Aus dem Institut für Hirnforschung der Universität Tübingen
Abteilung Hirnforschung
Abteilungs Leiter: Professor Dr. R. Meyermann
Sektion für Immunpathologie des Nervensystems Leiter: Professor Dr. H. Schlüsener

Cellular transport and binding proteins of oligonucleotides and small-interfering RNA in microglial cells

Inaugural-Dissertation
zur Erlangung des Doktorgrades
der Humanwissenschaften

der Medizinischen Fakultät
der Eberhard Karls Universität
zu Tübingen

vorgelegt von

Zhiren Zhang, aus Wuhan, China

2006

Dekan: Professor Dr. C. D. Claussen

1. Berichterstatter: Professor Dr. H. Schlüsener

2. Berichterstatter: Professor Dr. B. Schloßhauer

Table of content

Abbreviations

AIF-1	Allograft inflammatory factor 1
BDNF	Brain-derived neurotrophic factor
BrdU	5-bromo-2´-deoxyuridine
CNS	Central nervous system
DAPI	4',6'-diamidino-2-phenylindole hydrochloride
dsRNA	Double-stranded RNA
EGF	Epidermal growth factor
FCS	Fetal calf serum
FGF	Fibroblast growth factor
FITC	Fluorescein isothiocyanate
HIV	Human immunodeficiency virus
hnRNPs	Heterogeneous nuclear ribonucleoproteins
IL1 β	Interleukin-1 β
IL6	Interleukin-6
IL12p40	Interleukin-12p40
iNOS	Inducible nitric oxide synthase
ip	Intraperitoneally
LPS	Lipopolysaccharide
NO	Nitric oxide
ODN	Oligonucleotides
$P2X_4R$	$P2X_4$ receptor
PAMPs	Pathogen-associated molecular patterns
PBS	Phosphate-buffered saline
PE	Phosphodiester
PFA	Paraformaldehyde
pi	Post injection
PKR	dsRNA-dependent protein kinase R
Poly(I:C)	Polyinosine-polycytidylic acid
PRR	Pattern recognition receptor
PS	Phosphorothioate

PVC	Perivascular cells
RNAi	RNA interference
SELEX	Systematic evolution of ligands by exponential enrichment
siRNA	Small interfering RNA
ssRNA	Single-stranded RNA
TLR	Toll like receptor
TNF-α	Tumor necrosis factor- α

Figures are numbered for each chapter separately. If not otherwise stated, the mentioned figure numbers refer to the figures in the same chapter.

Summary

Microglia are the major component of the cellular immune system in the central nervous system. Microglial cells are involved in almost all neuropathological processes and are therefore considered prime targets for gene therapy. With the knowledge of nucleic acids and gene regulation growing a class of new clinically relevant drugs, therapeutic nucleic acids, are rapidly developed. But poor in-vivo stability, low permeability and potential unspecific effects are big obstacles for potential therapeutic applications. Understanding of cellular uptake, transport and potential unwanted effects of therapeutic nucleic acids in microglia is thus important for applications in central nervous system diseases.

Our data support a receptor-mediated uptake mechanism for single-stranded oligonucleotides (ODN) (Chapter 2) and small interfering RNA (siRNA) (Chapter 5) in microglia because cellular uptake is dose-, time-, temperature-, modification-, and energy-dependent and inside cells they mainly localize to the cytoplasm with spot pattern. Further unmodified siRNA was showed to co-localize with endosomes after uptake. Cellular uptake is the prerequisite for the activity of most therapeutic nucleic acids. Increasing uptake might be a good way to enhance the efficiency of therapeutic nucleic acids. Here we provide evidence that a 3'-end polyG motif can enhance phosphodiester (PE) CpG-ODN uptake resulting into increased immunomodulatory activity in microglial cells (Chapter 3). Such effects are dependent on the location of the polyG motif and the backbone modification of ODN.

Inside cells, nucleic acids have the potential to sequence-unspecifically interact with certain cellular proteins (Chapter 2, 3 and 5). Such interactions are strongly backbone-modification dependent and to a much lesser degree of sequence dependent. Most ODN binding-proteins are RNA or DNA binding proteins, which are important for chromosome organization, transcription regulation and RNA processing. The sequence-unspecific interaction of nucleic acids with cellular binding proteins might influence the physiological function of these proteins. We observed that siRNA could bind to PKR and trigger enzymatic activity. In addition the binding proteins might affect intracellular nucleic acid

distribution. Three membrane proteins were identified as ODN binding-proteins that indicated they might be involved in nucleic acid uptake.

We observed that peripheral application of nucleic acids could have unwanted effects on the central nervous system. In Chapter 6, it is shown that a significant but transient increase of activated microglia was induced in rat spinal cord after peripheral administration of immunostimulatory nucleic acides, poly (I:C) and R848.

Our findings will contribute to rational design and evaluation of nucleic acid-based therapeutic strategies.

Chapter 1. General introductions

The last decades witnessed an enormous increase in information about nucleic acids. The elucidation of many disease related molecular pathways, together with the sequencing of human genome, make nucleic acids not only targets for disease intervention but also therapeutics to interfere with disease related processes. Now nucleic acids find a wide range of applications in fields such as biotechnology, molecular biology, diagnosis and therapy. Nucleic acids therapeutics represents a new paradigm for drug discovery. Based on different mechanism several groups of therapeutic nucleic acids were developed. The FDA approved one therapeutic nucleic acid and many others are in clinical trials.

1. Classification of therapeutic nucleic acids

Nucleic acids with potential therapeutic effects might be divided into the following three major groups. First, antisense nucleic acids, including small interfering RNA (siRNA) and catalytically active oligonucleotides (ODNs) referred to as ribozymes; Second, immunomodulatory nucleic acids, including ODNs containing a CpG motif, double-stranded RNA (dsRNA) and single-stranded RNA (ssRNA); and third, aptamers, structured nucleic acids that form binding pockets for specific ligands.

1.1 Antisense nucleic acids

Antisense technology was first suggested and implemented by Paterson et al., in 1977 and then one year later the first therapeutic options for antisense ODNs were explored as treatment for Rous sarcoma virus infection (1, 2). Since then, significant progress has been made in antisense technology. Vitravene, which targets the CMV IE-2 gene, was the first antisense ODNs based drug approved

by the FDA. Many other antisense drugs have shown promising activities in clinical trials (3).

Antisense nucleic acids are valuable tools to inhibit the expression of the targeted gene in a sequence specific manner. Although the basic principles are similar, several different antisense strategies can be discerned. Briefly, antisense ODNs pair with their complementary mRNA and inhibit mRNA transcription by steric blockade of the ribosome and/or activating the RNase H, which cleaves the RNA moiety of a DNA.RNA heteroduplex and therefore results in degradation of targeted RNA (4). Ribozymes and DNAzymes are another type of antisense nucleic acids, which have enzymatic activity themselves (5). They bind to substrate RNA through Watson-Crick base pairing which offers sequence-specific cleavage of transcript.

siRNA is a newly discovered type of antisense nucleic acids. siRNA, dsRNA 21- to 23- nucleotides (nt) long and with two nt 3' overhangs, has been shown to mediate powerful sequence-specific gene silencing in mammalian cells through RNA interference (RNAi). RNAi is an evolutionary conserved post-transcriptional gene silencing mechanism that proceeds through a two-step process. In the first step, long dsRNAs are recognised and digested by an RNase III enzyme, Dicer, to generate siRNA. Subsequently, these siRNAs, which act as a guide to ensure specific interaction with the target transcript, are incorporated into the RNA-induced silencing complex, which cleaves the corresponding transcript. Due to its powerful gene knockdown activity and specificity siRNA has been a widely used tool for determining gene function or for therapeutic purposes (6, 7).

1.2 Immunostimulatory nucleic acids

Immunostimulatory nucleic acids form another big type of therapeutic nucleic acids, all of which belong to the toll-like receptor (TLR) ligands. TLRs are the key receptors that allow mammals to detect the presence of infection by recognizing specific microorganism derived elements, which are the so-called pathogen-associated molecular patterns (PAMPs). The release of PAMPs during an infection provides a “danger signal” to the TLRs and triggers defensive innate immune response (8). Thirteen mammalian TLR paralogues have been identified so far and most of their specific ligands have been identified (9, 10). TLR3 is implicated in virus-derived dsRNA (11). TLR7 (murine) and TLR8 (human) recognize GU-rich single-stranded RNA (ssRNA) (12). The natural ligand for TLR9 is ODNs containing CpG motif (13). Activation of TLR signalling pathways by different ligands leads to the expression of many genes that function in host defence, including inflammatory cytokines, chemokines, major histocompatibility complex, co-stimulatory molecules and multiple effector molecules.

