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Epidermal cell culture model with tight stratum corneum as a tool for dermal gene delivery studies

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Abstract

The purpose of this study was to evaluate the feasibility of organotypic cultures of rat epidermal cells as a tool to study non-invasive dermal gene delivery. Also, a novel transfection method employing liposomal pre-treatment of stratum corneum (SC) was evaluated. Rat epidermal cells were cultured on Transwell tissue culture inserts and formation of stratum corneum barrier was evaluated in permeability studies with two model compounds. Transfections were performed with naked pCMV-SEAP2 plasmid and 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP)/dioleylphosphatidylethanolamine (DOPE)/DNA lipoplexes. Naked DNA was administered on the stratum corneum of the cell culture model with or without prior treatment of the stratum corneum with DOTAP/DOPE liposomes. Transfection was evaluated non-invasively by monitoring concentrations of secreted alkaline phosphatase (SEAP) in the culture medium of the basolateral compartment at 24-h intervals. Transfection with lipoplexes produced significant gene expression in rat epidermal keratinocyte (REK) epidermal culture model. Likewise, delivery of naked DNA on stratum corneum after DOTAP/DOPE liposome pre-treatment produced gene expression. Naked DNA alone did not result in detectable gene expression. In dermal gene delivery studies REK epidermal culture model is a suitable tool that includes tight stratum corneum and allows transgene expression in viable epidermis and non-invasive sampling of secreted gene product in the basolateral compartment. Liposomal pre-treatment of the stratum corneum augments transfection of viable epidermis.

Keywords: Gene delivery; Dermal; Cell culture model; Liposome

1. Introduction

Gene therapy has great potential in the medical treatment, including also several dermal treatment targets (e.g. psoriasis, melanoma and wounds) (Kopp et al., 2004). Topical application is preferably used gene delivery method into the skin. It is more convenient and also more efficient than parenteral gene delivery in some instances (Fynan et al., 1993; Shi et al., 1999). In addition to the skin conditions, genes can be used also for dermal delivery of systemic treatments, for example, percutaneous DNA vaccination. In that case, gene encoding a specific antigen is delivered into the dendritic cells in the skin to develop immune response against infectious pathogens and tumors. Several delivery methods have been used for DNA vaccination: gene gun (Fynan et al., 1993), adenoviruses and liposomes (Shi et al., 1999), electroporation (Babiuk et al., 2003) and naked DNA (Kang et al., 2004). Due to the emerging interest on dermal gene therapy, improved methods are needed for the DNA delivery. After its topical application onto the skin, DNA must be transferred across the limiting barrier of stratum corneum (SC) into the viable cells below (e.g. epidermal, dermal or dendritic cells), which then express the transgene.

Gene delivery can be studied using cellular in vitro methods or in vivo in animals or humans. Gene delivery studies with non-differentiated skin cells do not give reliable information because the major barrier of stratum corneum is not present. In vitro studies using pieces of human cadaver skin are widely used in transdermal drug delivery investigations (Kang et al., 2004; Suhonen et al., 2003). This model contains the stratum corneum but the dead tissue does not express transgenes and, therefore, it is not suitable for gene delivery studies. Percutaneous DNA transfections have been tested in animals but in vivo studies are laborious and not always suitable for mechanistic experimentation. Leakiness of the skin (usually 10–100 times higher

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permeability than in human skin) is the major drawback of animal models, such as hairless mice and rats. On the other hand, experiments with human volunteers are not ethical in the early phase of gene transfer studies.

Epidermal cell culture models derived from keratinocytes offer an alternative in vitro research method for dermal gene delivery. Cell cultures are cost-effective, easy to care and ethically acceptable. Several commercial cell models of human skin have been introduced. For example, EpiDerm® (MatTek Corporation, MA, USA), EpiSkin® (Episkin SNC, Lyon, France) and SkinEthic® (Laboratoire SkinEthic, Nice, France) have been used in permeability and irritation studies with chemicals (Lotte et al., 2002; Faller and Bracher, 2002). Primary or early passage human keratinocytes form stratified differentiated epidermis in these models, but unfortunately the commercial skin models are much more permeable than the human skin (Schmook et al., 2001).

