厚生労働科学研究費補助金

医療機器開発推進研究事業

核酸をコアとするナノ微粒子による薬物・免疫治療システムの開発に関する研究

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主任研究者　　西川 元也

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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

主任研究者　　西川元也　京都大学大学院薬学研究科准教授

研究要旨　前年度までの検討において、それぞれ半分ずつ相補的な 3 種類のオリゴヌクレオチド（ODN）を用いて調製した Y 型 2 本鎖 ODN（Y-ODN）が、通常の一本鎖あるいは二本鎖 ODN と比較して有意に高いサイトカイン産生能を有することを明らかにした。そこで本年度は、ODN の免疫活性化能をさらに増強することを試みた。Y-ODN を順次連結することで構築した世代数 3 のデンドリマー様 DNA は、マクロファージ様細胞 RAW264.7 への添加により Y-ODN と比較して有意に高い腫瘍壊死因子α産生を誘導した。フォスフォロチオエート（PS）型 ODN による安定化の影響を検討したところ、非メチル化 CpG 配列（CpG モチーフ）を含む ODN 鎖を PS 型とすることで、細胞からのサイトカイン産生は有意に増大した。一方、腫瘍内に浸潤する免疫担当細胞を標的とした DNA－抗癌剤デリバリーについて、プラスミド DNA－ドキソルビシン（DXR）結合体の有用性を評価した。CpG モチーフを含む DNA を用いて作製した DXR 結合体は、それぞれ単独の場合と比較して有意に高い結腸癌細胞増殖抑制効果を示した。また、CpG モチーフを含まない DNA では抑制効果は低く、CpG モチーフによる免疫細胞活性化が癌細胞増殖抑制に有効であることが示された。肝転移モデルマウスへの投与によっても、DNA-DXR 結合体投与により CpG モチーフ依存的な血中インターロイキン 12 濃度の増加が検出された。以上より、担癌モデルマウスで認められた DNA-DXR 結合体の高い抗腫瘍効果には、DXR の殺細胞効果に加えて CpG モチーフにより誘導されるサイトカインの関与が示された。

A. 研究目的

腫瘍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することで腫瘍組織への薬物ターゲティングが可能である。これまでに、リポソームやナノスフェアなどをキャリアとした研究が精力的に行われた結果、臨床で利用可能な製剤が開発されるに至っている。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抗腫瘍免疫療法にも多大な関心が寄せられている。DNA ワクチンの場合には、免疫担当細胞で抗原が直接発現することに加えて、DNA 中の非メチル化 CpG 配列（CpG モチーフ）による免疫担当細胞の活性化が抗腫瘍免疫誘導を増強するとされる。そこで本研究では、DNA をコアとしたナノ粒子を新たに設計・構築し、これに抗癌剤と CpG モチーフとを組み込むことで、化学療法及び免疫療法を同時に実現可能な治療システムの開発を試みる。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癌細胞をはじめとする異物を排除する機能が低下し、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報告されている。CpG モチーフは自然免疫を活性化することから、核酸をコアとする薬物キャリアを用いることで、腫瘍組織中に浸潤した免疫担当細胞の活性化による抗腫瘍免疫の誘導が期待される。また、DNA はドキソルビシン（DXR）など DNA インターカレータとの結合能を有することから、核酸ナノ粒子を用いることで、抗癌剤を腫瘍組織へターゲティングすることも可能と考えられる。抗腫瘍免疫を活性化するとともに抗癌剤による殺細胞効果が期待される本ナノサイズ DDS は、メカニズムの異なる 2 つの癌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を利用した抗癌剤 DDS とは異なり、相乗的かつ高い抗腫瘍効果が期待される。

B. 研究方法

(1) Y-ODN 連結によるデンドリマー様 DNA の開発：それぞれ半分ずつ相補的な 3 種類の 30 塩基 ODN を当モルずつ混合し、95 ℃で加熱後徐々に冷却することで Y-ODN を調製した。このとき配列中に強力な CpG モチーフ GACGTT を挿入することで Y-CpG ODN を設計した。4 種類の異なる Y-ODN（1 種類の Y-CpG ODN を含む）を作製し、DNA ligase を用いて順次結合することで、世代数 3 のデンドリマー様 DNA（G3-dendritic CpG DNA：G3）を構築した。

(2) PS 型 ODN を含む Y-ODN の調製：CpG モチーフを Y0a 鎖に 1 つ含む Y-CpG ODN を設計した。Y-CpG ODN を構成する ODN（Y0a、Y0b、Y0c）それぞれについてリン酸ジエステル結合（PO）を PS に置換した ODN(S)を用意した。また、CpG モチーフを含む Y0a 鎖に関しては、両末端から 3 個の結合のみを置換した ODN(S_3)も設計した。PO 型及び PS 型 ODN を種々組み合わせて Y-CpG ODN を調製した。

(3) コレステロール修飾 CpG ODN（CpG ODN-Chol）の合成：CpG モチーフを含む 1 本鎖 ODN にアミンを導入し、cholesteryl chloroformate を反応させることで CpG ODN-Chol を合成した。

(4) ODN の物性評価：各種 ODN の見かけのサイズは光散乱光度法により測定した。ポリアクリルアミドゲル電気泳動により Y-ODN 形成及び血清中での安定性を、サーマルサイクラーにより融解温度（Tm）を測定した。

(5) 免疫活性化能の評価：マウスマクロファージ様細胞株 RAW264.7 細胞を播種し、24 時間後に Y-ODN または G3 を種々の濃度（2、6、18 μg/ml）で添加した。活性化の指標として、上清中腫瘍壊死因子（TNF）-α及びインターロイキン（IL）-6、IL-12 濃度を ELISA 法により測定した。

(6) DNA-DXR の調製：CpG モチーフを含有する DNA（CpG DNA）として pcDNA3.1、含有しない DNA（non-CpG DNA）として pCpG-mcs を選択し、DXR と重量比 10:1 で混合し、室温で 1 時間静置することで結合体を調製した。

(7) DNA-DXR 結合体による癌細胞増殖抑制：トランズウェル上段に RAW264.7 細胞を、下段にホタルルシフェラーゼ（Luc）を安定に発現するマウス結腸癌細胞 colon26/Luc を播種した。培養 24 時間後に DXR または DNA、DNA-DXR を RAW 細胞側に添加し、48 時間培養後ルシフェラーゼ活性を指標に癌細胞数を測定した。

(8) 担癌マウスでの DNA に対する免疫応答：colon26/Luc (1×10^5 cells/mouse) を BALB/c 雄性マウスの門脈内に移植することで肝転移モデルマウスを作製した。移植 8 日後、担癌マウスに DNA あるいは DXR、DNA-DXR 結合体を静脈内投与し、6 時間後の血清および肝臓中 IL-12 濃度を ELIZA 法により測定

した。CpG DNA 及び non-CpG DNA を用いることでサイトカイン産生における CpG モチーフの関与について評価した。

C. 研究結果

(1) Y-ODN 及び G3 によるサイトカイン産生：電気泳動により、一部不完全ではあるもののデンドリマー様 DNA の形成が確認された。得られた G3 の見かけのサイズは約 23nm であり、腫瘍組織への受動ターゲティングに適したサイズであることが確認された。RAW264.7 細胞に添加により産生される TNF-α濃度は、Y-CpG ODN と比較して有意に高く、特に低濃度（2 μg/ml）では約 5 倍高い TNF-αが検出された。