Unmethylated CpG dinucleotides within specific flanking bases (referred to as CpG motifs) are immunostimulatory and are at a much higher frequency present in bacterial than in vertebrate DNA. Bacterial DNA or synthetic ODN with CpG motifs are recognized as danger signal by the innate immune cells via TLR9 to trigger a Th1-polarized immune response. CpG-ODN can trigger B cells, plasmacytoid dendritic cells, monocytes, macrophages and natural killer cells to proliferate, mature, up-regulate several costimulatory molecules and to secrete a variety of Th1-promoting cytokines, chemokines and immunoglobulins. Based on their strong immunostimulatory activity, a number of CpG-ODNs are at various stages of preclinical and clinical evaluation as adjuvant or therapeutic agents for cancer and allergic diseases (14, 15, 16).

Polyinosine-polycytidylic acid (poly (I:C)) is a synthetic dsRNA that activates the innate immune response through TLR3 (11). Poly (I:C) is one of the most potent type I interferon (IFN) inducers and can activate monocytes to produce tumor necrosis factor-α, interleukin-6, interleukin-1β and interleukin-12 and promotes dendritic cell maturation (17, 18). It has already been used in clinical trials in cancer (19, 20), chronic fatigue (21) and human immunedeficiency virus infection (22, 23). Further, it is also often used as a model of viral infection.

R848 (resiquimod; S-28463) is guanine nucleotide analogue and activates innate immune system selectively via TLR7/8 (12). Similar to poly (I:C), R848 induces production of interferon-α, interleukin-12, tumor necrosis factor-α and other cytokines (24, 25). It has been used in clinical trials of human papillomavirus infection and is considered in the therapy of cancers and as vaccine adjuvant (26).

1.3 Aptamers

ssDNA and RNA can fold into a variety of conformations and directly interact with cellular proteins and other ligands. Such studies have opened a new research field, the selection and design of “aptamers”. Nucleic acid aptamers are short, single-stranded ODNs or their modified analogues, which avidly and specifically interact with targeted ligands through their 3-dimensional structure. Aptamers can be selected out of a large combinatorial ODN library through an *in vitro* evolution process termed SELEX (Systematic Evolution of Ligands by Exponential Enrichment) (27, 28). Since 1990, a wide variety of aptamers targeted to ligands ranging from small molecules to complex mixtures have been isolated. Most selected aptamers have shown high specificity to and affinity for their ligands and are potential detection and/or diagnostic reagents.

Furthermore, some aptamers specifically inhibit biological functions of targeted proteins, resulting in potent therapeutic candidates in disease models (29).

One of the most studied aptamers in the therapeutic field is the anti-VEGF aptamer EYE001 (formerly referred to as NX1838). EYE001 is a chemically modified RNA aptamer with high specificity, high affinity, and nuclease resistance. Promising *in vitro* and *in vivo* results against disease related with angiogenesis indicate EYE001 might be a potent therapeutic reagent (30). Recently, clinical phase IA and phase II trials have shown that EYE001 anti-VEGF therapy is a promising treatment for various forms of ocular neovascularization, including age-related macular degeneration (31, 32).

2. Several major considerations for applications of therapeutic nucleic acids

Although conceptually elegant, the prospect of using nucleic acids as treating reagents remains tantalizing uncertain. Preclinical and clinical observations raised concerns about the stability, permeability and selectivity of the nucleic acid application.

2.1 Stability

In order for therapeutic nucleic acids to exert their effects, they must reach their targets. But the unmodified nucleic acids are rapidly degraded by both endonucleases and exonucleases in the blood and in cells. To remedy this hurdle, different kinds of modified nucleic acids were introduced to resist nuclease degradation (33). One of the easiest modifications to synthesize are phosphorothioates (PS) ODNs, which replace a non-bridging oxygen on the phosphate backbone with sulfur, producing a PS linkage. Such modification

greatly increases the ability of ODNs to retard nuclease degradation. Further, this modification is a substrate for RNasH. In addition, this modification also enhances binding with proteins, which can increase its uptake efficiency by cells but also might cause unwanted effects. Due to their stability and relative easy synthesis, PS ODNs have been the most widely studied and used ODNs to date (34, 35).

2.2 Permeability

As nucleic acids are polyanions, they cannot passively diffuse across cell membranes. So, poor cell permeability is another major obstacle for therapeutic nucleic acids. Antisense nucleic acids usually designed to interact with DNA or RNA must enter cells to exert their effects. For immunostimulatory nucleic acids, as the TLR3, TLR7/8 and TLR9 are all inside cells, it is also necessary for them to pass cell membranes (36). But for aptamers, dependent on the location of the targets, it may be necessary or unnecessary to enter cells.

But usually cellular uptake of ODN is a crucial aspect for ODN-based therapeutic agents. Notably in the brain, where transfection agents increasing ODN-uptake cannot be used, the spontaneous uptake of ODN is relevant to the design of ODN-based therapeutics. Uptake of ODN thus appears a limiting step in ODN activity.

Although many carrier agents to improve nucleic acid uptake have been developed it is important to understand the mechanisms that control the cellular uptake of naked nucleic acids. A large number of experiments strongly suggest that naked nucleic acids can enter into cells and exert their corresponding effects. But the mechanism is not clear yet. Most studies support that receptor mediated endocytosis is the major pathway for nucleic acids entering into cells but the receptors that mediated this process are still not fully identified (37, 38).

2.3 Unwanted effects

Since PS nucleic acids are the most widely applied nucleic acid in both, laboratory and clinic, concerns of their unspecific binding to proteins have been raised. As mentioned above, PS-analogues have an enhanced ability to bind to proteins, which cannot only increase cellular uptake efficiency but might also cause unwanted effects. *In vitro* PS ODN cause transcription factor Sp1 activation, platelet aggregation, inhibit cell proliferation and migration, perturb cell-surface receptor binding and induce changes in cell morphology (39, 40, 41, 42). In clinical studies, PS ODN caused thrombocytopenia, fatigue, fever, rashes, leukopenia, and complement activation (34). In addition, PS ODN might be neurotoxic at therapeutic concentrations higher than 1 μM (43), causing paralysis and necrosis in the spinal cord (44). Such sequence-independent effects are probably due to binding of PS ODN to multiple cellular proteins (41, 45). Until now, only few PS ODN binding proteins have been identified and much more work is needed.

Our Purposes

Microglial cells derived from the mononuclear-phagocyte lineage are the brain's ubiquitous but normally inconspicuous immune effector cells. They constitute the brain's autochthonous source of monocytic cells and thus are part of its intrinsic immune system (47). Microglia respond rapidly to subtle, acute and chronic pathological stimuli and are prominently involved in all major central nervous system pathologies ranging from acute events such as bacterial infections to neuro-inflammatory and degenerative diseases such as Alzheimer's disease. These unique features make microglia a most suitable therapeutic target (48). But until now, little is know about the uptake, cellular distribution and binding proteins of therapeutic nucleic acids in microglia.

Therefore we have studied cellular transport and binding proteins of PS ODN and siRNA in microglia.

References

(1) Paterson, B. M., Roberts, B. E., Kuff, E.L. *Proc. Natl. Acad. Sci. USA.* **74,** 4370-4374 (1977).

(2) Zamecnik, P. C., Stephenson, M. L. *Proc. Natl. Acad. Sci. USA.* **75,** 280-284 (1978).

(3) Opalinska, J. B., Gewirtz, A. M. Nat. *Rev. Drug Discov.* **1,** 503-514 (2002).

(4) Crooke, S. T. *Biochim. Biophys. Acta.* **1489,** 31-44 (1999).

(5) Schubert, S., Kurreck, J. *Curr. Drug Targets.* **5,** 667-681 (2004).

(6) Hannon, G. J. *Nature.* **418,** 244-251 (2002).

(7) Tijsterman, M., Ketting, R.F., Plasterk, R.H. *Annu. Rev. Genet.* **36,** 489-519 (2002).

