrich Co. Ltd., Gillingham, UK. HaCaT cells (derived from transformed human epithelium) [18] were grown in Dulbecco’s minimum essential medium supplemented with 10% foetal bovine serum and 1% penicillin-streptomycin solution. Cells were grown in a humidified incubator at 37°C, with a 5% carbon dioxide atmosphere. For experiments investigating the presence of drugs, hydrocortisone, methotrexate or infliximab was added to the culture medium, diluted to the relevant concentration, as previously described [19–21].

Plasmid DNA Preparation
The β-galactosidase (β-gal) expression plasmid pCMVβ®, containing the Escherichia coli LacZ gene under the control of the cytomegalovirus immediate-early promoter, was used in transfection of HaCaT cells. A QIAGEN plasmid mini kit was used in plasmid preparation, as per the manufacturer’s instructions (QIAGEN Ltd., Crawley, UK). The concentration and purity of DNA were measured using UV-vis spectroscopy at 260 and 280 nm. Plasmid was stored at −70°C before use in transfection studies.

Experimental Conditions
Previous studies have shown that variation in DNA:liposome ratio, exposure time, and pH of the medium can affect transfection efficiency [22]. However, there is no general universal optimum set of conditions for all cell types and transfectants, and therefore conditions must be optimised for each specific combination.

DNA: Lipofectamine® Ratio. Cells were transfected using plasmid DNA and Lipofectamine® at varying relative concentrations. The Lipofectamine® variant used was Lipofectamine® 2000. The DNA concentration in each well was maintained at 1 μg in 25 μl OptiMEM reduced-serum medium. The volume of Lipofectamine® was varied from 1 to 10 μl and made up to a total of 25 μl with OptiMEM. This produced DNA:Lipofectamine® ratios (μg/μl) of 1:1, 1:2, 1:5, and 1:10. Whilst it would be preferable to express these ratios as weight, molar or charge equivalents, the manufacturer of Lipofectamine® does not provide the concentration of their transfection reagent as it is deemed proprietary information. The manufacturer’s protocol however recommends DNA: Lipofectamine® ratios of 1:2 to 1:5, and so our ranges of ratios were selected to include these recommended ratios and explore the use of Lipofectamine® at lower (1:1 μg/μl) and higher (10:1 μg/μl) relative amounts.

Incubation Time of Transfected HaCaT Cells. HaCaT cells were transfected using plasmid DNA and Lipofectamine® in a ratio of 1:5 β-gal expression was measured on day 0 (4 h after transfection), and days 1, 3, 5, 7, 9, and 11 after transfection.

pH of Culture Medium. The physicochemical properties of the solution in which DNA-liposome complexes form and in which they interact with cells affect transfection. Furthermore, skin pH may vary, particularly in the context of use of additional creams and emollients. 1 M NaOH or 5 M HCl was added to the OptiMEM reduced-serum medium used in transfection to achieve solutions of pH 5, 6, 7, 8, and 9. pH was measured using a pH probe. HaCaT cells were transfected using the relevant medium and a DNA:Lipofectamine® ratio (μg/μl) of 1:5, and gene expression was measured 24 h after transfection.

Presence of Drugs. HaCaT cells were grown before and after transfection in culture medium containing hydrocortisone, infliximab, or methotrexate, at the relevant concentrations. The DNA:Lipofectamine® ratio used was 1:5. Mean transgene expression was calculated for each drug treatment on days 0 (4 h), 1, 3, 5, 7, 9, and 11 after transfection.
fixative solution for 10 min at room temperature. Cells were then rinsed twice with PBS, before the addition of 150 μl staining solution to each well, ensuring even coverage of the cells. Cells were incubated covered by the staining solution for 2 h at 37°C in 5% CO₂.

Quantification of Transgene Expression
Cells were viewed at a magnification of ×200. Random fields of view were photographed using an Olympus Moticam™ 2300 attached to the microscope. 10 fields of view per well were photographed in tests of DNA:Lipofectamine® ratio (3 wells for each ratio tested; n = 30) and 1 per well in all other tests (24 wells for each value tested; n = 24). From each photograph, the number of stained cells (expressing β-gal) were counted and expressed as a percentage of the total cells; these values were used to calculate the average percentage transfection for each data set.