It has been recently demonstrated that continuous rat epidermal keratinocyte (REK) cell line forms differentiated epidermal culture model with properly tight stratum corneum (Pasonen-Seppänen et al., 2001a,b). Extensive drug permeability studies (with 18 drugs at log P range of −4 to +4) showed that the permeability of this epidermal model corresponds reasonably well with human skin (Suhonen et al., 2003).

In this study, the feasibility of organotypic REK culture model, with previously shown good barrier properties was evaluated as a tool for dermal gene delivery studies. Transgene expression was investigated with pCMV-SEAP2 plasmid that encodes for secreted alkaline phosphatase (SEAP). We show that both 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP)/dioleylphosphatidylethanolamine (DOPE) lipoplexes and stratum corneum pre-treatment with DOTAP/DOPE are able to elicit gene expression in the viable epidermis after topical DNA administration.

2. Materials and methods

2.1. REK culture

The rat epidermal keratinocyte culture derived from newborn rat epidermal keratinocytes was originally isolated by Baden and Kubilus (1983). The continuous REK cell line used in these studies was a generous gift from Dr. Donald, K. MacCallum, Ann Arbor, MI, USA. REK culture was maintained and subcultured as described earlier (Pasonen-Seppänen et al., 2001a). Minimal essential medium (MEM) was acquired from Gibco (Life Technologies, Paisley, UK) and fetal bovine serum (FBS) was acquired from HyClone (Logan, UT, USA). Penicillin–streptomycin solution, L-glutamine and trypsin–EDTA solution were acquired from Sigma (St. Louis, MO, USA).

2.2. Epidermal culture model

Organotypic epidermal cell cultures were grown on Transwell tissue culture inserts (24 mm diameter and 3.0 μm pore size) obtained from Costar (Cambridge, MA, USA). Collagen from rat tails was isolated and collagen supports on tissue culture inserts were prepared as described earlier (Pasonen-Seppänen et al., 2001b). Earle's balanced salt solution (EBSS) was obtained from Sigma, sodium hydroxide from FF-chemicals (Yli-Ii, Finland) and 7.5% sodium bicarbonate solution from Gibco.

Confluent REK cultures were subcultivated on collagen-supported Transwell tissue culture inserts and maintained for 3 weeks as described recently (Suhonen et al., 2003) with minor modifications. On the first day, 2 ml of cell suspension (200,000 cells/ml) was applied onto the apical side and 2 ml of Dulbecco's MEM (DMEM, obtained from Gibco) including 10% FBS (HyClone), 4 mM L-glutamine (Sigma) and 50 μg/ml streptomycin and 50 U/ml penicillin (Sigma), was applied onto the basolateral side of the Transwell culture inserts. On the fourth day, the air-lift conditions were achieved by removing medium from the apical side of the inserts and culture medium on the basolateral side of the inserts was replaced with 1.5 ml of fresh medium. On the fifth day, culture medium on the basolateral side was replaced with medium supplemented with 40 μg/ml ascorbic acid (Sigma). On the seventh day and thereafter every day during 2 weeks, medium was replaced with fresh culture medium supplemented with ascorbic acid.

2.3. Permeability studies

The permeability characteristics of the epidermal culture model were regularly evaluated in order to validate the proper formation of the barrier. Permeation of two different probe compounds, mannitol (hydrophilic) and corticosterone (lipophilic), respectively, was studied in epidermal culture model as described earlier (Pasonen-Seppänen et al., 2001b). Studies were performed in two-chamber diffusion cells (Side-Bi-Side, Crown Glass Company, Cambridge, NJ, USA) having 3 ml compartment volumes and 0.64 cm^2 effective diffusional area. Phosphate-buffered saline (DPBS, 10×, pH 7.4) was obtained from Cambrex BioScience (Verviers, Belgium). Radiolabeled mannitol (D-(1-^{3}H(N))-mannitol, 26.3 Ci/mmol) and radiolabeled corticosterone (1,2,6,7-^{3}H(N))-corticosterone, 70.0 Ci/mmol) were acquired from NEN Life Science Products (Boston, MA, USA). Both radiolabeled permeants had higher than 97% purity. Samples from donor and receiver compartments were mixed with 3 ml of scintillation cocktail (UltimaGold, Packard, Bioscience, Groningen, The Netherlands) and analyzed by liquid scintillation counter (WinSpectral 1414, Wallac, Finland) for 10 min. The condition of the epidermal culture models was monitored during the experiment visually and possible air bubbles were removed from the diffusion area. Permeability coefficients P (cm/s) were determined at steady state for the model compounds as:

$$P = \frac{J}{AC_D} \quad (1)$$

where J is the flux (dpm/s/cm^2) at steady state, A the diffusional area (cm^2) and C_D is the concentration (dpm/cm^3) in the donor compartment.