(8) Iwasaki, A., Medzhitov, R. *Nat. Immunol.* **5,** 987-995 (2004).

(9) Barton, G. M., Medzhitov, R. *Science.* **300,** 1524-1525 (2003).

(10) Ulevitch, R. J. *Nat. Rev. Immunol.* **4,** 512-520 (2004).

(11) Alexopoulou, L., Holt, A.C., Medzhitov, R., Flavell, R.A. *Nature.* **413,** 732-738 (2001).

(12) Heil, F., Hemmi, H., Hochrein, H., Ampenberger, F., Kirschning, C., Akira, S., Lipford, G., Wagner, H., Bauer, S. *Science.* **303,** 1526-1529 (2004).

(13) Hemmi, H., Takeuchi, O., Kawai, T., Kaisho, T., Sato, S., Sanjo, H., Matsumoto, M., Hoshino, K., Wagner, H., Takeda, K., Akira, S. *Nature.* **408,** 740-744 (2000).

(14) Krieg, A. M. *Annu. Rev. Immunol.* **20,** 709-760 (2002).

(15) Rothenfusser, S., Tuma, E., Wagner, M., Endres, S., Hartmann, G. *Curr. Opin. Mol. Ther.* **5,** 98-106 (2003).

(16) Dalpke, A., Zimmermann, S., Heeg, K. *Immunobiology.* **204,** 667-676 (2001).

(17) Akiyama, Y., Stevenson, G. W., Schlick, E., Matsushima, K., Miller, P. J., Stevenson, H. C. *J. Leukocyte Biol.* **37,** 519-523 (1985).

(18) Manetti, R., Annunziato, F., Tomasevic, L., Gianno, V., Parronchi, P., Romagnani, S., Maggi, E. *Eur. J. Immunol.* **25,** 2656-2661 (1995).

(19) Robinson, R. A., DeVita, V. T., Levy, H. B., Baron, S., Hubbard, S. P., Levine, A. S. *J. Natl Cancer Inst.* **57,** 599-603 (1976).

(20) Salazar, A. M., Levy, H. B., Ondra, S., Kende, M., Scherokman, B., Brown, D., Mena, H., Martin, N., Schwab, K., Donovan, D., Dougherty, D., Pulliam, M., Ippolito, M., Graves, M., Brown, H., Ommaya, A. *Neurosurgery.* **38,** 1096-1101 (1996).

(21) Strayer, D. R., Carter, W. A., Brodsky, I., Cheney, P., Peterson, D., Salvato, P., Thompson, C., Loveless, M., Shapiro, D. E., Elsasser, W. *Clin. Infect. Dis.* **18 (Suppl. 1),** S88-S93 (1994).

(22) Carter, W. A., Strayer, D. R., Brodsky, I., Lewin, M., Pellegrino, M. G., Einck, L., Henriques, H. F., Simon, G. L., Parenti, D. M., Scheib, R. G. *Lancet.* **1,** 1286-1292 (1987).

(23) Gillespie, D., Hubbell, H. R., Carter, W. A., Midgette, P., Elsasser, W., Mullaney, R., Strayer, D. R. *In Vivo.* **8,** 375-380 (1994).

(24) Dockrell, D. H., Kinghorn, G. R. *J. Antimicrob. Chemother.* **48,** 751-755 (2001).

(25) Buates, S., Matlashewski, G. *Antimicrob. Agents Chemother.* **45,** 1137-1142 (2001).

(26) Sauder, D. N., Smith, M. H., Senta-McMillian, T., Soria, I., Meng, T. C. *Antimicrob. Agents Chemother.* **47,** 3846-3852 (2003).

(27) Ellington, A. D., Szostak J. W. *Nature.* **346,** 818-822 (1990).

(28) Ellington, A. D., Szostak J. W. *Nature.* **355,** 850-852 (1992).

(29) Breaker, R. R. *Nature.* **432,** 838-845 (2004).

(30) Carrasquillo, K. G., Ricker, J. A., Rigas, I. K., Miller, J. W., Gragoudas, E. S., Adamis, A. P. *Invest. Ophthalmol. Vis. Sci.* **44,** 290-299 (2003).

(31) Eyetech Study Group. *Retina.* **22,** 143-152 (2002).

(32) Eyetech Study Group. *Ophthalmology.* **110,** 979-986 (2003).

(33) Micklefield, J. *Curr. Med. Chem.* 8, 1157-1179 (2001).

(34) Fiset, P. O., Gounni, A. S. *Rev. Biol. Biotech.* **1,** 27-33 (2001).

(35) Juliano, R. L., Alahari, S., Yoo, H., Kole, R., Cho, M. *Pharm Res.* **16,** 494-502 (1999).

(36) Nishiya, T., DeFranco, A. L. *J. Biol. Chem.* **279,** 19008-1901 (2004).

(37) Juliano, R. L., Yoo, H. *Curr. Opin. Mol. Ther.* **2,** 297-303 (2000).

(38) Tari, A. M., Lopez-Berestein, G. *Curr. Opin. Investig. Drugs.* **2,** 1450-1453 (2001).

(39) Perez, J. R., Li, Y., Stein, C. A., Majumder, S., van Oorschot, A., Narayanan, R. *Proc. Natl. Acad. Sci. U S A.* **91,** 5957-5961 (1994).

(40) Wang, W., Chen, H. J., Schwartz, A., Cannon, P. J., Stein, C. A., Rabbani, L. E. *J. Clin. Invest.* **98,** 443-450 (1996).

(41) Rockwell, P., O'Connor, W. J., King, K., Goldstein, N. I., Zhang, L. M. *Proc. Natl. Acad. Sci. U S A.* **94,** 6523-6528 (1997).

(42) Anselmet, A., Mayat, E., Wietek, S., Layer, P. G., Payrastre, B., Massoulie, J. *FEBS Lett.* **510,** 175-180 (2002).

(43) Agrawal, S. (1991) *Prospects for Antisense Nucleic Acid Therapy of Cancer and AIDS*, 1st Ed., Wickstrom, E., Wiley-Liss, New York, pp 143-158.

(44) Wojcik, W. J., Swoveland, P., Zhang, X., Vanguri, P. *J. Pharmacol. Exp. Ther.* **278,** 404-410 (1996).

(45) Brukner, I., Tremblay, G. A. *Biochemistry* **39,** 11463-11466 (2000).

(46) Aldskogius, H. *Expert Opin. Ther. Targets* **5,** 655-668 (2001).

(47) Kreutzberg, G. W. *Trends Neurosci.* **19,** 312-318 (1996).

(48) Nakajima, K., Kohsaka, S. *Curr. Drug Targets Cardiovasc. Haematol. Disord.* **4,** 65-84 (2004).

Chapter 2.

Uptake, intracellular distribution, and novel binding proteins of immunostimulatory CpG oligodeoxynucleotides in microglial cells

Zhiren Zhang[a], Toni Weinschenk[b], Hermann J. Schluesener[a]

[a]Institute of Brain Research, University of Tuebingen, Calwer Street 3, Tuebingen D-72076, Germany

[b]Institute for Cell Biology, Department of Immunology, University of Tuebingen, Auf der Morgenstelle 15, Tuebingen D-72076, Germany

Journal of Neuroimmunology. 160, 32–40 (2005)

Abstract

Microglial cells are central components of the innate immune system of the brain and contribute to inflammatory and degenerative processes. DNA with unmethylated CpG dinucleotides is a potent stimulant of microglial cells. We have analyzed uptake, intracellular distribution, and cellular binding proteins of CpG oligdeoxynucleotides (ODNs) by the microglial cell line N9. The uptake of CpGODN is concentration-, time-, and temperature-dependent, but, interestingly, independent of the CpG dinucleotides. After internalisation, CpG-ODN localized to the cytoplasm and showed a typical speckled distribution pattern. We further purified the cellular binding proteins of CpG-ODN and identified several binding proteins by tryptic digestion and mass spectrometry. Most of the CpG-ODN binding proteins are RNA processing enzymes, which are important for RNA splicing, export, and stability. Further, we identified a protein, pigpen, which has not been observed in microglial cells, so far. These proteins apparently bind CpG-ODN with low selectivity, as binding is independent of CpG dinucleotides. Interference of immunostimulatory and therapeutic oligonucleotides with proteins and enzymes of RNA transport and processing has not been described so far and might affect the physiological functions of these proteins and also might influence cellular localization of therapeutic ODN. These findings are helpful in understanding the cellular fate of ODN and the nonsequence-specific effects of ODN and for rational design and evaluation of ODN-based therapeutic strategies.

