Kinetic Modeling of Plasmid DNA Degradation in Rat Plasma

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ABSTRACT A major obstacle in gene delivery is the transport of intact plasmid DNA (pDNA) to target sites. We sought to investigate the kinetic processes underlying the degradation of pDNA in a rat plasma model, as this is one of the main components responsible for the clearance of pDNA after intravenous administration. We further sought to construct a complete kinetic model to describe the degradation of all three topoforms (supercoiled, open circular, and linear) of pDNA in a rat plasma model. Supercoiled pDNA was incubated in isolated rat plasma at 37°C in vitro. At various time points, the plasma was assayed by electrophoresis for the amounts of supercoiled, open circular, and full-length linear pDNA remaining. The calculated amounts remaining were fit to linear differential equations describing this process. In this model, pDNA degradation is considered to be a unidirectional process, with supercoiled degrading to open circular and then to the linear topoform. The calculated kinetic parameters suggested that supercoiled pDNA degrades in rat plasma with a half-life of 1.2 minutes, open circular pDNA degrades with a half-life of 21 minutes, and linear pDNA degrades with a half-life of 11 minutes. Complexation of pDNA with liposomes resulted in a portion of the supercoiled plasmid remaining detectable through 5.5 hours.

INTRODUCTION

Biotechnology is a rapidly growing area in the pharmaceutical sciences. One approach to achieving a therapeutic effect would be to have a protein made in vivo, utilizing the body's own mechanisms to produce a competent protein. Gene therapy is one potential route by which to accomplish this goal. Gene therapy also potentially offers the treatment of genetic and end-stage diseases. The replacement of mutated DNA via gene therapy can result in the production of a competent protein. These potentials make gene therapy a rapidly advancing area in therapeutics.

Plasmid DNA templates in the supercoiled topoform have been associated with a more rapid rate of expression when compared to open circular pDNA, and this is particularly observable within the first few hours after transfection (1). By 24 hours after delivery of pDNA, this difference becomes less significant, with open circular and supercoiled pDNA expressing similar levels of transgene, and both expressing significantly greater expression levels than linear pDNA (2).

Early studies revealed that serum nucleases play a role in the rapid clearance of genomic DNA from the circulation of injected animals (3). Recent studies on the kinetics of pDNA have used radiolabeled pDNA for detection (2,4). However, the radiolabeling procedure involves nick translation, eliminating the possibility of maintaining the supercoiled topoform. Furthermore, this method does not differentiate degraded pDNA from intact plasmid, thus yielding an overestimation of the true half-life of the intact pDNA. Other studies on the kinetics of supercoiled and open circular pDNA have been only qualitative, citing the presence of pDNA topoforms at various time points (4-6).

Thierry et al. studied the stability of pDNA in the bloodstream of mice after intravenous injection (5). Their results indicated that supercoiled plasmid was not detectable in the plasma or red blood cell fractions 1 minute after injection of pDNA. Kawabata et al. found that the supercoiled pDNA was completely converted to the open circular topoform within 5 minutes when incubated in mouse whole blood (6). Little other information on the kinetics of pDNA is available. The exact kinetics underlying this rapid degradative process are not fully understood.

It is necessary to study the effects of naked pDNA in order to begin understanding the importance of the degradation of pDNA in the blood, and to allow

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a foundation upon which comparisons of delivery vehicles can be made. Naked pDNA has been shown to remain in the plasma fraction of blood (4). For these reasons, we sought to investigate the kinetic processes underlying the stability of pDNA in a rat plasma model. We further sought to construct a complete kinetic model to describe the degradation of all three topoforms of pDNA in a plasma model. This model will allow a prediction of the time course of potential tissue exposure to the transcriptionally active supercoiled and open circular pDNA topoforms.

METHODS

Phenol: chloroform: isoamyl alcohol (25:24:1, v/v/v), Tris, boric acid, ethylenediaminetetraacetic acid (EDTA), and agarose were purchased from Sigma Chemical Company (St. Louis, MO). Ethidium bromide (electrophoresis grade) was purchased from Fisher Biotech (Fair Lawn, NJ). Competent JM109 bacteria (Promega, Madison, WI) were transformed according to the manufacturers directions with the pGL3 control plasmid (Promega). Plasmid DNA was isolated from overnight cultures using the Plasmid Maxi-Prep kit (Quiagen, Valencia, CA), and was >95% supercoiled by agarose gel analysis.