Statistical Analysis
Data are expressed as the percentage of cells stained positive for gene expression. Statistical analysis was performed using one- and two-way ANOVAs to compare expression between data sets and experiments. Tukey’s multiple comparison test and Bonferroni’s post tests were done to determine significance, and the null hypothesis was rejected at p < 0.05.

Results

Optimisation of DNA:Lipofectamine® Ratio
Knowledge of the optimal ratio of plasmid DNA to liposome is necessary prior to the development of any gene therapy-based product for the skin. Variation in the DNA:Lipofectamine® ratio had a significant effect on transfection efficiency, and was dose dependent (fig. 1). The ratio of DNA:Lipofectamine®, producing the greatest percentage transfection was 1:5. This led to a mean transfection of 2.56% of cells (p < 0.001 vs. control). Ratios of 1:2 and 1:10 produced much lower percentage expression (0.86 and 0.85%, respectively) but were still significant with respect to control (p < 0.01). The percentage of cells expressing the gene product following incubation with the 1:5 ratio was significantly higher compared to both 1:2 and 1:10 (p < 0.001). The level of expression from a 1:1 (μg/μl) ratio was not significant. No β-gal expression was seen with any of the negative controls, indicating that treatment with DNA or Lipofectamine® alone, or with neither, is not sufficient for transfection.

Optimisation of Incubation Time for HaCaT Transfection
Determination of the time over which a cell must be exposed to a DNA-liposome complex may be particularly important in vivo, as this may be limited by natural processes, such as desquamation of the epidermis. This
will also likely have a bearing on dosing and reapplication schedules. The greatest expression was seen on day 3 after transfection (fig. 2, 3a), with 7.60% of cells transfected. This result was significant with respect to day 0 (p < 0.001), but not significant compared to days 1 or 5, on which 6.51 and 5.81% transfection was achieved, respectively. Days 9 and 11 after transfection did not show significant results (fig. 2).

**Optimisation of pH for Transfection of HaCaT Cells**

The physicochemical properties of the solution in which the anionic DNA and cationic liposomes interact with each other and with cells are some of the most important factors affecting transfection. We found that a transfection environment at pH 7 (fig. 3b) led to the greatest β-gal expression (fig. 4), with 12.43% of cells transfected. This was significant with respect to both pH 6 (p < 0.001) and pH 8 (p < 0.001), at which 5.43 and 7.10% transfection was achieved, respectively.

**Effect of Drugs on Transfection Efficiency**

The effect of three of the drugs commonly used to treat PG on the efficiency of reporter gene expression into keratinocytes was studied. Cells were grown and transfected in medium containing hydrocortisone, infliximab, methotrexate or no drug, and gene expression was measured on days 0–11, as previously described. Treatment of cells with hydrocortisone gave the greatest transfection efficiency on all days except day 7 (fig. 5). The enhanced transfection with hydrocortisone was significant compared to no drug treatment on days 1, 5 and 11 after transfection (p < 0.05, p < 0.05, p < 0.01, respectively). Similarly, percentage transfection in hydrocortisone-treated cells was significant compared to infliximab-treated cells on the same days (p < 0.001, p < 0.05, p < 0.01, respectively). Hydrocortisone caused significantly greater expression compared to methotrexate on all days where gene expression was apparent. Treatment with methotrexate produced significantly lower gene expression on days 1, 3, 5, and 7 than in all other groups, and significantly less expression on days 9 and 11 than treatment with hydrocortisone (p < 0.05, p < 0.001, respectively). Transfection achieved with infliximab was generally lower but not significantly different from that achieved with no drug treatment on any day. The highest percentage transfection was achieved on day 3 in all treatment groups (including no drug treatment) except hydrocortisone-treated cells (fig. 3c), in which the greatest β-gal expression (8.91%) was seen on day 1 after transfection (fig. 5). This was not significant compared to day 3 or 5 after transfection (p > 0.05), but was significant with respect to day 0 (p < 0.001) and day 7 onwards (p < 0.05). The highest transfection rate in cells treated with infliximab (fig. 3d) was seen on day 3 after transfection (fig. 5). This was significant with respect to day 0 (p < 0.001), but not compared to days 1 or 5 after transfection (p > 0.05). Interestingly, the level of expression increased from day 9 to day 11 after transfection in both the hydrocortisone- and infliximab-treated cells, but this difference was not statistically significant (p > 0.05) and may have been due to the chance distribution of transfected cells in the random fields of view selected. Treatment of cells with methotrexate (fig. 3e) produced the highest transfection rate on day 3 after transfection (fig. 5), which was significant compared to days 0–1 (p < 0.001) and days 7–11 (p < 0.001), but not compared to day 5 (p > 0.05). No β-gal expression was seen in methotrexate-treated cells on day 11, in contrast to cells treated with other drugs.