(2) PS 型 ODN 含有 Y-ODN によるサイトカイン産生：Y-CpG ODN による TNF-α産生は、CpG モチーフを含む ODN の両末端から 3 個あるいは全てのリン酸結合を PS 型とすることで有意に増大した。CpG ODN(S)と CpG ODN(S_3)との間に顕著な違いは認められなかった。CpG ODN 鎖を PS 型 ODN とすることで、血清中での安定性が増大したことから、活性化能の増強は安定性の増大によることが示唆された。また、PS 型 ssCpG ODN よりも高いレベルの TNF-αが得られたことから、PS 型 ODN の場合にも Y 型形成によりサイトカイン産生が増大することが示された。一方、CpG モチーフを含まない ODN を PS 型に置換した場合のサイトカイン産生は、全て PO 型 ODN で調製した Y-CpG ODN と同等であった。また、3 種類の ODN を PS 型とした場合には有意なサイトカイン産生低下が観察された。このとき、Tm 値は有意に低下したことから、全ての ODN 鎖を PS 型とすることで Y 型が形成されにくくなる可能性が示された。

(3) CpG ODN-Chol によるサイトカイン産生：CpG ODN-Chol は、コレステロール導入数及び導入部位依存的に自己会合体を形成することが明らかとなった。RAW264.7 細胞に添加することで産生される TNF-αは、コレステロール未修飾 CpG ODN よりも圧倒的に高いことが示された。アミン結合体や rhodamine 修飾体、あるいはコレステロールと CpG ODN との混合物では高いサイトカイン産生は認められなかったことから、CpG ODN-Chol による高いサイトカイン産生には ODN に対するコレステロール修飾が必須であることが示された。

(4) DNA-DXR による癌細胞増殖抑制：CpG DNA の RAW264.7 細胞への添加により、colon26/Luc 細胞の増殖は若干抑制されたが、non-CpG DNA 添加では増殖抑制効果は認められなかった。DXR 添加により colon26/Luc 細胞の増殖は顕著に抑制され、CpG DNA の併用によりその抑制効果は増大した。

(5) 担癌マウスでの DNA-DXR に対する応答性の評価：colon26/Luc 細胞をマウス門脈内に移植することで作製した肝転移モデルマウスに対し、CpG DNA あるいは non-CpG DNA を静脈内投与したところ、CpG DNA 投与の場合に限り投与量依存的に血中 IL-12 濃度の上昇が観察された。CpG DNA-DXR に関しても同様の検討を行ったところ、CpG DNA の場合と比較すると低いものの、有意な IL-12 産生が認められた。

D. 考察

CpG DNA 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、Toll-like receptor-9 (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 PS 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プラスミド DNA などの PO 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。さらに最近の研究から、CpGモチーフがないDNAを認識してインターフェロンβ産生を誘導する機構の存在も明らかとされてきた。これらの情報は、DNAが免疫活性化剤として利用可能であることを意味すると同時に、その臨床応用にはまだまだ基礎情報集積の必要性を示唆するものである。

本研究では、核酸（DNA）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てきた。昨年度の検討において、ODNをY型に組み上げることで、RAW264.7細胞からのサイトカイン産生を有意に増大可能であることを見出した。そこで本年度は、この知見をもとにより強力な免疫活性型DNAの開発に取り組んだ。Y-ODNを順次連結した世代数3のデンドリマー様DNA（G3）は、Y-CpG ODNと比較して有意に高いサイトカイン産生を示した。設計上の理由により、今回開発したG3には最外殻の12個のY-ODNにのみ各2個のCpGモチーフが含まれている。従って、1 CpGモチーフ当たりの活性は、デンドリマー様構造とすることで飛躍的に高まることが示唆された。この活性増大が、Y-ODNの場合に認められた細胞による取り込み増大に起因するかについては今後の検討が必要である。今回開発したG3は、腫瘍組織へのデリバリーに適したサイズ（約20 nm）であることから、G3を基本骨格としてナノ粒子を開発することで、効率的な腫瘍ターゲティングが実現可能と考える。

昨年度の検討から、通常のdsDNAと比較するとY-ODNは若干不安定であることが示唆されたため、PS型ODNの利用による安定化について検討した。その結果、1種類のODNをPS型としたY-ODNの場合にはTm値の有意な低下は認められず、血清中での安定性は増大する傾向が認められた。一方、2あるいは3種類のODNをPS型とした場合には、Tmの有意な低下が認められた。CpG ODNを部分的あるいは完全にPS型とすることで有意なサイトカイン産生の増大が認められた反面、Tm値の低下が認められたODNではサイトカイン産生量も低いことが示された。以上の結果は、Y-ODNによる免疫活性化においてODNの安定性は重要であるものの、それ以上にY-ODNの構造安定性が重要であることが示された。

一方、コレステロール修飾CpG ODNを用いた検討からは、適当な化学修飾を施すことによる活性増強の可能性が示された。デンドリマー様DNAには修飾可能なDNA末端が数多く存在し、G3ではその数は24個である。従って、化学修飾を利用して種々の構造を導入することで、DNA構造体の高機能化が期待される。腫瘍デリバリーにおいては、ポリエチレングリコール修飾など動態制御のための構造付加による活性増強も考えられることから、こうした末端修飾の可能性も含めて、このボトムアップ型DNAナノ粒子の設計・開発をさらに進める予定である。

昨年度までの検討において、プラスミドDNAとDXRとの結合体を投与することで、肝臓での癌細胞増殖を効率よく抑制可能であることを示した。この増殖抑制に、CpGモチーフによる免疫活性化が関与するかについて検討したところ、CpG DNA結合体の場合に限り高いIL-12産生が認められた。CpG DNA-DXR結合体を投与した場合には、IL-12レベルは若干低くなる傾向が認められ

たが、これは免疫担当細胞に対するDXRの影響が出たものと推察される。CpG DNAとDXRとの併用による癌細胞増殖抑制は、培養細胞を用いた検討においても認められた。プラスミドDNAと比較するとデンドリマー様DNAは嵩高いこと、そのため内部に空間が存在することが推定される。従って、DNAからのDXR放出はデンドリマー様DNAのほうが緩徐であることが想定される。今後、デンドリマー様DNAを利用したシステム開発を行う予定である。

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、ODNのY型化、さらにはデンドリマー様構造体の構築により免疫活性化能の増大が得られた。また、DXRをDNAに結合することで高い抗腫瘍効果を得た。

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

雑誌

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IMMUNOLOGY ORIGINAL ARTICLE

Enhanced immunostimulatory activity of oligodeoxynucleotides by Y-shape formation

Makiya Nishikawa, Mitsuhiro Matono, Sakulrat Rattanakiat, Nao Matsuoka and Yoshinobu Takakura

Department of Biopharmaceutics and Drug Metabolism, Graduate School of Pharmaceutical Sciences, Kyoto University, Sakyo-ku, Kyoto, Japan

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Correspondence: M. Nishikawa, Department of Biopharmaceutics and Drug Metabolism, Graduate School of Pharmaceutical Sciences, Kyoto University, Sakyo-ku, Kyoto 606-8501, Japan. Email: makiya@pharm.kyoto-u.ac.jp
Senior author: Y. Takakura, email: takakura@pharm.kyoto-u.ac.jp

Summary

DNA containing unmethylated CpG dinucleotides (CpG DNA) is a potent activator of innate and acquired immune responses. Although the sequence-specific immunostimulatory activity of CpG DNA has been extensively explored, little information is available about the importance of the stereochemical properties of CpG DNA. In this study, Y-shaped oligodeoxynucleotides (Y-ODNs) were prepared using three ODNs with the halves of each ODN being partially complementary to a half of the other two ODNs. Y-ODN induced greater amounts of tumour necrosis factor-α and interleukin-6 from RAW264.7 macrophage-like cells than did conventional single-stranded ODN (ssODN) or double-stranded ODN (dsODN). The Y-ODN was less stable in serum than dsODN, but greater amounts of Y-ODN were taken up by macrophage-like cells compared with dsODN. A newly designed Y-ODN containing three potent CpG motifs generated significantly higher levels of cytokines compared with dsODN containing the identical sequences. These results indicate that the Y-shaped form of ODN is a novel, reproducible and reliable approach to enhancing the immunostimulatory activity of ODNs.