Keywords: Microglia; CpG oligonucleotides; Mass spectrometry; Oligonucleotides binding proteins; hnRNPs

1. Introduction

Bacterial oligdeoxynucleotide (ODN) and synthetic ODN with hexameric motifs consisting of central unmethylated CpG dinucleotides are immunostimulatory and can trigger Th1-polarized immune response. CpG dinucleotides flanked by two 5' purine and two 3' pyrimidines are strongly immunostimulatory. Such CpG-ODN can activate, in vitro and in vivo, a variety of immune cells, including B cells, plasmacytoid dendritic cells, monocytes, macrophages, and natural killer cells. They can induce a wide variety of Th1-promoting cytokines, such as interleukin-12 and interferon-γ, and up-regulate several costimulatory molecules. Owing to their strong immunostimulatory activity, a number of CpG-ODN are at various stages of preclinical and clinical evaluation as antitumor, antiviral, antibacterial, and anti-inflammatory agents, and as adjuvants in immunotherapy (Krieg, 2002; Rothenfusser et al., 2003; Dalpke et al., 2001).

Despite these different experimental and therapeutic applications, little is known about cellular binding proteins of CpG-ODN. It has been reported that CpG-ODN binds to the pattern recognition receptor Toll-like receptor-9 (TLR-9) after internalization (Hemmi et al., 2000), and CpG-ODN uptake is the rate-limiting step for CpG-ODN activity (Manzel et al., 1999). The mechanisms of cellular uptake and intracellular binding/distribution of CpG-ODN are not well understood and some intermediate molecules might be involved (Modlin, 2000; Medzhitov, 2001).

To overcome the problem of rapid degradation of unmodified ODN in serum and inside cells, ODN analogs have been used in clinical research to increase resistance to cellular nucleases. Among the most commonly used analogs are phosphorothioate (PS) ODN, which has been shown to possess a favourable profile for therapeutic development with respect to hybridisation affinity, nuclease stability, pharmacokinetic distribution, and cellular uptake. However, PS modification leads to a greater degree of protein binding compared to diester ODN, which determines much of the toxicological characteristics of this class of ODN. In vitro PS ODN causes transcription factor Sp1 activation, platelet aggregation, inhibition of cell proliferation and migration, perturbation of

cell surface receptor binding, and induction of changes in cell morphology (Perez et al., 1994; Wang et al., 1996; Rockwell et al., 1997; Anselmet et al., 2002). In clinical studies, PS ODN caused thrombocytopenia, fatigue, fever, rashes, leukopenia, and complement activation (Yuen and Sikic, 2000). In addition, PS ODN might be neurotoxic at therapeutic concentrations higher than 1 μM (Agrawal, 1991), causing paralysis and necrosis in the spinal cord (Wojcik et al., 1996). Such sequence-independent effects are probably due to binding of PS ODN to multiple cellular proteins (Rockwell et al., 1997; Brukner and Tremblay, 2000).

Microglial cells are the brain's effector cells of the innate immune system and play a key role in all major central nervous system pathologies. Microglial cells can be activated by CpG-ODN in vitro and in vivo and express TLR-9 (Dalpke et al., 2002a,b; Takeshita et al., 2001a,b; Schluesener et al., 2001; Carpentier et al., 2000).

The object of the present study was to examine the binding, uptake, and intracellular distribution of CpG-ODN by microglial cells and, finally, to analyze their binding proteins by mass spectrometry.

2. Materials and methods

2.1. Synthetic ODN

All ODNs used in this study were obtained from MWGBiotech (Ebersberg, Germany). Sequences of ODN are as follows: CpG3: 5'-TCCATGACGTTCCTGATCGT-3', CpG-inverse3: 5'-TCCATGAGCTTCCTGATGCT-3'; CpG1826: 5'-TCCATGACGTTCCTGACGTT-3' and CpGinverse1826: 5'-TCCATGAGCTTCCTGAGCTT-3'. PS ODN, unmodified ODN, FITC-labelled ODN, biotinlabelled ODN, and unlabelled ODN were used. The difference between CpG3 and CpG1826, or between CpG-inverse3 and CpG-inverse1826, only exists at the four nucleotides located at the 3' end.

2.2. Cell cultures

Murine N9 microglial cells were cultured in RPMI 1640 with 10% heat-inactivated fetal calf serum (FCS) with penicillin and streptomycin at 100 U/ml (Gibco, Grand Island, NY) at 37°C in 5% CO_2.

2.3. Cytofluorometric analysis of ODN uptake

A total of 10^6 microglial N9 cells in 10 ml of RPMI 1640 with 10% FCS were seeded into a 10-cm^2 Petri dish and cultured for 24 h. Afterwards, the medium was removed and cells were washed two times with FCS-free RPMI 1640 and then incubated with FCS-free RPMI 1640 at 37°C for 1 h to remove residual FCS. After incubation, the medium was replaced by serum-free RPMI 1640 containing FITC-ODN at indicated concentrations. Cells were cultured in the dark for the time indicated. Then, incubation was terminated and cells were incubated with PBA buffer (phosphate-buffered saline (PBS), 2% BSA, and 0.1% sodium azide) at 4°C for 10 min to remove cell surface binding of ODN. Thereafter, cells were washed twice with PBS and detached from dishes with trypsin-EDTA. After neutralizing the trypsin with the medium containing FCS, cells were collected by centrifugation at 1000 rpm for 5 min. After washing, cell fluorescence intensity was quantified by flow cytometry (Dalpke et al., 2002a,b).

For inhibition experiments, sodium azide (0.1%) (LeGrow et al., 1999), dextransulfate (0.14 mg/ml), polyvinylsulfate (0.14 mg/ml), or fucoidan (0.14 mg/ml) (Liang et al., 2001) were added, respectively, 15 min prior to addition of FITC-ODN.

For pulse experiments, microglial cells were washed with PBS after incubation with FITC-ODN and then incubated in medium containing 10% FCS as indicated. After incubation, other steps were the same as described above (Dalpke et al., 2002a,b). All experiments were performed in triplicates and presented are typical result.

2.4. Fluorescence microscopy

A total of 10^5 N9 cells in 0.4 ml of RPMI 1640 were seeded onto six-well chamber slides (Lab-Tek Chamber Slide; Nalgene Nunc International Naperville, USA) and cultured at 37°C, 5% CO2 for 24 h. Afterwards, medium was removed and cells were washed two times with FCS-free RPMI 1640 and then incubated with FCS-free medium at 37°C for 1 h to remove residual FCS. After incubation, cells were washed once with PBS and then FITC-ODNs (0.2 μM) in FCS-free RPMI 1640 were added and cells were cultured for 1 h at 37°C. After removal of the medium, cells were washed with PBS two times and then fixed with 2% paraformaldehyde (PFA) for 15 min at room temperature. After a further wash with PBS, cells were mounted in a mounting medium containing DAPI (Vectashield Mounting Medium with DAPI; Vector Laboratories, Burlingame, USA), and then observed by fluorescence microscopy. All experiments were performed in triplicate and typical results are presented.

2.5. Analysis of nitric oxide (NO) induction

A total of 5 x 10^5 cells in 500 μl of medium were seeded in 48-well plates and cultured for 24 h. After replacement of the medium by medium without phenol red, stimulants were added at indicated concentrations and cells cultured for 48 h. The concentration of NO in supernatants was measured by standard Griess assay (Sigma, Munich, Germany). Briefly, 50 μl of supernatants was mixed with an equal volume of Griess reagent (1% sulphanilamide and 0.1% naphthyl-ethylenediamine dihydrochloride in 2.5% phosphoric acid). After 10 min of incubation in the dark at room temperature, the absorbance of the chromophore formed was measured at 560 nm using a multiplate reader. Nitrite concentration was calculated by comparison to a standard calibration curve with sodium nitrite (Wang et al., 2002). All experiments were performed in triplicate.