**Discussion**

PG is an uncommon yet debilitating skin disorder, for which there is currently no specific or targeted therapy. Existing treatments are used with varying success and expose patients to potentially severe side effects. There is therefore a need for the development of novel therapies, of which gene therapy may be promising. The skin is an
Fig. 3. Representative photographs showing β-gal expression analysis of keratinocytes measured on day 3 after transfection (no drug treatment, a), cultured and transfected at pH 7 (b), cultured and transfected in medium containing hydrocortisone on day 3 after transfection (c), infliximab on day 3 after transfection (d), and methotrexate on day 3 after transfection (e). Blue colour (in the online version only) indicates cells expressing β-gal after staining. Original magnification ×200.
accessible target for gene therapy, both for direct in vivo application of therapeutic agents, as well as ex vivo transfection and transplantation of target cells to affected areas [23, 24]. However, given its dynamic nature, involvement of multiple native and migratory cell types, and constant renewal, successful transfection of the involved cells is a challenge. Here, we have defined some of the environmental parameters for the optimal transfection of keratinocytes, the cells thought to play a pivotal role in the disease pathophysiology. Furthermore, we have determined the effect of the concurrent use of three of the drugs currently used in the treatment of PG, which will impact on the clinical application of future gene therapy strategies for this disease.

The relative concentrations of DNA and Lipofectamine® are critical to transfection efficiency. The concentration of Lipofectamine® becomes limiting when it is low relative to that of DNA, and DNA is not taken up into cells. Skin cells are one of the few targets in which naked DNA may be taken up [25], but in most reported cases the DNA was present at high concentrations and chemical enhancers were present [26]. In this instance this seems unlikely, as supported by the lack of transfection in the control system lacking Lipofectamine®. Where the concentration of Lipofectamine® is high relative to that of DNA, the transfection efficiency may be similarly reduced because the amount of DNA is limiting, or due to Lipofectamine® toxicity. Several liposomes, including Lipofectamine®, have been shown to be cytotoxic [27, 28], increasingly so at higher concentrations [27], due to liposome-induced apoptosis through caspase activation and reactive oxygen species generation [28, 29]. In addition, DNA-liposome complexes can tend to form large aggregates, particularly at higher lipid concentrations [22]. Our finding of an optimum DNA:Lipofectamine® ratio of 1:5 is supported in the literature in various cell lines [22, 30–32].

The time from introduction of transfection reagents to measurement of transfection had a significant impact on gene expression. The time required for the uptake of DNA-liposome complexes has been demonstrated to be 6 h [33], which supports our finding of a lack of transgene expression at 4 h (day 0), and the increase by 24 h (day 1) after transfection. Caplen et al. [22] found that under standard transfection conditions, transfection efficiency increased as the duration of incubation of cells with complexes increased. Previous studies have reported peak gene expression to occur within the first 48 h after trans-
fection [34], and 48–96 h (day 2–4) after transfection [35, 36]. Furthermore, a precise time period of 3 days was reported by de la Monte and Wands [37] as showing maximal gene expression. In our study, cells were transfected and incubated with the transfection reagents for 5 h, under standard conditions. Following this, the reagents were replaced with fresh, complete growth medium. A longer duration from transfection to measurement of gene expression allows more time for transcription and translation of the DNA once it has been taken into the cell, leading to greater gene expression, as shown by the increase in expression from day 0 to day 3. Following this time of peak expression, as the DNA is cleared, the measured gene expression will reduce. Due to limiting biological processes, in vivo any given cell may only be exposed to complexes transiently, and so reapplication of gene therapy products at optimal intervals would be necessary.