Keywords: CpG motif; immunostimulatory activity; oligodeoxynucleotides; Toll-like receptor 9; Y-shape formation

Introduction

Bacterial DNA contains unmethylated CpG dinucleotides (CpG motifs) that induce a potent immunostimulatory response upon recognition by the Toll-like receptor 9 (TLR9) expressed on dendritic cells, B cells and macrophages.[1,2] Synthetic oligodeoxynucleotides (ODNs) containing CpG motifs (CpG ODNs) can mimic the immunostimulatory activity of bacterial DNA and exhibit similar immune responses.[3,4] Once activated by bacterial DNA or CpG ODN, i.e. CpG DNA, immune cells secrete various cytokines, including interleukin-6 (IL-6), IL-12, interferon-α/β (IFN-α/β), IFN-γ and tumour necrosis factor-α (TNF-α), and increase the expression of various costimulatory molecules.[5,6] Thus, CpG DNA can induce T helper type 1 cytokine production; this promotes a cytotoxic T-lymphocyte response with enhanced immunoglobulin production, which has been used in the treatment of a broad spectrum of diseases, including cancer, viral and bacterial infections, allergic diseases and inflammatory disorders.[1,7–9]

The immunostimulatory activity of DNA has been extensively investigated using ODNs with varying base combinations, and several rules have been proposed. The activity of CpG DNA depends on the flanking sequences, and an ODN containing a GACGTT hexameric nucleotide motif strongly stimulates the immune system in rodents.[10,11] Other parameters have also been reported to be important for immunostimulatory activity of DNA, e.g. TpC dinucleotide on the 5′ end, pyrimidine-rich on the 3′ side of the motif and the presence of two or three CpG motifs in a sequence.[1] Thus, the optimal sequence of CpG ODN for activating mouse or human immune cells was elucidated by examining many possible base combinations. Although a few reports have shown that aggregation of ODNs increases their immunostimulatory activity,[12,13] the stereochemical effects of CpG ODN on immunostimulatory activity have hardly been explored.

DNA possesses many desirable chemical and physical properties as a polymeric material and much progress has been made in DNA computing [14,15] and DNA nanotechnology.[16–19] Recently, Li *et al.* established a reproducible method for constructing dendrimer-like DNA, by connecting Y-shaped DNA (Y-DNA) composed of three ODNs.[20] This unique-structured DNA has been applied

to various experimental settings, including nanobarcodes.[21] However, the biological and immunological characteristics of such structured DNA preparations have not been examined. Their unique structure may be recognized differently by immune cells.

In the present study, Y-ODN was prepared using three ODNs with the halves of each ODN being partially complementary to a half of the other two ODNs. Then, the immunostimulatory activity of Y-ODN was examined using RAW264.7, a mouse macrophage-like cell line. Here, we show that newly designed Y-ODN containing three potent CpG motifs can be a powerful immunostimulatory compound through increased uptake by immune cells.

Materials and methods

Chemicals

Dulbecco's modified Eagle's minimum essential medium (DMEM), RPMI-1640 medium and phosphate-buffered saline (PBS) were obtained from Nissui Pharmaceutical (Tokyo, Japan). Fetal bovine serum (FBS) was obtained from MP Biomedicals (Eschwege, Germany). Opti-modified Eagle's medium (Opti-MEM) was purchased from Invitrogen (Carlsbad, CA). DNase I and 20-base-pair (bp) DNA ladder were purchased from Takara Bio (Otsu, Japan). Polymyxin B sulphate salt was purchased from Sigma Chemical Co. (St Louis, MO). All other chemicals were of the highest grade available and were used without further purification.

Cell cultures

RAW264.7 macrophage-like cells were grown in RPMI-1640 medium supplemented with 10% heat-inactivated FBS, 0·15% $NaHCO_3$, 100 units/ml penicillin, 100 μg/ml streptomycin and 2 mM L-glutamine at 37° in humidified air containing 5% CO_2. Cells were then plated on 24-well culture plates at a density of 5×10^5 cells/ml and cultured for 24 hr. B16-BL6/Luc, a clone of murine melanoma B16-BL6[22] that stably expresses the firefly luciferase gene,[23] was grown in 5% CO_2 in humidified air at 37° with DMEM supplemented with 10% FBS, 100 IU/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine. They were then plated on 24-well culture plates at a density of 2×10^4 cells/ml and cultured for 8 hr.

Oligodeoxynucleotides

Phosphodiester ODNs (Table 1) were purchased from Invitrogen. For cellular uptake studies, Y0a labelled with fluorescein at the 5′ end was used for all fluorescein-labelled ODN preparations.

Table 1. Oligodeoxynucleotide (ODN) sequences used for preparation of single stranded-, double stranded- and Y-shaped ODNs

Name	Sequence (5′→3′)
Y0a	TGACTGGATCCGCATGACATTCGCCGTAAG
Y0b	TGACCTTACGGCGAATGACCGAATCAGCCT
Y0c	TGACAGGCTGATTCGGTTCATGCGGATCCA
r.Y0a	TGACCTTACGGCGAATGTCATGCGGATCCA
r.Y0b	TGACAGGCTGATTCGGTCATTCGCCGTAAG
r.Y0c	TGACTGGATCCGCATGAACCGAATCAGCCT
Y0a(CpG)	TGACGACGTTCGCATGACATTCGCCGTAAG
Y0b(CpG)	TGACCTTACGGCGAATGACCGAATCAGCCT
Y0c(CpG)	TGACAGGCTGATTCGGTTCATGCGAACGTC
Y0a(CpG_3)	TCGACGTTTCCGCATGACATTCGCCGAACG
Y0b(CpG_3)	TCGACGTTCGGCGAATGACCGAATCAAACG
Y0c(CpG_3)	TCGACGTTTGATTCGGTTCATGCGGAAACG
r.Y0a(CpG_3)	TCGACGTTCGGCGAATGTCATGCGGAAACG
r.Y0b(CpG_3)	TCGACGTTTGATTCGGTCATTCGCCGAACG
r.Y0c(CpG_3)	TCGACGTTTCCGCATGAACCGAATCAAACG

All ODNs have a phosphodiester backbone. The 'r.' indicates that the sequence is complementary to the ODN with four base overhangs at both 5′ ends, and each of these complementary ODNs was used to obtain double-stranded ODN. The potent immunostimulatory CpG motif (GACGTT) is underlined.

Preparation of Y-ODN and double-stranded ODN

Y-ODN was prepared by mixing equimolar amounts of three 30-base ODNs as reported previously.[20] In brief, three ODNs dissolved in an annealing buffer [10 mM Tris–HCl, pH 8·0, 1 mM ethylenediaminetetraacetic acid (EDTA) and 50 mM NaCl] were mixed in sterile Milli-Q water at a final concentration of 0·5 mM for each ODN. Mixtures were incubated at 95° for 5 min, 65° for 2 min, 62° for 1 min, then slowly cooled to 4°. Formation of Y-ODN was confirmed by 21% polyacrylamide gel electrophoresis (PAGE) at 200 V for 1·5–2 hr. Double-stranded ODN (dsODN) with four base overhangs at both 5′ ends was prepared by addition of a complementary ODN (r.Y0a, Table 1) to Y0a ODN.

