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核酸をコアとするナノ微粒子化による薬物・免疫治療システムの開発に関する研究

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核酸をコアとするナノ微粒子化による薬物・免疫治療システムの開発に関する研究

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研究要旨　これまでに開発した核酸をコアとするナノ粒子化技術を利用し、癌を対象とする薬物・免疫治療システムの開発を試みた。今年度は、DNA による抗腫瘍免疫の誘導に焦点を当て、CpG DNA によるサイトカイン産生および腹膜播種モデルにおける抗腫瘍効果について検討した。CpG モチーフを多く含むプラスミド DNA（pDNA）の添加により、マウス脾臓マクロファージおよび肝非実質細胞から腫瘍壊死因子（TNF）-αが産生された。pDNA をカチオン性リポソームとの複合体とすることで、これら細胞に加えて腹腔マクロファージからもサイトカイン産生が認められた。新たにデザインした CpG オリゴヌクレオチド（CpG DNA）をカチオン性リポソーム複合体としてマウス腹腔内に投与したところ、腹腔中に TNF-αおよびインターロイキン 12 が検出されるとともに、マウス腹膜播種モデルにおいて高い抗腫瘍効果が得られた。一方、それぞれ半分ずつ相補的な 3 種類の DNA を用いることで形成される Y 型（分岐型）2 本鎖 DNA（Y-DNA）に CpG モチーフを組み込んだ Y-CpG DNA は、同じ塩基配列からなる通常の 2 本鎖 CpG DNA よりも有意に高い TNF-αを誘導した。得られた情報をもとに免疫活性化能の高い DNA－カチオン性化合物複合体を構築し、その表面を親水性ポリマーで被覆することによりナノ粒子化を実現することで、腫瘍組織へのターゲティングが可能なナノ粒子の開発を目指す。

A. 研究目的

癌組織では血管透過性が亢進し、通常血管壁を透過しないサイズの高分子・微粒子が集積することから、サブミクロン、特に直径 100 nm 以下のナノ粒子を利用した癌組織への薬物ターゲティングが期待されている。これまでにも、リポソームやナノスフェアなどをキャリアとした膨大な研究が行われ、その一部についてはすでに臨床使用可能な製剤が開発されている。しかしながら、依然として癌に対する薬物治療が十分に達成されているとは言い難い。一方、癌抗原を投与することで生体に備わる免疫機構を活性化する癌免疫治療も注目を集めている。DNA ワクチンの場合には、DNA 中の非メチル化 CpG 配列、いわゆる CpG モチーフ、による免疫担当細胞の活性化が、抗原による抗腫瘍免疫効果を増大することが示されている。そこで本研究では、これまでに開発したプラスミド DNA（pDNA）のナノ微粒子化技術、またはナノサイズのデンドリマー様 DNA 構造体を用いることで、抗癌剤を癌組織へターゲティング可能なナノ粒子を設計・構築し、癌への薬物ターゲティングが可能な新規ナノ粒子型ドラッグデリバリーシステムを開発する。近年、腫瘍組織に集積するマクロファージなどの免疫担当細胞は、腫瘍組織環境下において本来の異物排除機能が低下し、各種メディエータを放出することで癌細胞の浸潤・転移、増殖を亢進することが指摘されている。DNA 中の CpG モチーフは自然免疫機能を活性化可能であることから、本研究で開発する

核酸をコアとするキャリアを用いることで腫瘍組織中の免疫担当細胞の活性化が期待できる。また、DNA はドキソルビシンなど DNA インターカレータをはじめとする各種抗癌剤との結合能を有することから、核酸をコアとするナノ粒子を用いることで、抗癌剤を効率よく癌組織へターゲティングすることも可能である。免疫機能を活性化しつつ抗癌剤による殺細胞効果が期待される本ナノ粒子デリバリーシステムは、メカニズムの異なる 2 つの治療戦略を融合した新規システムであり、リポソームなど生物学的に不活性なキャリアとは異なり、相乗的かつ高い抗腫瘍効果が期待できる。

B. 研究方法

(1) マウスへの DNA 投与後の TNF-α産生：CpG モチーフを多く含む DNA としてルシフェラーゼ発現 pDNA を、CpG モチーフが少ない DNA としては牛胸腺 (CT) DNA およびメチル化 pDNA を用いた。検討には ICR マウスを用い、クロドロネート含有リポソームを静脈内投与したマウスをマクロファージ除去マウスとした。カチオン性リポソームには N-[1-(2,3-dioleyloxy)propyl]-n,n,n-trimethylammonium chloride (DOTMA) と cholesterol をモル比 1:1 で混合した DOTMA/Chol リポソームを作製し、DNA とリポソームを電荷比 1:2.24 (-:+) で混合することでカチオン性リポソーム複合体を調製した。各 DNA を、単独またはカチオン性リポソーム複合体として静脈内投与し、経時的に血清中および各臓器中の TNF-α濃度を ELISA 法により測定した。