2.4. Plasmid DNA

The reporter gene plasmid pCMV-SEAP2 encoding secreted alkaline phosphatase under the control of the cytomegalovirus gene enhancer/promoter was constructed by Mr. Mika Reinisalo at the University of Kuopio, Finland. The plasmid DNA was amplified using *Escherichia coli* and purified by column separation with Qiagen Mega Kit (Qiagen, Hilden, Germany). The concentration of plasmid DNA was evaluated by absorbance at 260 nm (Cary 50 Bio Spectrophotometer, Varian, Australia).

2.5. Liposomes

Cationic liposomes are widely used as non-viral vectors for gene delivery. We prepared cationic DOTAP/DOPE liposomes at molar ratio 1:1 with final DOTAP concentration of 3.2 mM. DOTAP and DOPE were obtained from Avanti Polar Lipids (Alabaster, AL, USA). The lipids were dissolved in chloroform (Sigma) to the concentration of 10 mg/ml. DOTAP solution (1030 μl) and DOPE solution (1152 μl) were mixed in glass test tube and subsequently chloroform was evaporated in vacuum evaporator (VV2011, Heidolph, West Germany) at 37 °C under nitrogen stream. Resulting lipid film was resuspended in sterile water and sonicated in the bath sonicator (Sonorex, Bandelin Electronic, Germany) at room temperature for 30 min until a translucent lipid solution was formed. The size of liposomes was determined by light scattering with NICOMP 380ZCS (Particle Sizing Systems, Santa Barbara, CA, USA).

2.6. Preparation of lipoplexes

Lipoplexes of pCMV-SEAP2 and cationic DOTAP/DOPE liposomes were prepared at +/− charge ratio of 2. The amount of plasmid DNA per epidermal culture insert was 4.7 μg. Needed volume of liposome solution was calculated according to Eq. (2)

$$V = \frac{NM}{C_L} R \tag{2}$$

where V is the volume of liposome solution (μl), N the amount of negative charges in DNA molecule (3.055 nmol/μg), M the amount of plasmid DNA (4.7 μg), C_L the concentration of liposome solution (nmol/μl) and R is the +/− charge ratio (+2). Lipoplexes were prepared aseptically in laminar flow hood by mixing calculated volumes of pCMV-SEAP2 plasmid solution and liposome solution in 1.5 ml eppendorf tubes. Lipoplexes were incubated at room temperature for 20 min before transfections.

2.7. Transfection protocol for cationic lipoplexes

Transfection studies were performed using epidermal culture models on Transwell tissue inserts as illustrated in Fig. 1. Firstly, both sides of epidermal culture models were washed with PBS. Fresh culture medium (2 ml) was applied on the basolateral side and 2 ml of 25 mM Hepes (Sigma)/133 mM NaCl buffer pH 7.4 was applied on the apical side of the inserts. Then, 500 μl of previously prepared cationic lipoplexes containing 4.7 μg plasmid DNA were placed on the apical compartment and incubated for 5 h at 37 °C in humified 95% air/5% CO_2 atmosphere.

2.8. Transfection protocol for liposome pre-treatment studies

The liposome pre-treatment of epidermal culture models was performed 12 h prior to transfections. Medium in the basolateral compartment was replaced with 2 ml of culture medium and 2 ml of 25 mM Hepes/133 mM NaCl buffer pH 7.4 was applied on the apical compartment. The amount of cationic DOTAP/DOPE liposomes equivalent to protocol for lipoplexes was added on the apical compartment and the culture models were incubated at 37 °C in humified 95% air/5% CO_2 for 12 h. Subsequently, liposomes were removed and the apical compartment was washed with 2 ml of PBS. The amount of naked plasmid DNA equivalent to protocol for lipoplexes (4.7 μg) was added on the apical compartment and inserts were incubated for 5 h. Transfection of naked DNA without liposome pre-treatment was performed as a control.