2.6. CpG-ODN-mediated protein purification

N9 cells cultured in flasks were rinsed with ice-cold PBS two times and then collected with a scraper. A total of 1.5 x 10^8 cells were sonicated and incubated in solubilization buffer (50 mM Tris-HCl, 150 mM NaCl, 1% Triton X-100)

containing a protease inhibitor cocktail (Sigma) for 2 h on ice. Lysates were cleared by centrifugation at 14,000g at 4°C for 5 min. An aliquot of the supernatants was used for protein purification.

One milligram (100 µl) of magnetic streptavidin microbeads (Dynal Biotech, Oslo, Norway) was coated with 200 pmol of biotin-labelled ODN by incubation in 1 ml of selection buffer (30 min, room temperature). The lysates were incubated with the ODN-coated magnetic microbeads in the presence of a 100-fold excess of tRNA (20 nmol) as an unspecific competitor in selection buffer (total volume 1.5 ml, 0°C, 15 min). The protein-ODN-magnetic bead-complex was recovered in a magnet stand and washed five times (first wash: 1 ml of selection buffer with 150 mM NaCl; second through fifth wash: 200 µl of selection buffer with 100 mM NaCl with 2 nmol tRNA). Proteins were removed from CpG-ODN-coated beads by heating in the loading buffer and analyzed by 10% polyacrylamide gel electrophoresis and detected by staining with Coomassie blue. Bands were analysed by tryptic digestion and mass spectrometry (Blank et al., 2001). This process is shown in Fig. 4J.

2.7. Protein identification

In gel, tryptic digestion was performed as described (Shevchenko et al., 1996) and modified as outlined below. Briefly, the protein band was excised from the gel, fully destained, and digested for 3 h with porcine trypsinase (sequencing grade, modified; Promega, Mannheim, Germany) at a concentration of 67 ng/µl in 25 mM ammonium bicarbonate, pH 8.1, at 37°C. Prior to peptide mass mapping and sequencing of tryptic fragments by tandem mass spectrometry, the peptide mixture was extracted from the gel by 1% formic acid followed by two changes of 50% methanol. The combined extracts were vacuum-dried until only 1-2 µl were left and the peptides were purified by ZipTip according to the manufacturers' instructions (Millipore, Bedford, MA, USA). MALDI-TOF analysis from the matrix a-cyano-4-hydroxycinnamic acid/nitrocellulose prepared on the target using the fast evaporation method (Arnott et al., 1998) was performed on a Bruker Reflex III (Bruker Daltonik, Bremen, Germany) equipped with a N2

337-nm laser gridless pulsed ion extraction, and externally calibrated using synthetic peptides with known masses. The spectra were obtained in positive ionization mode at 23 kV.

Sequence verification of some fragments was performed by nanoelectrospray tandem mass spectrometry on a hybrid quadrupole orthogonal acceleration time of flight tandem mass spectrometer (Q-Tof; Micromass, Manchester, UK) equipped with a nanoflow electrospray ionization source. Gold-coated glass capillary nanoflow needles were obtained from Protana (Type Medium NanoES spray capillaries for the Q-Tof; Micromass, Odense, Denmark). Database searches (NCBInr, nonredundant protein database) were done using the MASCOT software from Matrix Science (Perkins et al., 1999).

3. Results

3.1. Characterizations of CpG-ODN uptake and distribution in microglial N9 cells

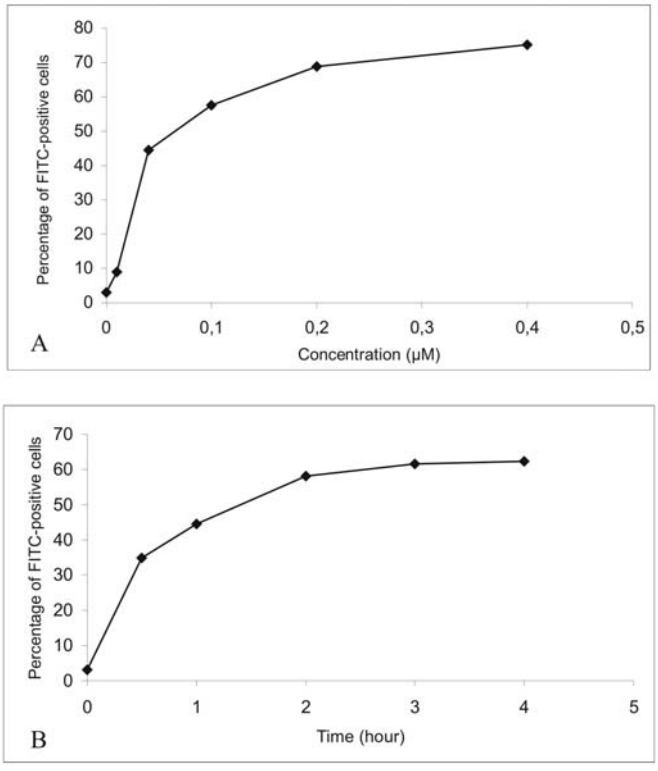


Fig. 1. PS CpG-ODN uptake in N9 cells is dose- and time-dependent.
(A) N9 microglial cells were cultured for 24 h and then washed with serum-free RPMI 1640 to remove residual FCS. PS FITC-CpG3 was added at indicated concentrations (in FCS-free RPMI 1640) and incubated for 1 h. Thereafter, cells were washed with PBA buffer and treated with trypsin–EDTA to remove cell surface-bound FITC-ODN. The fluorescence of N9 cells was measured by flow cytometry.
(B) N9 microglial cells were cultured for 24 h and then washed with serum-free RPMI 1640 to remove residual FCS. PS FITC-CpG3 0.2 (μM) was added (in FCS-free RPMI 1640) and incubated at 37 °C for various times. Thereafter, cells were washed with PBA buffer and treated with trypsin–EDTA to remove cell surface-bound FITC-ODN. The fluorescence of N9 cells was measured by flow cytometry.

FITC-labeled PS CpG3-ODN, PS CpG-inverse3 ODN, and unmodified CpG3-ODN were used in this study to assess uptake by microglial N9 cells. Notably, ODNs with inverted CpG motifs were used as control of selectivity.