(2) DNA 添加による培養マクロファージからの TNF-α産生：定法に従い、ICR マウスから常在性腹腔マクロファージ、脾臓マクロファージ、肝非実質細胞、腎メサンギウム細胞を単離した。対照にはマウスマクロファージ様細胞株 RAW264.7 を用いた。pDNA または CT DNA を単独あるいはカチオン性リポソーム複合体として細胞に添加し、一定時間培養後の上清中 TNF-α濃度を ELISA 法により測定した。別途、各細胞群での Toll-like receptor-9（TLR9）の発現を real time PCR により評価した。

(3) CpG DNA 複合体による抗腫瘍効果：新たに 26-mer の天然型 CpG DNA を設計した（5'-TCGACGTTTTGACGTTTTGACGTTTT-3'）。対照には CpG モチーフを含まない ODN（random DNA、GpC DNA）を用いた。癌細胞の体内分布および増殖を定量的に評価するために、ルシフェラーゼ安定発現癌細胞株 B16-BL6/Luc および colon26/Luc を樹立した。腹腔内に癌細胞を移植することで腹膜播種モデルマウスを作成し、その腹腔内に各 DNA を単独または DOTMA/Chol リポソーム複合体として投与した。移植 7 日後に腹腔内臓器を摘出し、臓器中ルシフェラーゼ活性を指標に癌細胞数を評価した。また、担癌マウスでの腹腔中 TNF-αおよびインターロイキン 12（IL-12）濃度を ELISA 法により測定するとともに、マウスの延命効果についても検討した。

(4) Y 型 DNA の構築と免疫活性化能の評価：それぞれ半分ずつ相補的な 3 種類の DNA を当モルずつ混合することで Y 型 DNA（Y-DNA）を得た。このとき 1 種類の配列中に CpG モチーフを挿入した Y-DNA（Y-CpG DNA）も設計した。対照には同じ塩基配列の 1 本鎖（ssDNA）および 2 本鎖 DNA（dsDNA）を用いた。各 DNA を RAW264.7 に添加し、一定時間培養後の上清中 TNF-α濃度を ELISA 法により測定した。

C. 研究結果

(1) DNA 静脈内投与後のマウス血漿中、臓器中 TNF-α濃度：マウスに pDNA を静脈内投

与した場合、25 μg の投与量では血清中TNF-αは検出されなかったが、250 μg とすることで血清中ならびに脾臓、肝臓で高レベルの TNF-αが検出された。CpG モチーフの少ない CT DNA では高投与量（250 μg）の場合でも TNF-αは検出限界以下だった。一方、カチオン性リポソーム複合体の場合には、25 μg DNA の場合にも CpG モチーフ依存的なTNF-αの産生が認められた。以上から、マウスに DNA を静脈内投与した場合には、CpG モチーフ数に依存した免疫活性化反応が惹起されること、またその活性化はカチオン性リポソーム複合体とすることで大幅に亢進することが明らかとなった。

pDNA 投与による炎症性サイトカイン産生におけるマクロファージの関与を明らかにするために、肝 Kupffer 細胞および脾臓マクロファージを機能的に除去したマウスを用いたところ、カチオン性リポソーム複合体投与による TNF-α産生はほぼ完全に消失した。一方、pDNA 単独投与の場合にはコントロール群と同程度の TNF-αが血清中に検出された。

(2) DNA による培養マクロファージからの TNF-αの産生：RAW264.7 では、CpG モチーフ数の少ない CT DNA を添加した場合と比較して、pDNA の添加により約 8 倍も高い TNF-αが産生された。脾臓マクロファージおよび肝非実質細胞においても、CpG モチーフに依存した TNF-α産生が認められた。一方、腹腔マクロファージおよび腎メサンギウム細胞からは、pDNA を添加しても TNF-α産生は認められなかった。各細胞での TLR9 発現は、脾臓マクロファージで最も高く、次いで肝非実質細胞、腹腔マクロファージの順であり、腎メサンギウム細胞では検出されなかった。従って、pDNA 単独に対するサイトカイン産生と、細胞における TLR9 発現との間には、正の相関が認められた。

カチオン性リポソーム複合体を添加した場合には腹腔マクロファージからも TNF-α の産生が認められた。しかしながら、その産生はカチオン性リポソームの種類・脂質組成に大きく影響されることが明らかとなり、DOTMA/Chol リポソームと他のカチオン性リポソーム（Lipofectin、Lipofectamine など）で大きく異なることが示された。

(3) CpG DNA 複合体投与による腹膜播種性癌転移抑制：CpG DNA 複合体投与後のマウス腹腔中サイトカイン濃度を測定したところ、生理食塩水投与（コントロール）群、random DNA 複合体投与群と比較して有意に高い TNF-α、IL-12 が検出された。そこで、B16-BL6/Luc または colon26/Luc 担癌マウスを用いて抗腫瘍効果を評価したところ、CpG DNA 複合体の投与により腹腔内臓器における癌細胞数がコントロール群の約 0.1%にまで減少した。これに伴い、生存日数も著明に延長した。この長期生存マウスに癌細胞を再移植したところ、50～70%程度のマウスが癌細胞を拒絶した。以上の結果から、複合体投与により一旦癌細胞の増殖が抑制されたマウスでは、全身的な癌免疫が誘導されており、再移植した同種の癌細胞は免疫反応により拒絶されたものと推察された。