Blood was isolated from male Sprague-Dawley rats (300-350 g) by cardiac puncture and immediately placed in heparinized test tubes (Vacutainer; Becton Dickinson, Franklin Lakes, NJ) on ice. Blood samples were centrifuged at 16,000 g for 5 minutes. For dilution experiments, plasma was diluted to 25 and 50% with PBS or PBS containing 0.1 mM EDTA. To analyze the effects of heat, plasma samples were incubated at 90°C for 10 minutes in sealed tubes before assay. 600 µl of plasma was removed and placed on ice until assay. Plasma samples were warmed to 37°C in a water bath and maintained at 37°C for the duration of the experiment. 12 µg of pDNA, in 10 mM Tris 1 mM EDTA buffer, was incubated in 37°C plasma and 50-µl samples were taken at the times indicated. 80 µl of phenol: chloroform: isoamyl alcohol (25:24:1, v/v/v) was immediately added to each sample, vortexed for 5 seconds at low speed, and placed on ice. Samples were centrifuged at 20,800 g for 10 minutes at room temperature. From the supernatant,

an aliquot of 15 µl was removed, 5 µl of 1 x loading dye (Promega,) added, and placed on ice until loaded on an agarose gel. A final volume of 10 µl was loaded on agarose gels.

Samples were loaded on 0.8% tris borate EDTA (TBE) buffer based agarose gels containing 0.001% ethidium bromide. Electrophoresis was carried out at 15 V/cm for 12 hours. Gels were visualized using an ultraviolet light box and photographed using a Kodak DC40 camera (Eastman Kodak, Rochester, NY). The amounts of supercoiled, open circular, and linear pDNA were calculated using Kodak Digital Science 1D Image Analysis Software (version 2.01; Eastman Kodak Company, Rochester NY) using a Lambda Hind III digest standard (Promega) as a reference.

Comparisons of the relative fluorescence of supercoiled pDNA versus linear pDNA were made by digesting supercoiled pDNA with the Hind III restriction enzyme (Promega) which has a single recognition sight in the plasmid. Spectrophotometrically equivalent amounts of linear and supercoiled pDNA were then loaded on agarose gels as described above and the relative fluorescence compared. It was found that on a weight-to-weight ratio, supercoiled pDNA was only 55% as fluorescent relative to linear pDNA by agarose gel analysis. To correct for this difference, supercoiled pDNA amounts were multiplied by 1.8 prior to analysis. This difference has been reported previously, and is likely due to the relative inaccessibility of ethidium bromide to the supercoiled topology (8).

Percentage of recovery was calculated by comparing phenol: chloroform: isoamyl alcohol (25:24:1, v/v/v) extracted versus nonextracted known amounts and analyzing on agarose gels as described above. Percentage of recovery using the phenol: chloroform: isoamyl alcohol method was found not to be dependent on topoform. Recovery was 90 (±6)% for supercoiled and 86(±13) % for linear pDNA.

All lipids were purchased from Avanti Polar Lipids (Alabaster, AL). Liposomes were prepared by mixing 1,2-dioleoyl-3-trimethylammonium propane

and dioleoyl phosphatidylethanolamine in a 1:1 molar ratio in chloroform and drying the mixture under nitrogen at 60° C in a Buchi Rotovapor. The dried lipid film was then reconstituted in sterile water at a concentration of 1 mg/ml and shaken in a 60° C water bath for 15 minutes. Liposomes were then sized by extrusion through a 0.05-μm polycarbonate membrane (Poretics Corporation, Livermore, CA).

THEORETICAL

The degradation of supercoiled pDNA was assumed to follow pseudo first-order kinetics. The model used is diagrammed in (Figure 1). In this model, pDNA degradation is considered to be a unidirectional process. The degradation of linear pDNA is considered to yield fragments of heterogeneous lengths, thus these products were not included in the fitted model. No elimination from any of the compartments is assumed to occur through routes other than degradation to the following topoform. Based on this model the following differential equations were derived to describe the following process:

$$\begin{aligned}\frac{dSC}{dt} &= -k_s \cdot SC \\ \frac{dOC}{dt} &= k_s \cdot SC - k_o \cdot OC \\ \frac{dL}{dt} &= k_o \cdot OC - k_l \cdot L\end{aligned}$$

The amounts of supercoiled, open circular and linear pDNA were then modeled using the integrated form of the following equations:

$$\begin{aligned}SC &= SC_0 \cdot e^{-k_s t} \\ OC &= k_s \cdot SC_0 \cdot \left(\frac{1}{k_s - k_o} \cdot e^{-k_o t} + \frac{1}{k_o - k_s} \cdot e^{-k_s t} \right) \\ L &= k_o \cdot k_s \cdot SC_0 \cdot \left(\frac{1}{(k_s - k_o)(k_o - k_l)} \cdot e^{-k_o t} + \frac{1}{(k_o - k_l)(k_l - k_s)} \cdot e^{-k_s t} + \frac{1}{(k_s - k_l)(k_l - k_o)} \cdot e^{-k_l t} \right)\end{aligned}$$

Figure 1. Kinetic model of plasmid DNA degradation in plasma. The model is considered to be a unidirectional process. SC, OC, and Linear represent the amounts of supercoiled open circular and linear plasmid, respectively, in each compartment. The rate constants k_s , k_o , and k_l represent the degradation constants for supercoiled open circular and linear plasmid respectively.