Dynamic light-scattering analysis

The apparent hydrodynamic sizes of Y-ODN, Y-ODN (CpG), Y-ODN(CpG_3), single-stranded ODN (ssODN; Y0a) and dsODN were measured by laser light scattering using a Malvern Zetasizer 3000HS (Malvern Instruments, Malvern, UK) equipped with a helium-neon laser (633 nm).

Cytokine release from RAW264.7 cells

RAW264.7 cells were washed three times with 0·5 ml PBS before use. Then, ssODN, one of three kinds of dsODN, Y-ODN, Y-ODN(CpG) or Y-ODN(CpG_3)

diluted in 0·5 ml Opti-MEM was added to cells. The cells were incubated for 8 hr (TNF-α) or 24 hr (IL-6), and the supernatants were collected and stored at −80° until use. To exclude the effect of contaminated lipopolysaccharide (LPS) on cytokine release, polymyxin B, an inhibitor of LPS, was added to samples at a final concentration of 50 μg/ml. The levels of TNF-α and IL-6 in supernatants were determined by enzyme-linked immunosorbent assay using OptEIA™ sets (Pharmingen, San Diego, CA).

Stability of Y-ODN and dsODN in serum

The Y-ODN and dsODN (10 μg/100 μl) were incubated with 50% non-heat inactivated FBS at 37°. After 0, 2, 4, 8, or 24 hr of incubation, the reaction was terminated by adding 2 μl 0·5 M EDTA solution per 10 μl of samples. ODNs were extracted with phenol/chloroform/isoamyl alcohol, and the extracts were run on a 21% polyacrylamide gel and stained with ethidium bromide. Before the extraction, a fixed amount of dsODN of 45 bp was added to each sample, and the intensity of the band on gels was used to validate the efficiency of the extraction step. The amount of remaining ODNs was estimated by a Cool Saver (ATTO, Tokyo, Japan).

Uptake of ODNs in RAW264.7 cells

Fluorescein-labelled Y0a ODN was used for the preparation of fluorescein-labelled (F-) ssODN, dsODN and Y-ODN. RAW264.7 cells were plated on 96-well culture plates at a density of 5×10^5 cells/ml and cultured for 24 hr. Cells were washed three times with 100 μl PBS, incubated with F-ODN for 1 hr at 37° or 4°, harvested, and washed three times with 100 μl PBS. Then, the intensity of fluorescence of the cells was analysed by flow cytometry (FACSCalibur; BD Biosciences, San Jose, CA) using CellQuest software (version 3.1; BD Biosciences).

Growth inhibition of B16-BL6/Luc cells

RAW264.7 cells (5×10^5 cells/ml) were mixed with each ODN (10 or 20 μg/ml) and incubated for 8 hr. Then, the conditioned medium was added to B16-BL6/Luc cells, and the cells were cultured for 48 hr. The number of B16-BL6/Luc cells was determined by measuring the luciferase activity of cell lysates in a luminometer (Lumat LB 9507, EG & G Berthold, Bad Wildbad, Germany) as previously reported.[23]

Statistical analysis

Differences were statistically evaluated by one-way analysis of variance (ANOVA) followed by the Fisher's protected least significant difference (PLSD) test for multiple comparisons. A P-value of < 0.05 was considered to be statistically significant.

Results

Physicochemical properties of Y-ODN

Equimolar amounts of three ODNs (Y0a, Y0b and Y0c) were hybridized to obtain Y-ODN, the putative structure of which is shown in Fig. 1(a). Figure 1(b) shows the gel electrophoresis of the DNA preparations. As reported in a previous study,[20] Y-ODN showed a single band, the mobility of which was less than that of ssODN or dsODN, supporting the assembly of all three ODNs to form Y-ODN. Similarly, newly designed Y-ODN containing a potent CpG motif, Y-ODN(CpG), was prepared with Y0a(CpG), Y0b(CpG) (identical to Y0b) and Y0c(CpG). The Y-ODN containing three potent CpG

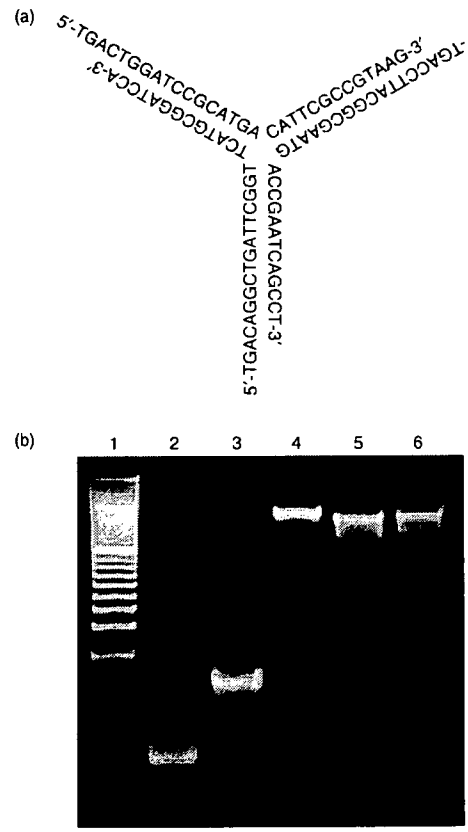

Figure 1. Formation of Y-shaped oligodeoxynucleotide (Y-ODN). (a) Putative structure of Y-ODN, which was prepared with three ODNs (Y0a, Y0b, Y0c) with the halves of each ODN being partially complementary to a half of the other two ODNs. (b) Polacrylamide gel electrophoresis analysis of single-stranded (ss), double-stranded (ds) and Y-ODN. Each ODN was run on 21% polyacrylamide gel at 200 V for 2 hr. Lane 1, 20-bp DNA ladder (Takara Bio); lane 2, ssODN; lane 3, dsODN; lane 4, Y-ODN; lane 5, Y-ODN(CpG); lane 6, Y-ODN(CpG_3).

motifs, Y-ODN(CpG_3), was also prepared with Y0a(CpG_3), Y0b(CpG_3) and Y0c(CpG_3). Polyacrylamide gel electrophoresis of the newly designed Y-ODN(CpG) and Y-ODN(CpG_3) showed one major band with a mobility similar to that of Y-ODN (Fig. 1b, lanes 5 and 6, respectively), suggesting a Y-shape formation of these ODNs containing CpG motifs. The apparent sizes were estimated to be 7·02 ± 0·22, 7·07 ± 0·40 and 7·09 ± 0·24 nm for Y-ODN, Y-ODN(CpG) and Y-ODN(CpG_3), respectively. Similarly, the sizes of ss-(Y0a) and dsODN were estimated to be 3·61 ± 0·68 and 6·86 ± 1·34 nm, respectively.