(4) Y-DNA による TNF-α産生：RAW264.7 への Y-CpG DNA の添加により、高レベルの TNF-αが産生された。このときの TNF-α濃度は、同じ配列からなる dsCpG DNA よりも有意に高かったことから、Y 型という特徴的な DNA 構造が TLR9 を介するサイトカイン産生を亢進する可能性が示された。以上より、CpG DNA を Y 型構造にすることで、その免疫活性化能が増強可能であることが示された。

D. 考察

pDNA や合成オリゴヌクレオチドに含ま

れる非メチル化CpG配列、いわゆるCpGモチーフが、マクロファージや樹状細胞に発現するTLR9に認識されることで種々のサイトカインが産生されることが明らかとされてから、その機構解析、さらには治療への応用に関する膨大な数の論文が発表されてきた。CpG モチーフにより産生されるサイトカインは、Th1型に分類される抗腫瘍サイトカインが中心であることから、その癌治療への応用が期待されている。しかしながら、TLR9を介するCpG DNA認識、さらにはサイトカイン産生に関しては、おもにホスホロチオエート型オリゴヌクレオチドを用いた検討が行われており、その結果が必ずしもpDNAなどのホスホジエステル型DNAには当てはまらないことも指摘されている。さらには、カチオン性化合物との複合体化により CpG DNA によるサイトカイン産生能は、一般的には増大する傾向にあるが、最近の報告では必ずしも一義的に規定されないことも証明されつつある。本研究では、核酸をコアとすることで、免疫担当細胞からの抗腫瘍サイトカイン産生を誘導し、これによる抗癌剤との相乗効果を目的としたシステム開発を行うが、このDNAによる免疫活性化能を最大限に引き出すことを目的に種々の検討を行った。

マウスでの検討においては、DNA を静脈内投与した場合にはCpG モチーフに依存した免疫活性化反応が誘導されることが示された。また、その活性化はカチオン性リポソームとの複合体とすることで増大することも確認された。クロドロネート含有リポソームを用いた検討から、肝 Kupffer 細胞、脾臓マクロファージなどの貪食細胞が、pDNA に内在するCpG モチーフに対して活性化し、TNF-αなどのサイトカイン産生を誘導することが示された。このことは、マウスから単離した初代培養マクロファージを用いた検討からも支持された。その一方で、カチオン性リポソーム複合体によるサイトカイン産生には、細胞依存的現象に加えて、リポソームの組成によっても顕著に異なることが明らかとなった。従って、サイトカイン誘導による抗腫瘍効果を最大化するためには、複合体化に用いるカチオン性化合物のスクリーニングが重要と考えられる。今後この点について検討を進める予定である。また、固形腫瘍へのデリバリーが達成された場合には、腫瘍関連マクロファージや腫瘍関連樹状細胞での反応性が重要と考えられることから、こうした細胞での評価も必要と思われる。

CpG DNAによるサイトカイン誘導が、抗腫瘍効果に十分であるかを明らかにするためにマウス腹膜播種モデルを用いて評価した。その結果、CpG DNA複合体を腹腔内投与することで有意な抗腫瘍効果が得られ、一部のマウスにおいては癌を拒絶可能なレベルの抗腫瘍免疫が誘導可能であった。投与したCpG DNA複合体の体内動態およびサイトカインを産生する細胞の同定、さらには静脈内投与による効果等について今後検討する予定である。

pDNA は、均一な物性および構造のポリマーであり、大量調製が容易であることから、ナノ粒子型の薬物・免疫治療システムのキャリアに適していると考えられる。しかしながら、ポリエチレングリコール修飾を組み合わせたナノ粒子化に際しては、その反応の厳密な制御が困難であることも想定されるため製剤の均一性低下が懸念される。そこで、デリバリーシステムのコアに用いる核酸として、デンドリマー様 DNA の利用を考えた。まず、その基本骨格であるY型DNAにCpGモチーフを組み込み、その免疫活性化能を評価したところ、驚いたことに通常の 2 本鎖CpG DNAよりも有意に高いサイトカイン産

生能を示した。今後その詳細な機構を解明するとともに、ボトムアップ型DNAナノ粒子の設計・開発を進めていく予定である。

E. 結論

CpGモチーフを含むDNAを用いることでTNF-αやIL-12などのTh-1型サイトカインを誘導可能であり、特にカチオン性リポソームとの複合体とすることで高い抗腫瘍免疫効果が得られた。一方、DNA 立体構造に関する検討において、CpG DNA をY型とすることによる免疫活性化能の増大が得られた。

F. 健康危険情報

なし

G. 研究発表

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H. 知的所有権の出願・登録状況

1. 特許取得

なし

2. 実用新案登録

なし

3. その他

なし

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3

Pharmacokinetics of Plasmid DNA-Based Non-viral Gene Medicine

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ABSTRACT

Non-viral gene therapy can be realized by optimization of the pharmacokinetic properties of both the vector and the encoded therapeutic protein. A major obstacle to its successful clinical application is the limited ability of plasmid

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DNA, the most convenient gene-coding compound, to distribute within the body after *in vivo* administration. Under normal conditions, plasmid DNA and its non-viral vector complexes have difficulty in passing through various anatomical and biological barriers. These characteristics greatly limit the number and distribution of cells transduced with the vector, because transgene expression only occurs in cells that are reached by the vector. New approaches to the design of vectors as well as the methods of administration, such as electroporation and a hydrodynamic delivery, have increased the transgene expression *in vivo*, suggesting that improved distribution of plasmid DNA is possible by these approaches. In this chapter, the basic pharmacokinetic properties of naked plasmid DNA under normal conditions are first reviewed, then the properties of both naked and complexed plasmid DNA are discussed under conditions where significant transgene expression takes place.