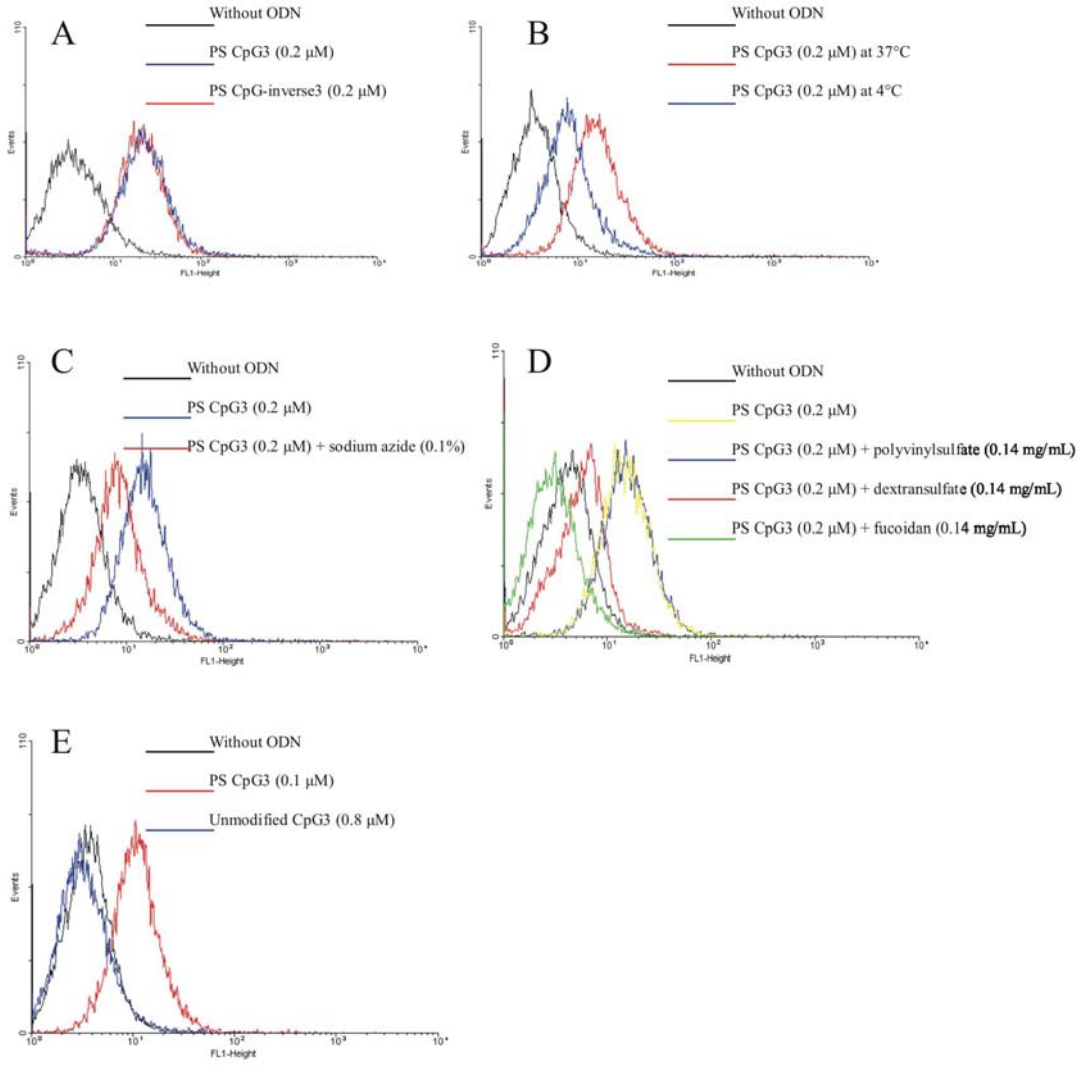


Fig. 2. CpG-ODN uptake in N9 cells is temperature- and modification-dependent and CpG motif-independent, and can be inhibited by sodium azide, fucoidan, and dextransulfate but not by polyvinylsulfate. N9 microglial cells were cultured in Petri dishes for 24 h. Then, cells were washed with serum-free RPMI 1640 to remove residual FCS. Thereafter, 0.2 µM FITC-ODN in FCS-free medium was added and cells were incubated for 1 h at 37 or 4 °C. Thereafter, cells were washed and treated with trypsin–EDTA to remove cell surface-bound ODN. Fluorescence of N9 cells was measured by flow cytometry. For inhibition experiments, 0.1% sodium azide, fucoidan (0.14 mg/ml), dextransulfate (0.14 mg/ml), or polyvinylsulfate (0.14 mg/ml) were added, respectively, 15 min prior to addition of FITC-ODN.

N9 microglial cell cultures were incubated with FITCCpG3-ODN at different concentrations and for different time periods. Thereafter, surface-bound ODNs were removed by trypsin-EDTA treatment and PBA buffer incubation. Dislodged

cells were analysed by flow cytometry. Uptake of PS FITC-CpG3-ODN increased at higher concentrations (Fig. 1A). Initial uptake of PS FITC-CpG3-ODN was fast, but after 2 h, it became slow (Fig. 1B). Microglial cells internalised the PS FITC-CpG3-ODN and control PS FITC-CpG-inverse3-ODN with the same efficiency (Fig. 2A).

Incubation of N9 cells at 4°C dramatically decreased PS FITC-CpG3-ODN uptake (Fig. 2B). Sodium azide, an inhibitor of cellular respiration and thus active transport, also significantly decreased the PS FITC-CpG3-ODN uptake (Fig. 2C). These findings strongly argue for an energy-dependent uptake of ODN.

Polyanions are polar, negatively charged molecules, and were used as competitors for PS FITC-CpG3-ODN uptake. Fig. 2D showed that uptake could be inhibited by dextransulfate and fucoidan, but not by polyvinylsulfate. N9 cells take up PS CpG3-ODN much more efficiently than unmodified CpG3-ODN (Fig. 2E).

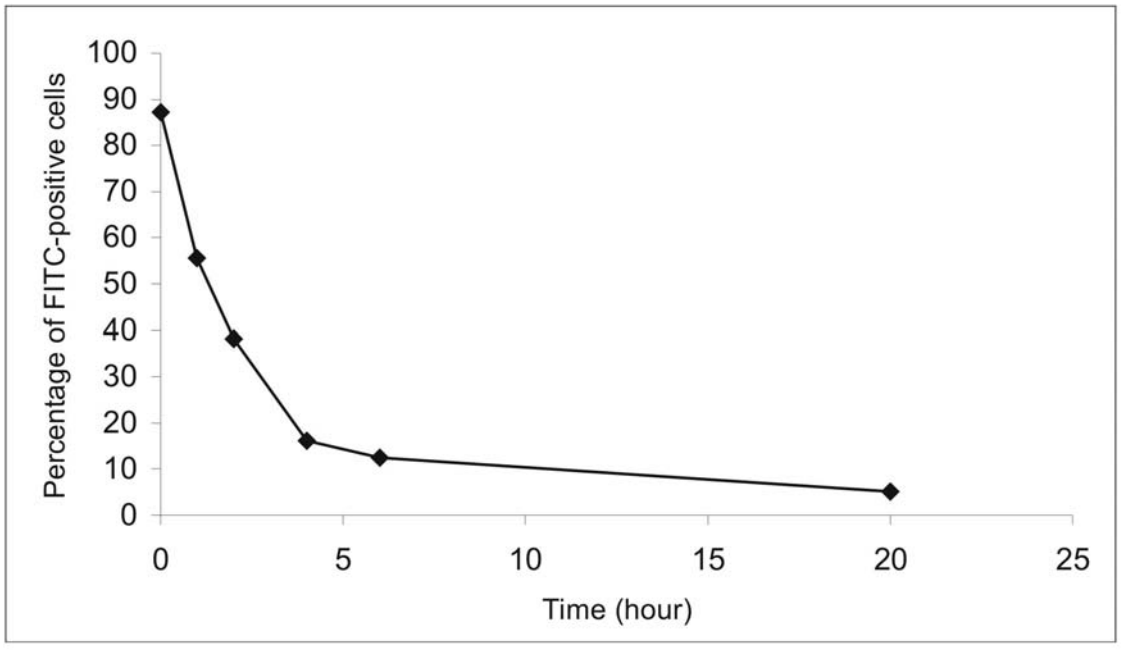


Fig. 3. Pulse experiment with PS FITC-CpG3-ODN in N9 Cells. N9 microglial cells were cultured in Petri dishes for 24 h. Then, cells were washed with serum-free RPMI 1640 to remove residual FCS. Then 0.2 µM PS FITC-CpG3-ODN in FCS-free medium was added and incubated with cells for 1 h at 37 °C. Thereafter, cells were washed and treated with trypsin–EDTA to remove cell surface binding ODN. After further incubations as indicated, fluorescence of N9 cells was measured by flow cytometry.

A pulse experiment shown in Fig. 3 revealed that after single pulse with PS FITC-CpG3-ODN, fluorescence intensity dropped with time, suggestive of degradation (or excretion) of ODN. After 4 h, the fluorescence in N9 cells had almost vanished.