Cytokine release from RAW264.7 cells by ODNs

Y0a, one of the three ODNs consisting of the Y-ODN, was used as ssODN. A complementary ODN (r.Y0a) to Y0a was hybridized to obtain dsODN with four base overhangs at both 5′ ends. Similarly, three kinds of dsODN were prepared by designing complementary ODNs to Y0b and Y0c. The mixture of these three kinds of dsODNs (dsODN × 3) contained exactly the same bases in the same structural configuration of four base overhangs at both 5′ ends as Y-ODN, representing a good control to evaluate the effects of the Y-shape formation on the immunostimulatory activity of ODNs. Figure 2(a) shows the TNF-α concentration in the culture media of RAW264.7 cells. The addition of ssODN, dsODN or dsODN × 3 to RAW264.7 cells induced only weak secretion of TNF-α at concentrations of 2 and 6 μg/ml. Increasing the concentration of these ODNs to 18 μg/ml slightly increased TNF-α secretion to levels of up to 400 pg/ml. In marked contrast, large amounts of TNF-α were secreted from RAW264.7 cells after the addition of Y-ODN and the amounts varied in a concentration-dependent manner. Figure 2(b) shows the IL-6 concentration in the culture media. Again, Y-ODN induced significantly greater amounts of IL-6 secretion from cells than the other ODNs at all concentrations examined. These results indicate that Y-ODN has a stronger immunostimulatory activity than conventional ssODN or dsODN, even though there are no potent immunostimulatory CpG motifs in the sequence.

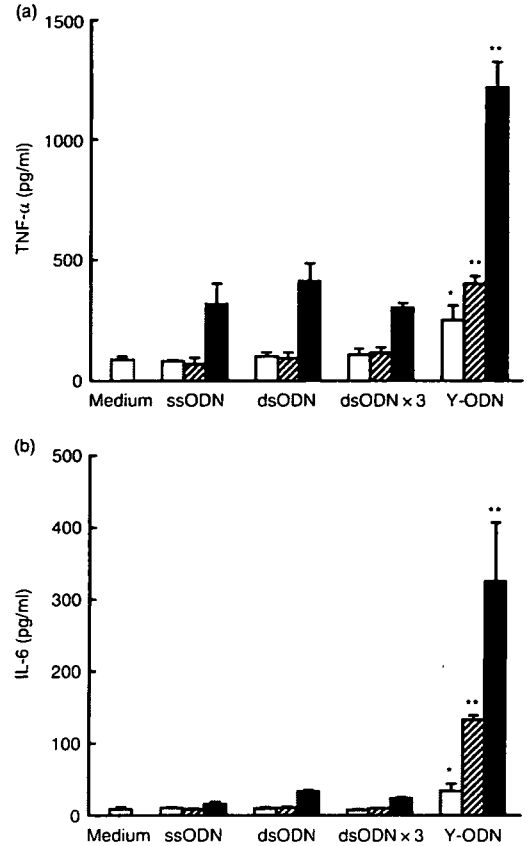

Figure 2. Secretion of cytokines from RAW264.7 cells after addition of oligodeoxynucleotide (ODN). Concentrations of (a) tumour necrosis factor-α (TNF-α) and (b) interleukin-6 (IL-6) in culture media were measured at 8 hr (TNF-α) or 24 hr (IL-6) after addition of each ODN to RAW264.7 cells at varying concentrations: (open bars) 2 μg/ml; (hatched bars) 6 μg/ml; (closed bars) 18 μg/ml. Results are expressed as the mean ± SD of three determinations. The experiment shown was a representative of three experiments with similar results. $^*P < 0.05$, $^{**}P < 0.01$, significantly different from single-stranded ODN (ss-ODN), double-stranded ODN (ds-ODN) and dsODN × 3 at the same concentration.

Stability of Y-ODN

Because the stability of ODN would affect their biological activity, the stability of dsODN and Y-ODN in 50% non-heat inactivated FBS solution was examined. Each ODN extracted was subjected to a PAGE analysis (Fig. 3a). The bands for 45-bp ODN, which was added to the mixtures just before extraction of ODNs, confirmed that the extraction efficiency of ODNs was almost identical in all samples. Both dsODN and Y-ODN were degraded with time in the FBS solution. A densitometric analysis of gel bands was performed in four identical experiments and the remaining amounts of dsODN and Y-ODN were plotted against the incubation time (Fig. 3b). Y-ODN showed a similar profile of degradation to dsODN, at least for the first 4 hr. Thereafter, it tended to be degraded more quickly than dsODN. Similar results were obtained when these ODNs were added to a solution containing DNase I (data not shown). These findings indicate that Y-ODN is degraded at a similar or slightly faster rate than dsODN under the conditions examined.

Cellular uptake of ODNs in RAW264.7 cells

To investigate whether the enhanced immunostimulatory activity of Y-ODN is mediated by an increased cellular

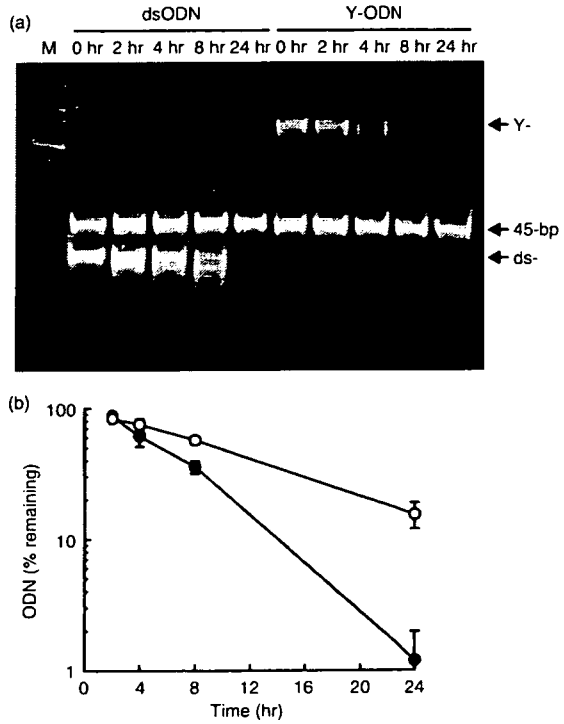

Figure 3. Stability of double-stranded oligodeoxynucleotide (dsODN) and Y-shape ODN (Y-ODN) in 50% non-heat inactivated fetal bovine serum (FBS). (a) The dsODN and Y-ODN were incubated in 50% non-heat inactivated FBS at 37° for the indicated times and the reaction was terminated by adding ethylenediaminetetraacetic acid. ODNs extracted were run on 21% polyacrylamide gel at 200 V for 2 hr and stained with ethidium bromide. 'M' represents 20-bp DNA ladder (Takara Bio). (b) The amounts of ODNs on the gel were estimated by Cool Saver. The remaining amounts of dsODN (O) and Y-ODN (●) were plotted against the incubation time. Results are expressed as the mean ± SD of four determinations.

uptake, the uptake of F-dsODN, F-dsODN × 3 and F-Y-ODN was examined in RAW264.7 cells. The uptake of these F-ODNs was greater at 37° than at 4° (data not shown) in all cases examined. A fluorescence-activated cell sorting (FACS) analysis was performed in three identical experiments and the mean fluorescence intensity (MFI) was measured at each concentration of ODN. MFI (37°–4°) was plotted against the concentration of ODN (Fig. 4). The MFI of cells treated with F-Y-ODN was significantly ($P < 0.05$) greater than the MFI of those treated with other F-ODN preparations, suggesting that an increased cellular uptake of Y-ODN contributes to the enhanced immunostimulatory activity of Y-ODN.