I. INTRODUCTION

A vector encoding a therapeutic gene can be a delivery vehicle for a protein, or its precursor form, because the protein translated from the gene encoded in the vector is the active form exhibiting the therapeutic effects after administration of the vector. Therefore, the pharmacokinetic properties of the translated product should directly influence the efficacy of any application of *in vivo* gene transfer.

If a protein is secreted from the transduced cells into the circulating blood where it exhibits its biological activity, the pharmacokinetic profile of the protein, not the vector, determines the effectiveness of *in vivo* gene transfer. This can be applied to various proteins including blood coagulation factors, erythropoietin, epidermal growth factor and hepatocyte growth factor. The concentration of these proteins in plasma, or in extracellular fluids, determines the therapeutic efficacy. These characteristics of the minor importance of the cell-type producing the protein offers investigators the option of choosing a suitable target for *in vivo* gene transfer. In gene therapy protocols for hemophiliacs, not only hepatocytes that produce the coagulation factors in healthy subjects, but also other cells such as fibroblasts or muscle cells have been investigated as target cells producing these factors (Herzog *et al.*, 1999; Kay *et al.*, 2000; Manno *et al.*, 2003; Roth *et al.*, 2001; Snyder *et al.*, 1999). In such cases, the level and duration of transgene expression is closely linked to the efficacy of gene transfer. These parameters governing the expression can be a function of the characteristics of the cells transduced, the vector applied, and the administration method (Nishikawa and Hashida, 2002). For example, muscle cells generally produce very prolonged transgene expression compared with other cells, such as hepatocytes and vascular endothelial cells. In addition, physiological properties, such as blood flow rate to tissues and the structure

of blood capillaries, can influence the therapeutic efficacy by altering the rate and extent of delivery of the protein from the transduced cells to the site of action. Therefore, the pharmacokinetic properties of the vector are extremely important in determining the efficacy of *in vivo* gene transfer for secretion proteins, although they have often received little attention in the past.

In contrast to this, intracellular proteins function at an appropriate place within a specific type of cell, therefore, they should be introduced to the appropriate cell by *in vivo* gene transfer. Because the translated protein may not distribute to the outside of the cells transduced, the pharmacokinetic properties of the protein have little effect on therapeutic efficacy. On the other hand, the pharmacokinetic properties of the vector are extremely important; the distribution of the vector to the target cell after administration is essential for transgene expression in the target cell. Genes for metabolic enzymes, structural proteins and transporters need to be delivered to the right target, otherwise, no therapeutic benefits will be obtained although some transgene expression is obtained. Therefore, vectors encoding those intracellular proteins require a well-controlled *in vivo* delivery system.

Thus, the pharmacokinetics—which is the study of the absorption, distribution, metabolism and excretion of vectors for *in vivo* gene transfer—is of significant importance for the development of useful vectors and protocols for *in vivo* gene therapy. Generally speaking, plasmid DNA-based non-viral vectors offer the advantages of safety and versatility over viral vectors (Nishikawa and Huang, 2001). So far, several promising results involving gene transfer using plasmid DNA-based approaches have been reported in preclinical and clinical settings (Morishita *et al.*, 2004). However, these are limited to gene transfer of secretion proteins where the pharmacokinetic behavior of vectors has less significance on the final output. In contrast, few significant improvements have been reported for gene therapy involving intracellular proteins, indicating the difficulties in controlling the pharmacokinetics of vectors, including plasmid DNA.

Gene transfer is expected to occur in cells reached by the vector directly or via the blood circulation. Although conventional pharmaceutical compounds are given by various routes, (e.g., oral, nasal, pulmonary, conjunctival, transdermal and rectal administration), vectors including plasmid DNA are rarely administered by these routes with an expectation of systemic absorption followed by gene transfer at sites in the body other than the administration site. In addition, in contrast to conventional chemical compounds, plasmid DNA is metabolized into fragments and excreted, and both these processes have been shown to contribute little to the overall therapeutic efficacy of plasmid DNA-based approaches. Therefore, distribution is the most important process in the pharmacokinetics of plasmid DNA.

In this chapter, we summarize the tissue distribution properties of plasmid DNA after its injection into the systemic circulation or tissues, in the free or complexed form with targeted or non-targeted non-viral vectors. The contribution of macrophages to the distribution of naked plasmid DNA is discussed in relation to the involvement of scavenger receptor-like mechanisms in the cellular uptake. Then, several options for the improved delivery of plasmid DNA are reviewed with an emphasis on changes in the pharmacokinetics of plasmid DNA.