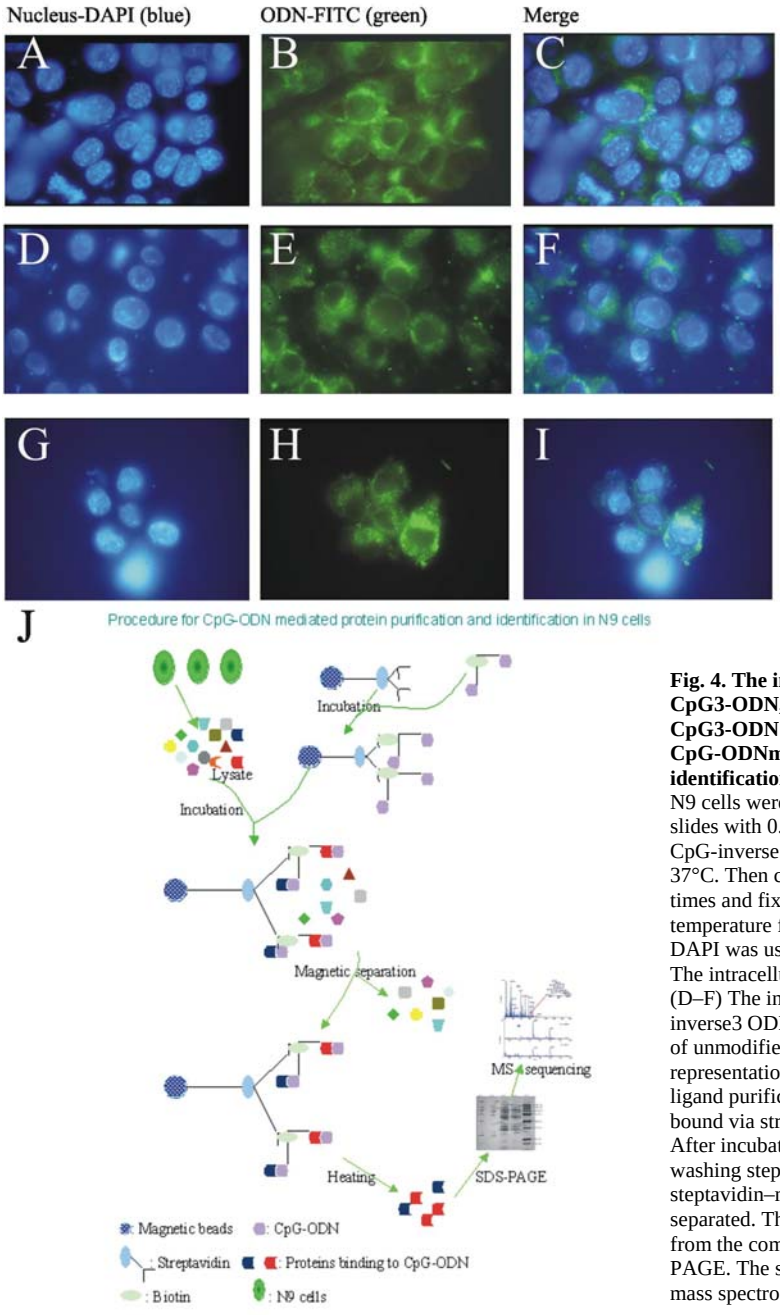


Fig. 4. The intracellular distribution of PS CpG3-ODN, PS CpG-inverse3, and unmodified CpG3-ODN in fixed N9 cells and procedure for CpG-ODNmediated protein purification and identification from N9 cells.
N9 cells were cultured on eight-well chamber slides with 0.2 μM FITC-ODN (PS CpG3, PS CpG-inverse3, or unmodified CpG3) for 1 h at 37°C. Then cells were washed with PBS for three times and fixed with PFA (2%) at room temperature for 15 min. Mounting medium with DAPI was used to show the cell nucleus. (A–C) The intracellular distribution of PS CpG3-ODN. (D–F) The intracellular distribution of PS CpG-inverse3 ODN. (G–I) The intracellular distribution of unmodified CpG3-ODN. (J) Schematic representation of the experimental procedure for ligand purification. Biotin-labelled ODNs were bound via streptavidin to magnetic microbeads. After incubation with cell lysates and several washing steps, the protein–CpG–ODN–biotin–steptavidin–magnetic beads complex was separated. The bound ligands were then recovered from the complex by heating and analysed by SDS-PAGE. The separated proteins were sequenced by mass spectrometry.

Fluorescence microscopy was used to study the intracellular distribution of FITC-CpG3-ODN (nuclei were counterstained with DAPI). The intracellular distribution pattern of FITC-ODN was independent of the CpG motif and

independent of modification. The ODN localized to the cytoplasm and showed a typical speckled pattern (Fig. 4).

3.2. CpG-ODNs can induce NO in microglial cells

As shown in Fig. 5, NO was induced in microglial N9 cells by PS CpG3-ODNs and lipopolysaccharide (LPS) but not by PS CpG-inverse3 ODNs, which suggested TLR-9 expression in N9 cells.

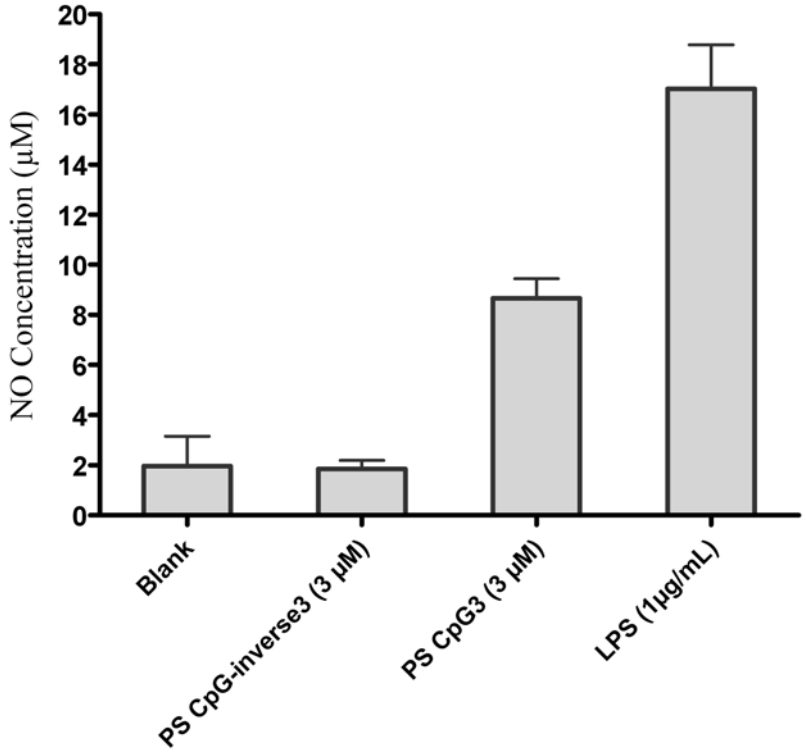


Fig. 5. CpG-ODNs can induce NO expression in microglia cells. A total of 5 x 10^5 cells in 500 µl of medium were seeded in 48-well plate and cultured for 24 h. After replacement of the medium with RPMI 1640 without phenol red, stimulants were added and incubated for 48 h. The concentration of NO in supernatants was measured by the standard Griess assay. Each bar represents the mean and S.E.M. of triplicates.

3.3. CpG-ODN-mediated binding protein purification and identification

To detect cellular-binding proteins of CpG-ODN in N9 microglial cells, we applied a method described recently (Blank et al., 2001) and presented schematically in Fig. 4J. Biotinylated ODN was used to capture binding protein from microglial N9 cells. After resolving the proteins by PAGE, some of the recovered peptides were identified by mass spectrometry.

Biotin-labelled PS CpG3-ODN, its CpG-inverse control, unmodified CpG3-ODN, its CpG-inverse control, unmodified CpG1826, and its CpG-inverse control were each used to purify binding proteins.

The spectrum of binding proteins is shown in Fig. 6 and no differences were observed between CpG-ODN and control ODN with inverted CpG motifs. However, differences were observed in respect to the modification of the ODN. The spectra of binding proteins for PS and unmodified ODN were different, as there were more proteins bands for PS ODN than for the unmodified ODN. Binding proteins for ODN were sequence-dependent because binding proteins for unmodified CpG3 and CpG1826 were different, although there were only several bases different between them. There were no binding proteins for unmodified CpG3 and its control (data not shown), but several binding proteins were identified for CpG1826 and its control. Our results indicate the complexity of cellular binding proteins for ODN.