Where SC , OC , and L are the amounts of supercoiled, open circular, and linear pDNA present at time t , respectively. SC_0 is the amount of supercoiled pDNA present at time $(t) = 0$. The constants k_o , k_s , and k_l represent the rate constants for the degradation of supercoiled, open circular, and linear pDNA respectively. The constants represent the activity of all enzymes acting in the degradation process. Nonlinear curve fitting and statistical analysis was carried out using Scientist (version 4.0, Micromath, Salt Lake City, UT). Area under the plasma concentration time curve was calculated using trapezoidal rule. Area under the terminal portion of the plasma concentration time curve was calculated by integration from the last concentration time point measured. Clearance was calculated from the product of the reported volume of rat plasma (7.8 ml) (7) and the terminal elimination rate constant (k).

RESULTS

A representative gel in which the three topoforms of plasmid are detectable is displayed in (Figure 2). In addition, at 60 minutes, the degradation products of linear pDNA are visible as a light smear running below the band. Under the conditions used in this experiment, limit of quantification was 0.9 ng/μl using the Lambda Hind III size standard. Plasmid amounts were calculated from agarose gel analysis using Kodak Digital Science 1D image analysis software (Eastman Kodak, Rochester, NY) as described in the methods section.

The observed and predicted values, based on the model displayed in (Figure 1), are plotted in (Figure 3).

Figure 2. Agarose gel analysis of pDNA degradation in isolated rat plasma. Lane 1, size standard; lane 2, 30 seconds; lane 3, 1 minute; lane 4, 2 minutes; lane 5, 3 minutes; lane 6, 5 minutes; lane 7, 10 minutes; lane 8, 20 minutes; lane 9, 30 minutes; lane 10, 45 minutes; lane 11, 60 minutes; lane 12, 80 minutes.

Figure 3. Experimental and fitted data based on the kinetic model described in the text. Data points represent actual experimental data. Lines represent values predicted by the model. The values are expressed as $\pm$ SD of 6 rats. Key: +supercoiled, x open circular, * linear.

Table 1. Kinetic Parameters of pDNA in Isolated Rat Plasma

Data represent the fitted values of $n = 6$ plasma samples ± 1 standard deviation.

Topoform	Rate constant	Value (min^{-1})	Standard deviation	Half-life (min)
Supercoiled	k_s	0.6	0.03	$1.2 (\pm 0.1)$
Open circular	k_o	0.03	0.002	$21 (\pm 1)$
Linear	k_l	0.06	0.008	$11 (\pm 2)$

Table 2. Data represent averages ± 1 standard deviation of $n = 6$ plasma samples.

AUC and Clearance Values of pDNA in Isolated Rat Plasma

Topoform	AUC ($\text{ng}/\mu\text{l} \cdot \text{min}$)	Clearance ($\mu\text{l}/\text{min}$)
Supercoiled	18	$360 (\pm 9)$
Open circular	310	$23 (\pm 1)$
Linear	130	$47 (\pm 5)$

Plasmid concentrations over time were well predicted by the model (coefficient of determination = 0.96). Kinetic parameters calculated based on the model are summarized in Table 1. Supercoiled pDNA degraded rapidly in the plasma with a half-life of $1.2 (\pm 0.1)$ minutes. Open circular plasmid was fairly stable, however, degrading with a half-life of $21 (\pm 1)$ minutes. Linear plasmid degraded more rapidly than the open circular topoform, but was fairly stable in comparison to the supercoiled plasmid degrading with a half-life of $11 (\pm 2)$ minutes. Open circular AUC was nearly 17 times larger than supercoiled, and 2.3 times larger than linear pDNA (Table 2).

No kinetics suggestive of enzyme saturation were observed under the experimental conditions tested. However, to ensure that saturation of plasma nucleases was not resulting in artificially low rate constant values, we analyzed the rate constants produced in dilute plasma (dilution was chosen because decreasing the dose of pDNA quickly results in a loss of sensitivity and sample sizes too large for loading). If saturation of plasma nucleases was occurring, we expected that the rate constants in dilute plasma should deviate from a linear relationship. Thus we tested the kinetics of pDNA degradation in 25 and 50% plasma. As displayed in (Figure 4), no deviation was observed in the degradation of supercoiled and open circular pDNA.