II. HOW TO TRACE PLASMID DNA *IN VIVO*

Plasmid DNA is chemically stable and easily handled. However, it is unstable after it enters the body. Incubation of plasmid DNA in freshly prepared mouse whole blood results in its degradation with an apparent half-life of about 10 min (Kawabata *et al.*, 1995). Furthermore, faster degradation of plasmid DNA has been detected after intravenous injection into mice. Nucleases mainly contribute to the degradation of plasmid DNA in the body; therefore, the degradation rate is a function of the type and amount of these enzymes in the compartment where the plasmid DNA distributes. For example, naked plasmid DNA injected into skeletal muscle may be considered stable for up to 4 h after intramuscular injection (Satkauskas *et al.*, 2001). As indicated in these previous studies, following administration, plasmid DNA is continuously degraded over time. Breaking down the linkages in plasmid DNA changes its size, a very important factor determining its pharmacokinetic behavior.

Degradation of plasmid DNA *in vivo* makes it very complicated to analyze the tissue distribution of 'biologically active' plasmid DNA. In an actual fact, the boundary between the active and inactive forms of DNA is not clear; degraded, linearized DNA fragments can be biologically active if they possess at least the minimal essential elements for transcription. Furthermore, the transcriptional activity of plasmid DNA depends on its functional form. Adami *et al.* (1998) reported that the open circular and linear forms of plasmid DNA were 90 and 10% as efficient as the supercoiled form. Although monitoring only the 'active' plasmid DNA is important for its therapeutic efficacy, the distribution of total plasmid DNA including the intact and degraded forms is discussed in most publications.

To avoid such a mix-up, the distribution of plasmid DNA in plasma can be examined by using analytical methods that can separate the different structural forms of plasmid DNA. A typical method is the separation of the different forms of plasmid DNA by agarose gel electrophoresis. Houk *et al.* (2001) reported that naked plasmid DNA injected into rats is successively

converted from the supercoiled to the open circular form, then the linear form with time. They reported that each form of the DNA had its own unique pharmacokinetic profile of elimination from plasma. Although this method can be used to accurately evaluate the plasma clearance of plasmid DNA, it is time-consuming, and is difficult to use for analyzing the distribution of DNA to tissues and organs. Another method is the polymerase chain reaction (PCR)-based detection of plasmid DNA (Oh *et al.*, 2001). In this case, a selected site of the DNA is amplified by PCR, so the integrity of the fragment is guaranteed. Because of the amplification, the sensitivity of this method is very high but, again, it is also time-consuming, and can only be easily applied to determine plasmid DNA in plasma or other body fluids, and not tissues. Real-time PCR can achieve a more rapid and quantitative determination of plasmid DNA in body fluids.

Using radioisotopes or fluorescent probes is one of the easiest, and most frequently used methods to trace the tissue distribution of plasmid DNA. Several techniques have been developed to this end. These include: ^{32}P-labeling by nick translation (Piatyszek *et al.*, 1988; Rigby *et al.*, 1977), covalent coupling of a fluorescent dye (Neves *et al.*, 2000; Slattum *et al.*, 2003), radioiodination of cytidine (Terebesi *et al.*, 1998), application of a fluorescent peptide nucleic acid clamp (Zelphati *et al.*, 1999), and metabolic labeling using ^{3}H-thymidine 5′-triphosphate (Wasan *et al.*, 1996). As discussed above, an important feature that should be taken into consideration is the changes in the structure of plasmid DNA produced by labeling. Recently, several labeling methods that do not significantly affect the transfection efficacy of plasmid DNA have been proposed (Nishikawa *et al.*, 2003; Slattum *et al.*, 2003; Zelphati *et al.*, 1999). The use of these techniques is suitable for studying the tissue distribution of plasmid DNA. We developed a residualizing radiolabel for plasmid DNA, in which ^{111}In is chelated to diethylenetriaminepentaacetic acid that is covalently conjugated to plasmid DNA through 4-[*p*-azidosalicylamido]butylamine (Nishikawa *et al.*, 2003). This labeling preserved the overall structure of plasmid DNA and its transcriptional activity was 40–98% that of the original, depending on the number of adducts introduced. Furthermore, compared with its ^{32}P-labeled counterpart, ^{111}In-labeled naked plasmid DNA showed a prolonged retention of radioactivity in the liver, the major organ taking up the DNA from the circulation, after intravenous injection into mice. This slow release of radioactive metabolites makes it easy to estimate the tissue uptake of plasmid DNA as well as its complex. We have shown that the level of transgene expression in the lung following the administration of polyethyleneimine/^{111}In-plasmid DNA complexes correlates well with the amount of radioactivity in the organ. Thus, residualizing radiolabels, which have been developed for proteins (Ali *et al.*, 1988; Deshpande *et al.*, 1990; Thorpe *et al.*, 1993), would be useful for evaluating

the tissue distribution of plasmid DNA and developing effective non-viral delivery methods.

III. PHARMACOKINETIC FEATURES OF NAKED PLASMID DNA AFTER INTRAVASCULAR INJECTION

It had been generally considered that naked plasmid DNA is unable to produce any significant transgene products *in vivo* due to its inability to enter cells and to its susceptibility to enzymatic degradation by various extracellular or intracellular nucleases (Barry *et al.*, 1999; Lechardeur *et al.*, 1999; Lew *et al.*, 1995; Pollard *et al.*, 2001). For example, when applied systemically via the vascular system, naked plasmid DNA is pharmacologically inactive and produces very little transgene expression, if any, because it is rapidly scavenged and degraded by the liver nonparenchymal cells, predominantly by Kupffer cells and the liver sinusoidal endothelial cells, as demonstrated in our own series of studies (Hisazumi *et al.*, 2004; Kawabata *et al.*, 1995; Kobayashi *et al.*, 2001).