2.9. Sampling and SEAP assay

Transfections were evaluated by determining the concentrations of gene product, SEAP, in the basolateral compartment

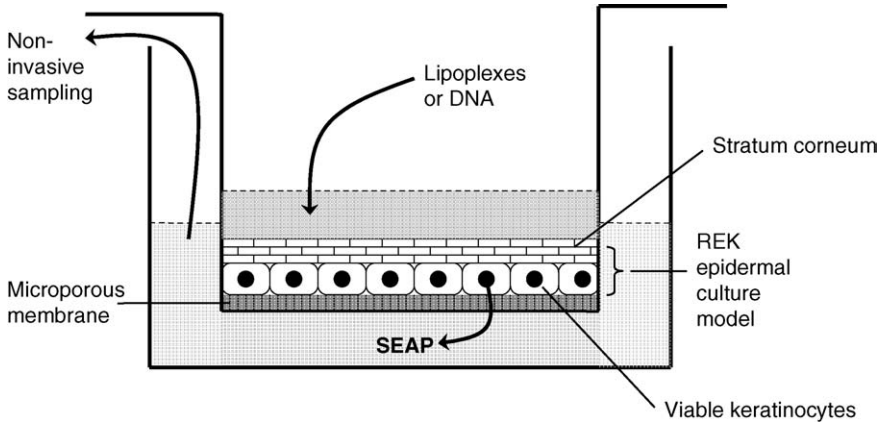

Fig. 1. Schematic picture of dermal gene delivery in REK epidermal culture model. Stratum corneum is illustrated as a brick wall-structure.

Table 1
Permeability coefficients (10^{-7} cm/s) of model compounds in epidermal culture model[a]

	Mannitol	Corticosterone
Intact culture model	0.51 ± 0.26	0.24 ± 0.04
Liposome pre-treated culture model	0.87 ± 0.43	0.31 ± 0.04

[a] Average ± S.D., $n = 5$.

(Fig. 1) at 24-h intervals. The first samples were taken immediately after incubation of 5 h. The samples of 100 μl were transferred to 1.5 ml eppendorf tubes and stored at −20 °C. Remaining medium from the basolateral compartment and solution from the apical compartment were removed. Both sides were washed with 2 ml of PBS and 1.5 ml of fresh culture medium was applied on the basolateral compartment. Thereafter, samples were taken, basolateral compartment was washed with PBS and fresh medium was changed at 24-h intervals during 21 days. The samples were analyzed with chemiluminescence assay using Great EscAPe^{TM} SEAP Reporter System Kit protocol (BD Biosciences Clontech, Palo Alto, CA, USA) and luminometer (Victor^{2TM} 1420 Multilabel counter, Perkin-Elmer Wallac, Finland).

3. Results and discussion

3.1. Permeability characteristics of the epidermal culture model

The formation of stratum corneum on epidermal REK model was evaluated in permeability studies with mannitol and corticosterone. Permeability studies were also performed on liposome pre-treated epidermal culture model in order to evaluate the effect of possible liposome fusion with stratum corneum on membrane permeability. Permeability coefficients are shown in Table 1. Our results demonstrate that in these experiments the REK model forms adequate barrier since permeability coefficients are close to the values of human skin and earlier data with REK model (Suhonen et al., 2003). Permeability measurements were not done in each culture insert, but our extensive prior studies (Pasonen-Seppänen et al., 2001a,b; Suhonen et al., 2003) indicate that the barrier formation in the REK cultures is reproducible. There is no reason to expect large variation or poor barrier formation in these cell cultures. The proper formation of stratum corneum in epidermal culture model is crucial, since it is the major barrier layer in the skin. These results gave us reliable starting point for dermal gene delivery studies.

It has been demonstrated that the re-organization of stratum corneum lipids by fused applied lipids can result in more permeable barrier (Kirjavainen et al., 1996). In our studies, the pre-treatment with DOTAP/DOPE liposomes did not change substantially the permeability of the model compounds in the epidermal cultures (Table 1). Permeability coefficient of corticosterone was slightly higher ($p < 0.01$, Mann–Whitney's U-test) in liposome pre-treated cultures than in untreated cultures. There was no significant change either in mannitol permeability. Our results suggest that pre-treatment with liposomes caused only minor changes in the integrity of stratum corneum of epidermal cell culture model.