Amongst factors influencing transfection, the physicochemical properties of the solution, in which DNA-liposome complexes form and interact with cells, are highly important. Cationic Lipofectamine® 2000 and anionic DNA must interact electrostatically in order to form complexes, and the state of protonation in the transfection system is an important rate-limiting step [38]. Furthermore, in gaining entry to the cell, positively charged complexes interact with the relative negative charge of the plasma membrane; entry of negatively charged complexes is prevented [39]. Local pH alters the state of protonation in the transfection system and has an important bearing on transfection efficiency. The sensitivity of liposomes to pH may additionally be taken advantage of in certain situations, by designing pH-sensitive liposomes and other nanoparticles that are stable at physiological pH but become unstable at lower pH such as those found in diseased tissue, releasing the DNA or drug they are carrying [40, 41]. This study has found the optimum pH for transfection of the HaCaT cell line, under the conditions used, to be pH 7. The optimum pH for transfection has been reported by other authors to be pH 9 [22], pH 8 [38], and pH 7 [39]. Optimum pH may, however, vary according to the liposome vector and to the solution, as well as cell line factors.

If a topical gene therapy is to be developed for conditions such as PG, additional external factors such as the effect of drugs that the patient may also be using for the condition must be taken into account, as these may influence the efficiency of gene transfer. This study is the first to investigate the effect on gene transfection of three drugs which are commonly used to treat PG, and has shown that a significantly positive effect was exerted by hydrocortisone. Hydrocortisone is a glucocorticoid, one of the most powerful immunosuppressive and anti-inflammatory groups of drugs [42], and is a first-line therapy for PG [2, 5, 43]. The effect of hydrocortisone on keratinocyte survival and proliferation is disputed. Some authors report promotion of proliferation [44, 45], while others report induction of apoptosis [42]. However, because transfection was reported as a proportion of total cells, this effect would have been minimal. Regardless of an effect on cellular proliferation, hydrocortisone has been shown to increase transfection efficiency in a dose-dependent manner [46, 47]. Lin et al. [48] reported a dose-dependent increase in transgene expression in multiple cell lines in vitro and in vivo when hydrocortisone was used with adenovirus vectors. This enhancement was shown when hydrocortisone was added before, together with, and after the vector. Corticosteroids play a major physiological role in the regulation of gene transcription and protein synthesis in all cells, and this may influence transgene expression in this way. Additionally, they may alter cell membrane fluidity and permeability due to their lipophilic nature, allowing entry of DNA:vector complexes [48].

Conversely, we showed that methotrexate significantly impaired transfection of keratinocytes. Methotrexate is cytotoxic, acting via the inhibition of purine and pyrimidine synthesis, hence its use in the treatment of inflammatory diseases [49]. In keratinocytes, methotrexate has been found to inhibit proliferation and alter cell morphology in vitro [50], and moreover to induce apoptosis [51, 52]. The cytotoxic effects of methotrexate could account for the lower β-gal expression in methotrexate-treated cells. All plates in this study were seeded to the same densities using cells from the same culture flask, and were incubated to grow for 24 h before transfection. Representative photographs of the methotrexate-treated plates do, despite this, show lower cell densities than plates that received other drug treatments. The presence of fewer cells could have reduced the likelihood of transfection. Dalby et al. [8] found that reporter gene activity declined as cell density decreased. In the present study, cells treated with methotrexate which had been transfected may subsequently have been killed, leading to a decreased percentage expression. Additionally, cells at low density are more susceptible to toxic effects of transfection [8]. There is scant work on the effects of methotrexate on transfection efficiency; however, Pallavicini et al. [53] found that treatment of cells with methotrexate led to an increased variability of the cellular location and...
amount of transfected genes and of transgene expression. These factors may have contributed to the significantly lower transgene expression in methotrexate-treated cells.