This was changed by Wolff *et al.* (1990), who showed that a direct injection of naked plasmid DNA into mouse skeletal muscle resulted in significant transgene expression. Furthermore, the group also obtained significant intravascular gene transfer with vector-free naked plasmid DNA by injecting it in a large-volume solution. They reported that a high level of transgene expression could be obtained in mouse liver by injecting naked plasmid DNA in hyperosmotic solution into the portal vein with transient occlusion of the outflow (Budker *et al.*, 1996). More recently, in 1999, Liu *et al.* (1999) and Zhang *et al.* (1999) reported an innovative finding that an astonishingly high level of transgene expression could be obtained in mouse liver and other major organs by a simple intravenous injection of naked plasmid DNA via the tail vein using a large volume of saline introduced at a high velocity.

Thus, naked plasmid DNA can be an effective non-viral vector with the advantage of simplicity, if a suitable method of administration is used. The differences in transgene expression observed when different administration methods are applied correlate with the altered pharmacokinetic features of plasmid DNA. Because plasmid DNA is a huge macromolecule with a high molecular weight of about 2,000 kD or more and a strong anionic charge, its distribution within the body is greatly limited. Therefore, the development of better non-viral vectors or administration methods requires an understanding of the fate of plasmid DNA *in vivo*. First, the tissue distribution characteristics of naked plasmid DNA are discussed in relation to its method of administration: normal intravenous injection, large-volume injection, the so-called hydrodynamics-based procedure (Liu *et al.*, 1999), and the procedure involving local application at a particular site.

A. Normal procedure

When naked ^{32}P-plasmid DNA was injected as a bolus into the tail vein of mice at a dose of 1 mg/kg (20 μg/20 g-body weight mouse), it rapidly disappeared from plasma and 60–70% of the injected dose was recovered in the liver within 5 min of injection (Kawabata *et al.*, 1995). The pharmacokinetic analysis of the tissue distribution based on a clearance concept (Nishikawa *et al.*, 1996) revealed that the apparent hepatic uptake clearance is close to the hepatic plasma flow rate. Under these conditions, naked plasmid DNA resulted in no transgene expression in major organs. This would be attributed to the extensive uptake and degradation of plasmid DNA by the liver nonparenchymal cells, such as Kupffer cells and liver sinusoidal endothelial cells. The hepatic uptake of plasmid DNA was dependent on the concentration and decreased on increasing the dose. In addition, we showed that the hepatic uptake of plasmid DNA is inhibited by calf thymus DNA, polyinosinic acid, dextran sulfate, and heparin, but not by polycytidylic acid and chondroitin sulfate (Kawabata *et al.*, 1995; Kobayashi *et al.*, 2001; Yoshida *et al.*, 1996). These findings indicate that plasmid DNA is taken up by scavenger receptors, which recognize polyanions in a charge- and/or structure-dependent manner (Terpstra *et al.*, 2000). However, we excluded the possibility of class A scavenger receptors (SRA) being involved in the uptake, based on the tissue distribution and uptake experiments using cultured macrophages from SRA-knockout mice (Takakura *et al.*, 1999). Another study using SRA-knockout mice supported the conclusion that SRA are not involved in the uptake of plasmid DNA (Zhu *et al.*, 2001).

The detailed characteristics of the uptake of plasmid DNA were examined using cultured cells. Various cells have the ability to take up plasmid DNA in a concentration- and temperature-dependent manner. The major cells responsible for the uptake of plasmid DNA are macrophages, as indicated in tissue distribution studies of plasmid DNA in which Kupffer cells, liver resident macrophages, make a large contribution to the clearance of plasmid DNA after systemic administration. Under *in vitro* culture conditions, mouse peritoneal macrophages efficiently took up plasmid DNA (Takagi *et al.*, 1998). Liver sinusoidal endothelial cells, another type of liver nonparenchymal cell, also possess the ability to scavenge a variety of polyanions circulating in the blood (Yamasaki *et al.*, 2002, 2003). A recent study of the tissue distribution of plasmid DNA in rats indicated that liver sinusoidal endothelial cells take up plasmid DNA as effectively as Kupffer cells (Hisazumi *et al.*, 2004). Liver sinusoidal endothelial cells isolated from rats showed a significant uptake and degradation of plasmid DNA in culture. Dendritic cells (DCs), another population of immune cells, are very important as far as both innate and acquired immunity are concerned. Again, the DC cell line, DC2.4, exhibits extensive uptake and degradation of plasmid DNA (Yoshinaga *et al.*, 2002). Primary mouse DCs

also exhibited similar processing of plasmid DNA to this cell line. We also observed that bovine brain microvessel endothelial cells, which constitute the blood-brain barrier, are also able to recognize plasmid DNA (Nakamura *et al.*, 1998).