3.2. Transfections

Long-term secretion of SEAP was achieved after non-invasive gene delivery to the epidermal culture model. Both lipoplex and liposome pre-treatment methods produced significant expression of SEAP reporter gene (Fig. 2). The pharmacokinetic parameters of SEAP secretion were calculated and they are outlined in Table 2. Transfection of naked DNA into untreated culture model did not produce detectable gene expression and no pharmacokinetic parameters were calculated in this case.

Transfection with DOTAP/DOPE lipoplexes produced the most efficient transfection. The peak of SEAP production was seen 96 h after transfection, when the concentration of SEAP in the basolateral samples was 0.15 μg/ml (Table 2). Secretion of SEAP protein continued for 12 days and the final cumulative amount of SEAP was 0.86 μg (Fig. 2B).

Liposomes are widely used as non-viral vectors in gene delivery and smaller (100–200 nm) liposomes are generally preferred. The mean size of free DOTAP/DOPE liposomes was 150 nm but the mean size of DOTAP/DOPE lipoplexes was about 900 nm. However, the size of lipoplexes may not be significant in dermal gene delivery since lipoplexes do not penetrate intact into the skin, but the lipids from lipoplexes may rather fuse to the stratum corneum thereby facilitating DNA delivery across stratum corneum.

Since intact liposomes do not probably permeate into the skin, we tested the application of liposomes separately prior to administration of DNA. Liposome pre-treatment of epidermal culture model before transfection with naked DNA turned out to be successful method. Lipid pre-treatment for 12 h resulted in SEAP secretion almost equivalent to lipoplex administration (Fig. 2A and B; Table 2). On the contrary, naked DNA without pre-treatment did not show any detectable transfection (Fig. 2). Presumably, the barrier function of untreated stratum corneum prevented transfection with naked DNA.

Table 2
Pharmacokinetic parameters of SEAP secretion in epidermal culture model after transfection with lipoplexes and pre-treatment method[a]

	C_{max} (μg/ml)	T_{max} (h)	Cumulative secretion of SEAP at 504 h (μg)	Rate of SEAP secretion (ng/h)	AUC (μg h/ml)
Lipoplexes	0.15 ± 0.03	96	0.85 ± 0.14	5.17 ± 0.78	20.35 ± 4.76
Liposome pre-treatment	0.10 ± 0.01	96	0.66 ± 0.03	3.33 ± 0.18	15.39 ± 1.68

[a] Average ± S.D., $n = 3$.