Within our study, despite maintaining controlled conditions, there is some variability in the percentage gene expression between different experiments, with experiments determining optimum ratios of DNA:Lipofectamine® 2000 showing lower transfection than those investigating timings, pH, and the influence of drugs. All experiments were subject to the same conditions, with gene expression measured at the same time point (except in those experiments investigating optimal timing). The variability between experiments is likely to be due to biological variability within the cultured cells, while importantly there is very limited variability within each experiment, as demonstrated by the narrow error bars within each. Additionally, the absolute percentages of gene expression are low, even at the greatest level achieved. This is a reflection of the semiquantitative method used, in which a coloured product arising from an enzymatic reaction was assessed visually. The greatest gene expression was seen on day 3 after transfection in all experiments, except those done in the presence of hydrocortisone, which produced the greatest transfection on day 1, producing further variability. However, the expression seen on day 1 in the hydrocortisone-treated cells was only marginally greater than that seen on day 3, and this difference was not significant. The earlier peak expression in these cells may have been due to biological variability within the cells, or may be a result of enhanced gene transcription and protein synthesis by the hydrocortisone itself.

This study has investigated some of the factors affecting transfection of keratinocytes in vitro and may be important in the development of gene therapy for PG and other skin diseases. However, there are numerous other variables in vivo that affect the application of these results and should also be considered. Due to the continuous regeneration of the epidermis, transfected cells are rapidly shed, and thus transgene expression is only short-lived [26]. This would make regular re-application essential. Moreover, successful gene delivery to excised human skin explants has been demonstrated using plasmid DNA-coated microneedles, without the need for a viral or liposomal vector [55], and several other physical methods of gene delivery exist [56]. This, together with the development of ex vivo skin models [57, 58], is promising for the future of gene therapy for skin conditions, and these models should be studied further as an intermediate between in vitro and in vivo studies. Moreover, healthy keratinocytes in culture systems are characteristically different from those in ulcers of PG, and disease models must be developed to study transfection if this work is to be relevant and practical in the treatment of PG.

As the pathophysiology of PG remains unclear, therapeutic genes are yet to be elucidated; this continues to be a key area for future work. There are several possible candidate genes. The majority of these may be involved in wound healing, such as keratinocyte growth factor [59], epidermal growth factor, or members of the fibroblast growth factor family [60]. Genes involved in immune regulation may also be investigated, after increasing evidence of an immune aetiology for PG [3, 61–66]. Particular immune targets may include genes encoding cell surface proteins involved in neutrophil trafficking, such as integrins CR3 and CR4 [63], and encoding interleukin-8, which recruits neutrophils to sites of infection and has been found to be overexpressed in PG ulcers and may be implicated in disease pathogenesis [62, 64]. PG is one feature of PAPA syndrome (pyogenic arthritis, PG and acne) [1, 67, 68], an autosomal-dominantly inherited disease. The genetic defect in this syndrome has been mapped to chromosome 15q [69] and is caused by mutations in the
PSTPIP1 gene, which encodes CD2 binding protein 1, involved in regulation of the inflammatory response [70, 71]. A further syndrome, PG, acne and suppurrative hidradenitis (PASH), has also been described in unrelated individuals, and may or may not have an underlying genetic component [72]. Further elucidation of these genetic disorders may help identify targets for gene therapy for PG.

In conclusion, we have identified certain conditions suitable for in vitro transfection of keratinocytes, which may be a prelude to the successful in vivo use of gene therapy as a treatment strategy for PG. Furthermore, we examined the effect of several drugs which are currently used in the management of this disease and in practice are likely to be used alongside any gene therapy agent. Gene therapy offers some hope for the treatment of PG, a severe disease with currently limited treatment options. This strategy is already being trialled for the treatment of other cutaneous conditions [73–76]. In vitro optimisation studies such as this, together with progression of understanding of the pathophysiological mechanisms behind PG, are necessary for a similar approach to the treatment of PG.

Acknowledgements

This study was supported by grants from the Bowel Disease Research Foundation and the Royal College of England.

Statement of Ethics

The authors have no ethical conflict to disclose.

Disclosure Statement

The authors have no conflicts of interest to disclose.

References


60 Werner S, Grose R: Regulation of wound healing by growth factors and cytokines. Physiol Rev 2003;83:835–870.


Gene Therapy for Pyoderma Gangrenosum