When the uptake of plasmid DNA by any of these types of cells was examined in the presence of neutral or anionic polymers, an almost identical profile of inhibition was observed. The uptake as well as binding were significantly inhibited by excess plasmid DNA, polyinosinic acid, dextran sulfate or heparin, but not by polycytidylic acid and dextran (Hisazumi et al., 2004; Nakamura et al., 1998; Takagi et al., 1998; Yoshinaga et al., 2002). This inhibition pattern resembles closely that of the *in vivo* study described above, supporting the hypothesis that these cells, i.e., macrophages, liver sinusoidal endothelial cells and DCs, make a major contribution to the clearance of plasmid DNA *in vivo*. The exact mechanisms underlying the uptake, including the receptors involved, are yet to be identified. Because of the small number of DCs in the body, their contribution to the distribution of plasmid DNA will be less significant than those of macrophages and liver sinusoidal endothelial cells.

B. Hydrodynamics-based procedure

Hydrodynamics-based gene delivery, involving a large-volume and high-speed intravenous injection of naked plasmid DNA, gives a significantly high level of transgene expression *in vivo* (Liu *et al.*, 1999). Because of the simplicity and the extraordinary efficiency of transgene expression among the current non-viral methods, it has attracted a lot of attention and has been used very frequently as an efficient, simple, and convenient transfection method for laboratory animals. Until recently, however, there was little published information on the pharmacokinetics of the injected plasmid DNA molecules and of the detailed mechanisms underlying the efficient gene transfer.

We investigated the *in vivo* pharmacokinetic characteristics of naked plasmid DNA in mice undergoing the hydrodynamics-based procedure (Kobayashi *et al.*, 2001), in comparison with the normal procedure (up to 200 μl for a 20-g mouse). The time-course profile and the degree of hepatic accumulation of ^{32}P-plasmid DNA were very similar for the hydrodynamics-based and normal procedures: In both cases, plasmid DNA was rapidly eliminated from the blood circulation and taken up mainly by the liver. While the hepatic accumulation profiles of ^{32}P-plasmid DNA were almost identical for the two injection procedures, marked transgene expression could be achieved by the hydrodynamics-based procedure but not by the normal one. The results of our confocal microscopic studies of liver sections indicated widespread intrahepatic distribution of fluorescein-labeled plasmid DNA following the hydrodynamics-based procedure. In addition, polyanions such as poly inosinic acid, dextran sulfate

and heparin, which significantly inhibit the hepatic uptake of plasmid DNA injected by the normal procedure, did not affect the hepatic uptake of plasmid DNA given by the hydrodynamics-based procedure. These results indicate that the hepatic uptake process of the plasmid DNA is different from that following normal intravenous injection where receptor-like mechanisms are most likely involved (Emlen *et al.*, 1988; Kawabata *et al.*, 1995; Kobayashi *et al.*, 2001; Yoshida *et al.*, 1996). Under normal conditions, the plasmid DNA molecules, injected slowly via the tail vein with a conventional volume of solution, enter the blood stream and are carried to the heart, and then distributed systemically. Due to the large amount of nucleases in the blood and other compartments such as on the surface of tissues (Emlen *et al.*, 1988), plasmid DNA injected by the normal procedure is likely to be rapidly degraded in the circulation and subsequently in liver cells after being recognized and taken up by liver nonparenchymal cells. On the other hand, part of the plasmid DNA injected by the hydrodynamics-based procedure is directly exposed to the liver cells and some of it is taken up by the cells in intact form before being mixed with blood. This may account for the observation that a high level of transgene expression can be obtained by the hydrodynamics-based procedure, but not by the normal one. In an actual fact, the persistent presence of a significant level of intact plasmid DNA has been demonstrated in the liver (Liu *et al.*, 1999). Also, delayed elimination of plasmid DNA from the plasma pool was observed in the hydrodynamics-based procedure, supporting the reduced degradation of plasmid DNA by nucleases (Kobayashi *et al.*, 2001).

Until recently, little was known about the mechanisms underlying efficient gene transfer by this procedure. Liu *et al.* (1999) demonstrated that a rapid injection and a large volume of plasmid DNA solution were required to obtain a high level of transgene expression. Following the first reports of the large-volume tail vein injection (Liu *et al.*, 1999; Zhang *et al.*, 1999), Budker *et al.* (2000) hypothesized that the cellular uptake mechanism of naked pDNA involved an active, receptor-mediated process. In addition, Lecocq *et al.* (2003) demonstrated in a subcellular distribution study using differential centrifugation methods that ^{35}S-plasmid DNA remained bound to the outside surface of the plasma membrane for at least 1 h after the hydrodynamics-based procedure, supporting the hypothesis that plasmid DNA was internalized slowly via a specific mechanism. On the other hand, we demonstrated in our *in vivo* pharmacokinetic studies of naked plasmid DNA, involving the normal or hydrodynamics-based procedure, that the hepatic uptake process appeared to be nonspecific (Kobayashi *et al.*, 2001). Liu *et al.* reported that β-galactosidase and Evans blue were efficiently delivered to hepatocytes by the hydrodynamics-based procedure (Zhang *et al.*, 2004). Notably, in their electron microscopic observations, identifiable membrane defects or pores were detected in the hepatocytes, which were generated by the high-pressure solution. Based on these