To further investigate the mechanism responsible for the observed degradation, and to further validate our assay (i.e., to ensure the assay was not causing degradation itself), we studied the degradation of pDNA in PBS diluted 25% heated plasma (90°C for 10 minutes) and PBS $\pm 0.1 \text{ mM}$ EDTA diluted 25% plasma. No degradation of supercoiled pDNA

Figure 4. Analysis of rate constants in dilute plasma. Degradation rate constants were modeled in PBS diluted rat plasma. Rate constants represent the fitted values of $n = 6$ rats/ time point. Key: diamond = k_s in dilute plasma, square = k_o in dilute plasma. The value of k_i is not reported due to the prolonged stability of linear plasmid in dilute plasma.

was observed in either case through 1 hour (data not shown). The degradation sensitivity to heat and EDTA provides evidence that the degradation observed in the assay is due to enzymatic processes.

To investigate the potential protective effects of cationic liposomes from plasma nucleases, we complexed a common liposomal delivery vehicle, DOTAP: DOPE (1:1 w/w), to pDNA (3:1 lipid: DNA, w/w). (Figure 5) displays that the supercoiled pDNA degraded no further after 20 minutes incubation, resulting in a portion of supercoiled pDNA remaining detectable at 5.5 hours (8.2% of the time equi 0 amount)

DISCUSSION

Previous reports on the kinetics of pDNA have been qualitative only, or they involved radiolabeling. These studies indicated that pDNA degrades within 5 minutes after incubation in whole blood in vitro or after intravenous injection (5,6). In this study we sought to quantitatively model the kinetics underlying the stability of pDNA in the plasma. The results revealed that supercoiled pDNA degrades in

plasma with a half-life of 1.2 minutes. Open circular pDNA was found to be more stable than the supercoiled topoform degrading with a half-life of 21 minutes. Linear pDNA degraded more rapidly than the open circular topoform (half-life = 11 minutes). These kinetics may be explained by the activity of various nucleases present in the plasma. Open circular plasmid must be nicked by endonucleases on each sister strand in the same location to generate linear pDNA. The supercoiled topoform degrades after a single nick to the phosphodiester backbone and thus degrades more rapidly. Linear pDNA would be accessible to both endonucleases and also exonucleases, possibly explaining its more rapid degradation than the open circular topoform.

Early investigations by Chused et al. suggested that nucleases may not play a major role in the degradation of tritiated KB cell DNA when intravenously injected in mice (9). However their assay was not able to identify the true activity of nucleases, given that their assay utilized genomic

Figure 5. Liposome-pDNA complexes were incubated in rat plasma for various time points. Lane 1, size standard; lane 2, 1 minute; lane 3, 2 minutes; lane 4, 5 minutes; lane 5, 10 minutes; lane 6, 20 minutes; lane 7, 30 minutes; lane 8, 60 minutes; lane 9, 2 hours; lane 10, 3 hours; lane 11, 5.5 hours.

DNA. Single strand cuts to the isolated genomic DNA would not yield small fragments and thus be undetectable by their method. This would yield an underestimation of true nuclease activity. Using the analytical procedures described here, single strand cuts to pDNA would lead to a degradation of the native supercoiled structure and be detectable using the method described here.

Investigations in mice by Thierry et al. suggested that the main nuclease activity was endonucleolytic based on the finding that the linear:supercoiled ratio increased over time and the supercoiled:open circular ratio remained identical (5). Our results suggest that linear pDNA follows more rapid degradation than open circular. This can be explained by endonuclease activity generating more free ends for degradation by exonucleases, exonucleases being more active than endonucleases, or topoform influencing the binding of these enzymes and thus affecting the reaction rate. Thus, endonucleases are not necessarily the major nucleases responsible for the degradation of pDNA in the circulation.

Liposome complexation with the pDNA revealed protection of the supercoiled topoform in the plasma from 20 minutes through 5.5 hours (Figure 5). No degradation of the supercoiled topoform was

observed after 20 minutes. This suggests that a fraction of the supercoiled pDNA is offered significant protection from plasma nucleases, and provides further evidence to suggest that liposome complexation may not only be a means by which to deliver pDNA to target sights, but also to specifically protect supercoiled pDNA from degradation.

In summary, this article presents a kinetic model describing the degradation of pDNA in rat plasma. A kinetic model is presented that we feel will be of use in the future as gene therapy moves toward clinical use. Furthermore, we provide evidence that supercoiled pDNA can remain stable in the plasma through 5.5 hours when complexed to cationic liposomes. Using the model derived, we are able to conclude that naked supercoiled pDNA degrades in rat plasma with a half-life of $1.2 (\pm 0.1)$ minutes, open circular with a half-life of $21 (\pm 1)$ minutes, and linear pDNA with a half-life of $11 (\pm 2)$ minutes.

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