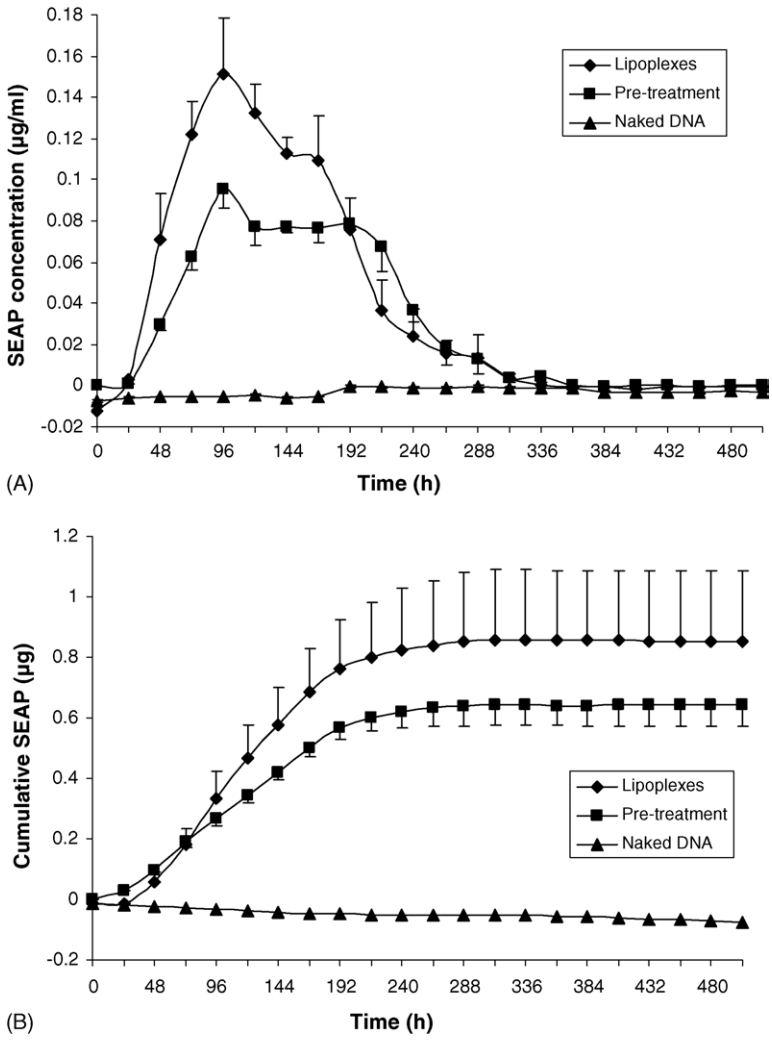

Fig. 2. Secreted alkaline phosphatase (SEAP) concentration (A) in epidermal culture medium and cumulative SEAP amount (B) after transfection of pCMV-SEAP2 with three different methods: lipoplexes, liposome pre-treatment plus naked DNA and naked DNA alone. Each point represents the average ± S.D., $n = 3$.

It seems that formation of lipoplexes is not required for topical transfection of keratinocytes. Prior administration of cationic lipid into the stratum corneum works as penetration enhancer for DNA (Fig. 2) in specific way, since the barrier of stratum corneum was not compromised to small neutral solutes (Table 1). Liposome pre-treatment could form basis of new formulations and clinical applications in dermal gene delivery. For example, semi-solid or liquid liposomal formulation could be applied on the skin for certain time period, followed by transfection with solution or gel with naked DNA. This could be easy and cheap method for dermal gene delivery in vivo. Liposome pre-treatment is probably more controlled method for penetration enhancement than lipoplexes as suggested by smaller standard deviations in the pre-treatment group (Fig. 2A). Problems associated with lipoplexes include the issues of complex size variation, reproducibility and stability. Efficacy of pre-treatment could be optimized by using different lipids, media and incubation conditions.

The lag time of 24 h was found in SEAP production (Fig. 2). This is likely due to DNA transport across stratum corneum, internalization, nuclear disposition in the epidermal cells and finally initiation of SEAP production and secretion. The secretion of SEAP was linear for 5 days (between 72 and 192 h) with both methods (Fig. 2B) and the rate of SEAP production with lipoplexes was slightly greater than with pre-treatment method (Fig. 2B; Table 2). The cumulative amount of SEAP with lipoplexes was 25% higher than with pre-treatment, but duration of gene expression was identical between the two methods and the expression was more stable with pre-treatment method (Fig. 2A).

It is generally accepted and also demonstrated in animal studies with mice (Fan et al., 1999) that gene expression after topical DNA application onto the skin is mainly localized in the hair follicles. Liposomes may also concentrate in lipid-rich hair follicles providing plasmid DNA with access into the skin. In our study only the delivery of DNA through stratum corneum was evaluated, since there are no hair follicles present in REK epidermal culture. The model demonstrates the possibility to deliver plasmid DNA directly through stratum corneum and to evoke gene expression in the keratinocytes contrary to animal studies where gene delivery is mainly located to the hair follicles.

4. Conclusions

The feasibility of organotypic REK culture model was demonstrated as a novel tool for non-invasive dermal gene delivery. This epidermal culture model is known to form tight stratum corneum and displays permeability characteristics comparable to the human skin. In gene delivery studies, REK epidermal culture allows the non-invasive use of any reporter gene that encodes secreting protein. Since no cell lysis is needed in sampling, this functional assay provides the evaluation of both the level and duration of gene expression independently from each epidermal culture on filter. Positive results were demonstrated with DOTAP/DOPE lipoplexes and interestingly also with simple DOTAP/DOPE pre-treatment followed by administration of naked DNA.

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