findings, they concluded that the hydrodynamics-based gene transfer was a nonspecific physical process. We also demonstrated transient hyperpermeability in the hepatic cellular membrane when using the hydrodynamics-based procedure (Kobayashi *et al.*, 2004a). Propidium iodide was effectively incorporated by the liver cells following the hydrodynamics-based procedure and the green fluorescent protein expressed beforehand and accumulating internally in the cytosol was dramatically eliminated from hepatocytes following a large-volume injection of saline. These results suggest a facilitated permeation of propidium iodide and green fluorescent protein through the cell membrane because these molecules are not supposed to cross the plasma membrane of viable cells. Nonspecificity in the cellular uptake process of plasmid DNA was further confirmed by a competitive study. A saturable amount of empty vector did not inhibit transgene expression in the liver following the hydrodynamics-based procedure (Kobayashi *et al.*, 2004a). Furthermore, fluorescein-labeled polystyrene microspheres of 50 nm in diameter were delivered into the intracellular compartment of hepatocytes following the hydrodynamics-based procedure in mice (Kobayashi *et al.*, 2004b).

C. Hydrodynamics at local sites

The principle of the hydrodynamics-based procedure is reproducible and applicable to an organ-restricted gene delivery method, where plasmid DNA is injected *in situ* into tissue-associated vessels, as demonstrated earlier by Wolff's group (Budker *et al.*, 1996, 1998; Zhang *et al.*, 1997, 2001). Zhang *et al.* (1997) showed that substantial amounts of transgene products were obtained by an injection of naked plasmid DNA-containing solution into the afferent and efferent vessels (i.e., the portal vein, hepatic vein, and bile duct) of the liver in mice, rats, and dogs. The hindlimb muscles were also successfully targeted by an intra-arterial injection of a large volume of naked plasmid DNA into the femoral artery (Budker *et al.*, 1998; Liang *et al.*, 2004; Zhang *et al.*, 2001). The amounts of plasmid DNA in hindleg muscles of mice were much greater after a large-volume injection of naked plasmid DNA than after a normal injection, suggesting that changes take place in the tissue distribution of plasmid DNA (Liang *et al.*, 2004).

IV. PHARMACOKINETIC FEATURES OF PLASMID DNA/NON-VIRAL VECTOR COMPLEX AFTER INTRAVENOUS INJECTION

Plasmid DNA is a huge macromolecule with a strong negative charge, and it has very limited access to the nucleus when administered by the normal procedure as described above. To facilitate the binding to cells, cationic vectors are often

used and optimization of the structure and function is a major challenge as far as non-viral vector (carrier) development is concerned.

Formation of a cationic complex of plasmid DNA greatly increases its interaction with the lung endothelial cells upon intravenous injection (Liu *et al.*, 1997; Mahato *et al.*, 1995). Not only the uptake but also the transgene expression is also increased in these cells after intravenous injection of cationic complexes. This is the result of a complicated series of events occurring *in vivo* after intravenous injection of plasmid DNA complex. Serum proteins (Li *et al.*, 1999) and blood cells (Sakurai *et al.*, 2001a,b) have been reported to affect the tissue distribution of intravascularly administered plasmid DNA complex. While plasmid DNA complex is able to avoid first-pass filtration in the lung, only a relatively small DNA complex can pass through the blood vessels and directly interact with the parenchymal cells of each tissue.

A. Lipoplex

An enormous number of cationic lipid/liposome systems have been developed to improve the transfection efficiency of plasmid DNA, some of which have been summarized in a recent publication (Audouy *et al.*, 2002). Cationic liposomes associate with plasmid DNA via an electrostatic interaction, resulting in the formation of a complex called lipoplex (Felgner *et al.*, 1997). The driving force for lipoplex to introduce genes into cells is its electrostatic binding to negatively-charged cellular membranes followed by endocytotic uptake. Some studies have shown that co-lipids, so-called helper lipids, in cationic liposomes are important determinants of the transfection efficiency.

In general, lipoplex is rapidly cleared from the blood circulation after intravenous injection and very little (only a few percent) of the lipoplex remains in the blood 10 min after injection (Mahato *et al.*, 1995, 1998). It immediately distributes to the lung, because it is the first-pass organ after intravenous injection. Then, significant amounts of lipoplex, once captured by the lung, redistribute to the liver. Several reports have suggested that intravenously administered lipoplexes are mainly taken up by liver Kupffer cells (Litzinger *et al.*, 1996; Mahato *et al.*, 1995; McLean *et al.*, 1997). The functional deletion of these cells by gadolinium chloride resulted in a reduced amount of lipoplex taken up by the liver (Sakurai *et al.*, 2002), indicating a significant contribution of Kupffer cells to the pharmacokinetics of lipoplex.

These characteristics of lipoplex, as far as tissue distribution is concerned, do not merely result from the physicochemical properties of lipoplex. A positively charged lipoplex surface would attract various types of blood components. Under *in vitro* conditions, the presence of serum has been shown to strongly affect, in most cases reduce, lipoplex-mediated gene transfer (Li *et al.*, 1999). Yang and Huang (1997) have shown that the inactivation of lipoplex by