Cytokine release from RAW264.7 cells by CpG ODNs

Activation of RAW264.7 cells by the newly designed Y-ODN(CpG) and Y-ODN(CpG_3) was examined under

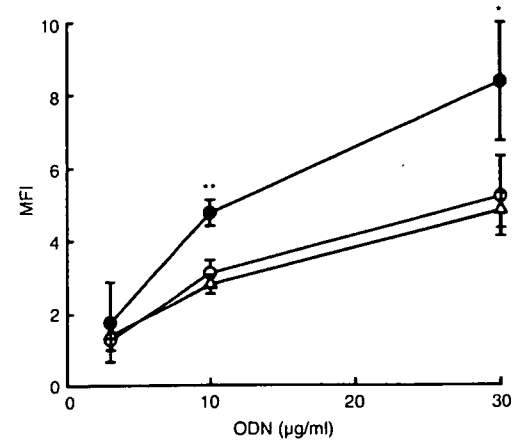

Figure 4. Uptake of fluorescein-labelled oligodeoxynucleotide (ODN) in RAW264.7 cells. Cells were incubated with fluorescein-labelled double-stranded ODN (dsODN) (O), dsODN × 3 (△) or Y-ODN (●) for 1 hr at 4° or 37°, and the amounts of ODN associated with cells were measured by flow cytometry. The mean fluorescence intensity (MFI) was plotted against the concentration of ODN. Results are expressed as the mean ± SD of three determinations. $^{*}P < 0.05$, $^{**}P < 0.01$, significantly different from dsODN and dsODN × 3.

the same conditions as above. Y0a(CpG_3), an ODN containing a potent CpG motif, was selected as ssODN with a CpG motif, ssODN(CpG_3). Then, dsODN(CpG_3) and dsODN(CpG_3) × 3 were prepared as described above. Again, dsODN(CpG_3) × 3 had the same sequence as Y-ODN(CpG_3). Contrary to ODNs with no potent immunostimulatory CpG motifs, any preparation of ODNs containing CpG motifs showed a marked secretion of TNF-α in a concentration-dependent manner (Fig. 5a). Approximately six-fold higher amounts of TNF-α were released from RAW264.7 cells treated with Y-ODN(CpG_3) compared with those treated with dsODN(CpG_3) × 3. The IL-6 concentration in the culture media was also measured, and similar results were obtained (Fig. 5b). All ODN preparations contained trace amounts of LPS, up to 2·5 EU/mg DNA, when measured by a *Limulus* test (Wako, Tokyo, Japan). To exclude the effects of contaminated LPS on cytokine release, polymyxin B was added to ssODN and Y-ODN(CpG_3). The addition of polymyxin B to Y-ODN(CpG_3) slightly reduced the level of TNF-α released from RAW264.7 cells (Table 2). However, the level was much greater than those obtained with the medium + LPS/polymyxin B or ssODN + LPS/polymyxin B, suggesting that contaminated LPS has little effect on the ODN-mediated cytokine release from RAW264.7 cells. These results suggest that the immunostimulatory activity of the CpG motif-containing ODNs can be significantly increased by the Y-shape formation. In addition, increasing the number of potent CpG motifs in Y-ODN is a useful approach to increasing the immunostimulatory activity of Y-ODN.

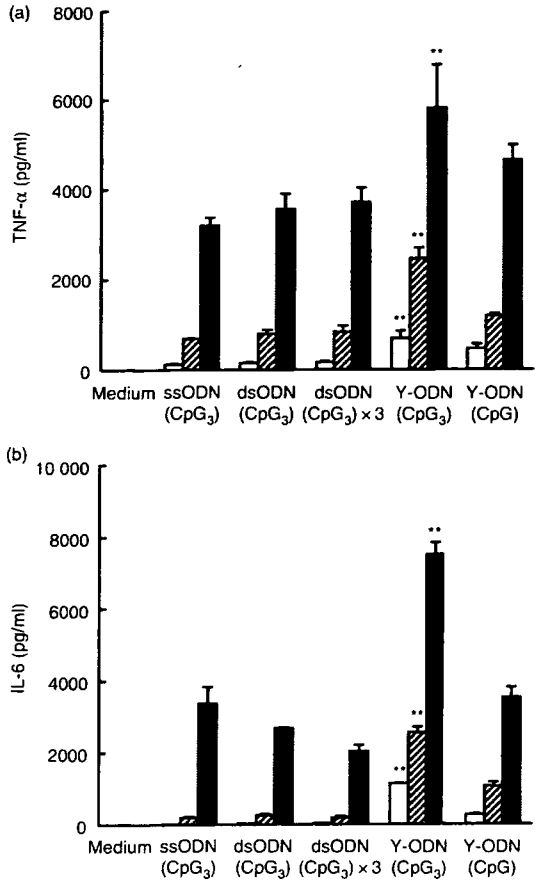

Figure 5. Secretion of cytokines from RAW264.7 cells after addition of CpG oligodeoxynucleotides (ODNs). Concentrations of (a) tumour necrosis factor-α (TNF-α) and (b) interleukin-6 (IL-6) in culture media were measured at 8 hr (TNF-α) or 24 hr (IL-6) after addition of each ODN to RAW264.7 cells at various concentrations: 2 μg/ml (open bars); 6 μg/ml (hatched bars); 18 μg/ml (closed bars). Results are expressed as the mean ± SD of three determinations. The experiment shown was representative of three experiments with similar results. $^{**}P < 0{\cdot}01$, significantly different from double-stranded ODN(CpG_3) 3 at the same concentration.

Effect of conditioned medium of ODN-treated RAW264.7 cells on proliferation of B16-BL6/Luc cells

To examine whether Y-ODN(CpG_3) is effective in inhibiting the proliferation of tumour cells, the conditioned medium of ODN-treated RAW264.7 cells was added to B16-BL6/Luc cells. The conditioned medium of Y-ODN(CpG_3)-treated RAW264.7 cells significantly inhibited the proliferation of tumour cells compared with the media of other ODNs (Fig. 6).

Discussion

CpG DNA activates immune cells expressing TLR9[1,2] and induces the cells to release a broad repertoire of chemokines and cytokines, including IL-6, IL-12, IFN-α/β, and TNF-α,[5,6] when it is sorted to endosomes after internalization via endocytosis.[24] CpG dinucleotide flanked by two purine bases on the 5′ side and two pyrimidine bases on the 3′ side, such as GACGTT, efficiently activates the murine immune system, whereas the optimal motif for humans is GTCGTT. Although the optimal sequences of CpG ODN for activating mouse or human immune cells have been identified by examining many possible base combinations, little is known about

Table 2. Effect of polymyxin B on tumour necrosis factor-α (TNF-α) release from RAW264.7 cells after addition of oligodeoxynucleotides (ODNs) with or without lipopolysaccharide (LPS)

	TNF-α (pg/ml)	
Compound	Control	+ polymyxin B
Medium	123 ± 11	394 ± 21
Medium + LPS	28 900 ± 700	448 ± 16
ssODN	278 ± 30	495 ± 23
ssODN + LPS	30 700 ± 300	557 ± 16
Y-ODN(CpG_3)	31 200 ± 700	27 400 ± 700
Y-ODN(CpG_3) + LPS	34 900 ± 1000	27 700 ± 1200

Polymyxin B, an inhibitor of LPS, was added to medium (Opti-MEM), single-stranded (ss) ODN or Y-ODN(CpG_3) at a final concentration of 50 μg/ml. LPS was added to samples at a final concentration of 1 ng/ml. Each sample was added to cells, and the TNF-α concentration in supernatants was measured after an 8-hr incubation. Results are expressed as the mean ± SD of three determinations.

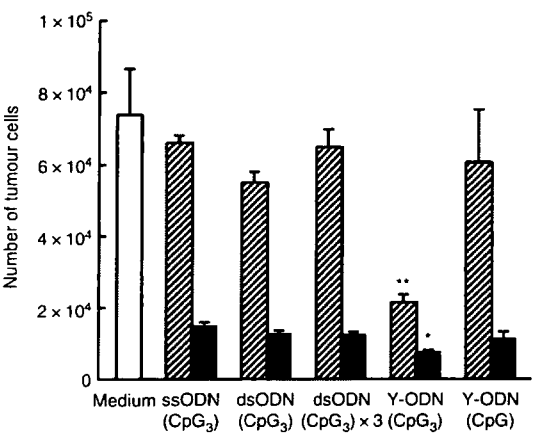

Figure 6. Growth inhibition of tumour cells by conditioned medium of RAW264.7 cells treated with oligodeoxynucleotides (ODN). Conditioned medium of ODN-treated RAW264.7 cells was added to B16-BL6/Luc cells, and the cells were cultured for additional 48 hr. Then, the number of cells was determined by measuring the luciferase activity of cell lysates. Results are expressed as the mean ± SD of three determinations. $^{*}P < 0{\cdot}05$, $^{**}P < 0{\cdot}01$, significantly different from other conditions.

the structural requirements of CpG ODN for the recognition of TLR9 and following the activation of immune cells. Phosphodiester ODN is rapidly degraded by nucleases in body fluids, such as serum, and intracellular compartments, which greatly limits the use of such ODNs as immunostimulatory agents. However, a recent study has shown that polyguanosine at the 3′ end of ODNs can increase the immunostimulatory activity of phosphodiester ODN.[12] Furthermore, it has been reported that the immunostimulatory effect was enhanced by using self-stabilized CpG DNA.[25–27] In the present study, we prepared Y-shaped ODNs, with or without immunostimulatory CpG motifs, and found that the Y-shape formation increased the immunostimulatory responses of ODNs in RAW264.7 macrophage-line cells, even though the stability was not increased by the Y-shape formation.

Y-shaped ODN (Y-ODN), the element of dendrimer-like preparations of ODN, was prepared using three 30-base ODNs. As reported in a previous paper,[20] Y-ODN showed a single band on polyacrylamide gels, the mobility of which was less than that of the bands for ssODN or dsODN (Fig. 1b). The addition of Y-ODN to RAW264.7 cells resulted in a significant secretion of both TNF-α and IL-6 in a DNA concentration-dependent manner, even though it had no potent immunostimulatory CpG motifs. DNA sequences other than potent immunostimulatory CpG motifs may trigger weak, but detectable, cytokine responses, and these could be involved in the immunostimulatory responses to ODNs without any potent CpG motifs. Although all ODN preparations contained trace amounts of LPS, up to 2·5 EU/mg DNA, this level of LPS induced only a little TNF-α secretion from RAW264.7 cells (data not shown). Furthermore, the addition of polymyxin B, an inhibitor of LPS, had little effect on the release of TNF-α from the Y-ODN(CpG_3)-treated RAW264.7 cells (Table 2). These results strongly support the idea that DNA is responsible for the cytokine response of cells to all ODN preparations under these experimental conditions, even though no potent CpG motifs were included in the sequences.

The RAW264.7 cells and other TLR9-positive cells secrete inflammatory cytokines on recognition of CpG DNA. TLR9 localizes in the intracellular compartments, such as the endoplasmic reticulum, and translocates to the lysosomal compartment when CpG DNA is taken up by the cells.[28] Therefore, CpG DNA should be transferred to such subcellular compartments to induce cytokine production. The level of cytokine release from TLR9-positive cells would be a function of variables, including the stability of DNA and the amount of DNA taken up by cells. Therefore, we examined whether the Y-shape formation of DNA increased the stability to nucleases and/or the cellular uptake. In the present study, we found that Y-ODN was less stable than dsODN. Y-ODNs have three terminals in one unit of the structure, whereas conventional dsODNs have two. This difference may explain the high susceptibility of Y-ODN to nuclease-mediated degradation.

In marked contrast, the Y-shape formation significantly increased the uptake of ODNs in RAW264.7 cells (Fig. 3b). A previous study demonstrated that increasing the length of ODN increases its endocytic uptake when ODNs with a length of 250 bp or less are used.[29] Because one unit of dsODN and Y-ODN contains 60 and 90 bases, respectively, this difference may be responsible for the greater uptake of Y-ODN compared with that of dsODN. However, the high immunostimulatory activity of Y-ODN could not be simply attributed to the increased uptake of Y-ODN because the increase in the level of cytokine release (three- to six-fold) by the Y-shape formation was greater than the increase in the uptake (about two-fold). Therefore, other factors, such as the affinity of ODN for TLR9 and intracellular localization, may also contribute to the increased immunostimulatory activity of Y-ODN.

To construct a new Y-ODN preparation with a potent immunostimulatory CpG motif, the GACGTT sequence, the most potent one in rodents, was inserted close to the 5′-terminal of Y0a, one of the components of Y-ODN, based on the information that TLR9 reads the CpG from the 5′-end of DNA.[30] Although the ssODN and dsODN with the CpG motifs were effective in inducing cytokines, such as TNF-α and IL-6, when added to RAW264.7 cells, the Y-ODN containing CpG motifs induced greater amounts of cytokines than these conventional CpG ODN preparations. Because all ODN preparations, i.e. ssODN(CpG_3), dsODN(CpG_3), dsODN(CpG_3) × 3 and Y-ODN(CpG_3), have identical numbers of potent CpG motifs, the Y-shape formation of ODN significantly increases the efficiency of cytokine production by CpG DNA. Furthermore, Y-ODN(CpG_3), which contained three potent CpG motifs in one unit, was much more effective in inducing TNF-α and IL-6 compared with Y-ODN(CpG) containing only one potent CpG motif. In accordance with the levels of cytokines, the conditioned medium of Y-ODN(CpG_3)-treated RAW264.7 cells showed a greater inhibitory effect on the growth of melanoma cells than other ODNs. Previous studies have demonstrated that CpG ODNs are a very strong activator of TLR9-positive cells, such as macrophages, B cells and dendritic cells, and have been considered as therapeutic agents against cancer, and infectious and allergic diseases.[1,31] As shown in the present study, cytokines possessing anti-proliferative activity of tumour cells, such as TNF-α, are induced by CpG ODNs. Therefore, cytokines released from CpG ODN-treated RAW264.7 cells would contribute to the growth inhibition of melanoma cells. These results indicate that highly potent immunostimulatory ODNs can be designed by increasing the number of CpG motifs in the sequences of Y-ODN.

The involvement of TLR9 in CpG DNA-mediated immune activation has been reported using $TLR9^{-/-}$ mice or cells isolated from those mice. Our preliminary experiments using $TLR9^{-/-}$ and wild-type mice suggested that Y-ODN(CpG_3) induces TNF-α production in a TLR9-dependent manner, although the level obtained was lower than those obtained with DNA–cationic liposome complexes. The involvement of TLR9 in the immune activation by Y-ODNs was therefore evident, but it should be further investigated in future experiments.

In conclusion, the Y-shape formation of ODN has been shown to be effective in inducing greater amounts of cytokines, such as TNF-α and IL-6, in macrophage-like, TLR9-positive cells than conventional ssODN or dsODN. These enhanced immunostimulatory effects of Y-ODN are, at least partly, associated with an increase in the uptake by TLR9-positive cells, but not with stabilization of ODN. CpG DNA has been explored as a therapeutic agent for cancer, asthma, allergy, and infectious diseases and as an adjuvant in immunotherapy, but it generally requires phosphorothioate or other chemical modification. Such modification may have disadvantages associated with systemic toxicity, such as a transient anticoagulant effect, activation of complement cascade, and inhibition of basic fibroblast growth factor binding to surface receptors, because of non-specific protein binding.[32] The findings of the present study provide a novel strategy for the development of potent immunostimulatory CpG ODN preparations free from such modification problems.

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Effects of Inflammatory Response on *In Vivo* Transgene Expression by Plasmid DNA in Mice

KEIKO KAKO, MAKIYA NISHIKAWA, HIROYUKI YOSHIDA, YOSHINOBU TAKAKURA

Department of Biopharmaceutics and Drug Metabolism, Graduate School of Pharmaceutical Sciences, Kyoto University, Sakyo-ku, Kyoto 606-8501, Japan

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ABSTRACT: To examine the effects of inflammatory response to plasmid DNA (pDNA) on transgene expression, serum tumor necrosis factor-α (TNF-α) was measured after intravenous injection of pDNA or calf thymus DNA (CT DNA) in the naked or complexed form with cationic liposomes (lipoplex). pDNA with many CpG motifs induced TNF-α production regardless of the forms. No significant TNF-α production was detected when CT DNA or methylated pDNA was injected. Clodronate liposomes and dexamethasone were used to deplete phagocytes or to inhibit inflammatory responses, respectively. Transient depletion of phagocytes, such as liver Kupffer cells and splenic macrophages, by clodronate liposomes slightly altered the tissue distribution of ^{32}P-pDNA lipoplex, but significantly reduced the TNF-α production and transgene expression. Dexamethasone significantly inhibited the initial transgene expression, but increased the duration of the expression slightly. Use of NF-κB activity-dependent plasmid vector suggested that the inhibition of NF-κB activation is involved in the reduced expression by these treatments. These findings indicate that tissue macrophages are closely involved in the CpG motif-dependent TNF-α production. It is also suggested that TNF-α activates NF-κB and increases transgene expression by pDNA having many NF-κB binding sites, but TNF-α also reduces transgene expression at later time periods, leading to short-term transgene expression. © 2007 Wiley-Liss, Inc. and the American Pharmacists Association J Pharm Sci

Keywords: plasmid DNA; gene delivery; lipids; lipoplexes; distribution; inflammatory cytokines; clodronate liposomes; dexamethasone; CpG motif

INTRODUCTION

Plasmid DNA (pDNA) expressing a therapeutic protein shows great promise in applications to *in vivo* gene therapy. Compared with viral vectors, pDNA possesses several advantages, including an excellent safety profile, essentially unlimited DNA carrying capacity, and simple large-scale production. However, transfection efficiency needs to be greatly improved for therapeutic applications.

Another concern using pDNA is the abundant presence of unmethylated CpG dinucleotides, or CpG motifs in the sequence. They are recognized by the mammalian immune system as a danger signal through a pathogen recognition receptor, Toll-like receptor 9 (TLR9).[1] After TLR9-positive cells, such as macrophages and dendritic cells, take up CpG motif-containing DNA, they secrete inflammatory cytokines, such as tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), and IL-12. It has been previously reported by our laboratory that primary murine macrophages and dendritic cells efficiently take up and degrade DNA, and then release inflammatory cytokines depending not only on the presence of the motif, but also on the formulation of DNA.[2–5] Because these cytokines

Correspondence to: Yoshinobu Takakura (Telephone: 81-75-753-4615; Fax: 81-75-753-4614; E-mail: takakura@pharm.kyoto-u.ac.jp)

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are cytotoxic, the excessive production of these cytokines is generally regarded as a side effect of nonviral vector administration and should be reduced as much as possible. Various approaches have been designed to reduce cytokine production, including reduction in the number of CpG motifs[6,7] and the use of PCR-amplified DNA fragments.[8,9]

On the other hand, biological responses, including cytokine production, may affect the level and duration of transgene expression after *in vivo* gene transfer. Of the cytokines induced by pDNA administration, TNF-α is a well-known activator of the transcription factor nuclear factor κB (NF-κB) that is present in the cytoplasm of a variety of cells.[10–12] Following activation, NF-κB translocates and accumulates in the nucleus, binds to DNA elements containing NF-κB biding sequence, and participates in the activation of transcription of various genes.[13] Genes activated by NF-κB include cell-surface molecules, such as immunoglobulin κ light chain, class I and II major histocompatibility complexes, and various cytokines. In addition, some viruses including cytomegalovirus (CMV) have also NF-κB binding sites in their enhancers, and viral production is stimulated by agents that activate NF-κB.[14]

These pieces of evidence have led us to form the following hypothesis: (1) TNF-α is secreted primarily by macrophages that recognize the CpG motifs in pDNA, (2) pDNA-induced TNF-α activates NF-κB in target cells, which, in turn, increases the transgene expression by pDNA in which any NF-κB responsive element is incorporated, and (3) pDNA-induced TNF-α plays a major role in cytokine-mediated cytotoxicity and suppresses transgene expression. In a previous study,[15] we have proved that NF-κB activation by pDNA-cationic liposome complex, or lipoplex, can be used to enhance lipoplex-mediated transgene expression by inserting NF-κB binding sites into a conventional pDNA. Although nonviral vectors could induce the release of TNF-α and other cytokines upon administration, depending on their physicochemical and biological properties, the overall effects of such inflammatory response on transgene expression are not yet fully understood. To develop a strategy for optimizing *in vivo* gene therapy using pDNA, it is important to investigate the correlation between inflammatory cytokine production and transgene expression after pDNA administration.

In the present study, therefore, we first investigated the cellular basis for the secretion of TNF-α in mice following administration of naked pDNA or its lipoplex. Then, the effects of TNF-α production on transgene expression were examined in mice treated with clodronate liposomes or dexamethasone to inhibit cytokine production. Correlation between NF-κB activation and transgene expression was also examined using an NF-κB activity-dependent pDNA.

MATERIALS AND METHODS

Chemicals

N-[1-(2,3-dioleyloxy)propyl]-*N*,*N*,*N*-trimethylammonium (DOTMA) was purchased from Tokyo Kasei (Tokyo, Japan). Cholesterol (Chol) was purchased from Nacalai Tesque (Kyoto, Japan). Lipopolysaccharide (LPS), dichloromethylenediphosphonic acid disodium salt (clodronate), and dexamethasone were purchased from Sigma (St. Louis, MO). [α-^{32}P]dCTP was purchased from Amersham (Tokyo, Japan). All other chemicals used were of the highest purity available.

DNA

pcDNA3 vector was purchased from Invitrogen (Carlsbad, CA). pGL3-control vector was purchase from Promega (Madison, WI). PathDetect® NF-κB *cis*-reporting pNF-κB-Luc and pLuc-mcs plasmids were purchased from Stratagene (La Jolla, CA, USA). pCMV-Luc encoding firefly *luciferase* gene was constructed based on pcDNA3 as described previously.[16] pcDNA3 contains 26 Pur-Pur-CpG-Pyr-Pyr sequences including two GACGTT that have been reported to be the most potent CpG motif in mice.[17] pGZB vector,[18] a CpG-reduced pDNA that has a backbone different from pCMV vectors, was kindly provided by Dr. Yew (Genzyme Corporation, Cambridge, MA). To construct pGZB-Luc, the firefly *luciferase* cDNA fragment amplified from pCMV-Luc was inserted into the *Sfi*I/*Eco*RI site of pGZB vector. pCpG-Luc was constructed by inserting the firefly *luciferase* cDNA fragment amplified from pCMV-Luc into the *Bgl*II/*Nhe*I site of pCpG-mcs vector (Invivogen, San Diego, CA). pCpG-Luc was amplified in *E. coli* GT115 and the other pDNA were amplified in *E. coli* DH5α and then isolated and purified using a Qiagen Endofree™ Plasmid Giga Kit (Qiagen GmbH, Hilden, Germany). The frequency of CpG dinucleotides was 6.0% and 3.1% for pCMV-Luc and pGZB-Luc, respectively, and these

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