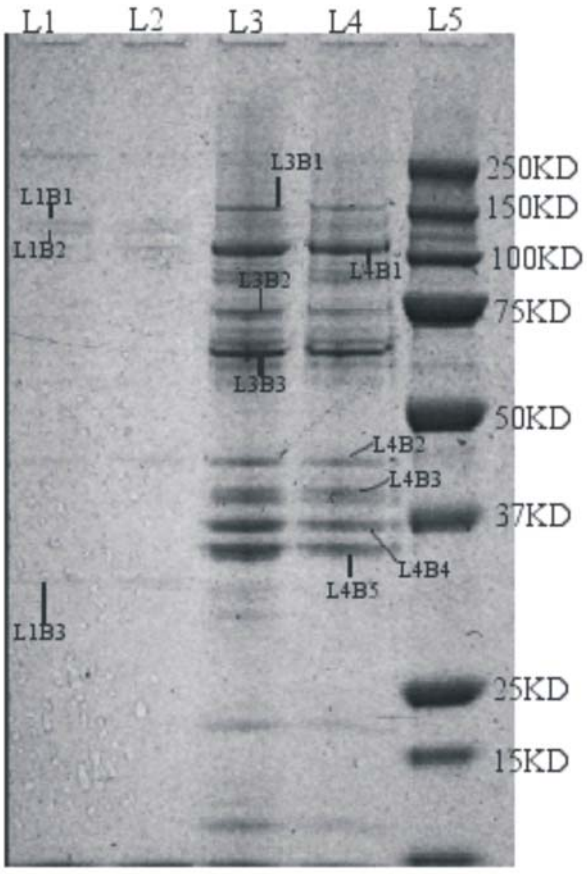


Fig. 6. Protein purification with CpG-ODN from microglial cells.
L1: Unmodified CpG-inverse1826; L2: unmodified CpG1826; L3: PS CpG-inverse3; L4: PS CpG3; L5: protein standards. The indicated bands were identified as: L1B1, pyruvate carboxylase; L1B2, ADP ribosyl transferase; L1B3, a protein with strong homology to human hnRNP A0; L3B1, DEAD/H box polypeptide 9; L3B2, DEAD/H box polypeptide 3; L3B3, pigpen; L4B1, hnRNP-U; L4B2, p45 AUF1; L4B3, p42 AUF1; L4B4, p40 AUF1; L4B5, p37 AUF1.

Mass spectrometry was used to identify the binding proteins and several microglial proteins were identified unequivocally. Eight of these identified biding proteins for PS ODN are RNA-binding proteins of three different families: the hnRNP U and different length variants of hnRNP D protein (AUF1, p45 AUF1, p42 AUF1, and p40, AUF1, and p37 AUF1). In addition, two members of the DEAD/H box polypeptides (3 and 9) and pigpen were identified.

Interestingly, three proteins, pyruvate carboxylase, ADPribosyl transferase, and a protein with strong homology to human hnRNP A0, were ligands for unmodified ODN only but not to PS ODN.

4. Discussion

Cellular uptake of ODN is a crucial aspect for most ODN-based therapeutic agents, like immunostimulatory ODN, antisense ODN, DNazymes, or aptamers. Notably in the brain, where transfection agents increasing ODN uptake cannot be used, the spontaneous uptake of ODN is relevant to the design of ODN-based therapeutics. Uptake of ODN thus appears as a limiting step in ODN activity. We demonstrated that uptake of PS CpG-ODN by microglial cells is dependent on ODN concentration and incubation time. ODN uptake is temperature-sensitive and can be blocked by sodium azide and is thus energy-dependent. This is in agreement with reports from other cellular systems (Krieg et al., 1995; Loke et al., 1989; Stein et al., 1993). Pulse experiment showed the rapid decrease of fluorescence intensity after uptake, which may be due to degradation (or excretion; Stein and Cheng, 1993).

Dextransulfate, fucoidan, and polyvinylsulfate are all polyanions and scavenger receptor ligands. Scavenger receptors have been identified as ODN ligands. In our study, CpG-ODN uptake in N9 cells can be inhibited by dextransulfate and fucoidan, but unexpectedly not by polyvinylsulfate, as it has been shown that all these three polyanions can inhibit the CpG-ODN uptake in a dosedependent manner in several other cells (Liang et al., 2001). Thus, the ligands mediating uptake in microglial cells might differ from other cells. An interesting finding was

that uptake of ODN by N9 microglial cells was CpG motifindependent. Our results show that PS as well as inverted CpG-ODN were effectively taken up by N9 cells and accumulated with the same speckled distribution pattern in the cytoplasm. In mammalian cells, CpG-ODNs activate immune cells via TLR-9, which is located at the lysosomal compartment (Hemmi et al., 2000; Takeshita et al., 2001a,b; Ahmad-Nejad et al., 2002). Our data here support that TLR-9 does not particularly contribute to CpG-ODN uptake and cellular localization because CpG-ODN and CpG-inverse ODN share the same uptake efficiency and distribution pattern.

Many novel cellular binding proteins of ODN have been identified in our study. Unanticipated binding effects of ODN to cellular proteins have been described from antisense therapeutics (Perez et al., 1994; Wang et al., 1996; Rockwell et al., 1997; Anselmet et al., 2002; Yuen and Sikic, 2000; Agrawal, 1991; Wojcik et al., 1996; Brukner and Tremblay, 2000).

Our results show that binding proteins for ODN are CpG motif-independent, but sequence- and modification-dependent. Clearly, no specific or selective recognition of the CpG motif was observed, although differences were observed between the spectrum of binding proteins from PS and unmodified ODN. This result is in accordance with cellular uptake. PS ODN has a much more efficient uptake than unmodified ODN (Fig. 2E).

Three binding proteins, pyruvate carboxylase, ADPribosyl transferase, and a protein with strong homology to human hnRNP A0, are identified as ligands for unmodified CpG1826 and its CpG inverse control.

The PS ODN binding proteins identified in our experiment are mostly RNA binding proteins with a typical RNA binding domain. Among them, hnRNP U and hnRNP D (including four isoforms: p45 AUF1, p42 AUF1, p40 AUF1, and p37 AUF1) belong to the heterogeneous nuclear ribonucleoproteins (hnRNPs) family. DEAD/H box polypeptide 9 and 3 are as well RNA binding proteins as pigpen.

The hnRNPs constitute a family of more than 20 nucleic acid binding proteins designated from A to U. These proteins are characterized by one or more

RNAbinding domains together with auxiliary domains that mediate protein–protein interactions. Members of the hnRNP family are known to shuttle between the nucleus and the cytoplasm, and have roles in many aspects of mRNA maturation/turnover, RNA processing, and telomere/telomerase regulation (Ford et al., 2002). Out of many hnRNPs, a few were found to bind to our ODN.

DEAD box proteins, characterized by the conserved motif Asp-Glu-Ala-Asp (DEAD), have ATP-dependent RNA helicase activity. These proteins are implicated in a range of cellular processes that involve regulation of RNA function, including translation initiation, RNA splicing, ribosomal assembly, and RNA secondary structure regulation (Hamm and Lamond, 1998).

Protein pigpen is also a RNA-binding protein. Pigpen is mainly expressed in endothelial cells, particularly in pathological proliferating endothelia. The exact function of pigpen is not clear yet but it might be involved in regulation of cell proliferation (Alliegro, 2001). PS-ODN has been reported to exert nonsequence-specific effects on in vitro smooth muscle cell proliferation (Wang et al., 1996).

Thus, the binding proteins identified are mostly RNA binding proteins with important roles in cellular RNA transport, maturation, splicing, stabilization, transcription, and translation. Binding of therapeutic ODN to these proteins might cause a variety of unwanted effects. Such effects might not be limited to one or several genes only, since global RNA processing might be impaired. In agreement with our observation, microarray analysis used to evaluate the specificity of antisense effect of PS ODN showed that, in addition to the target gene, the expression of many other genes was also changed (Fisher et al., 2002). Cho et al. (2001) also used DNA microarray to analyze antisense effect of PS ODN and also observed global changes in gene expression profile.

Further, ODN binding to these cellular proteins can influence cellular ODN localization, modifying or even abrogating the therapeutic effects of ODN. As the binding proteins identified here have different cellular localization patterns and even shuttle between nucleus and cytoplasm, and such binding is ODN sequencedependent, different ODNs that bind with different binding proteins

should have a selective tendency to localize in different cellular compartments. Lorenz et al. (2000) observed a nucleocytoplasmic shuttling of ODN, which shares characteristics with active transport and is carrier-mediated. Passive nuclear accumulation of PS ODN was also observed (Leonetti et al., 1991; Clarenc et al., 1993). Such different localization patterns should have a close relationship with different cellular binding proteins.

Taken together, our data indicate that CpG-ODN are taken up by microglial cells, but this process is not selective for the CpG motif, and could be considered more as a transport process for oligonucleotides in general. Several RNA-binding proteins as binding proteins of ODN have been identified. Thus, it appears that oligonucleotides, in general, and CpG-ODN, in particular, might interfere with cellular RNA processing machinery.

This bears the intrinsic problem of interference with an essential cellular pathway of cellular RNA processing and foreign ODN localization. The binding of foreign therapeutic ODN with cellular binding proteins should be taken into account when designing rational ODN-based therapeutic reagents and when considering mechanisms and sites of action. However, considering endogeneous cellular binding proteins, ODN uptake and cellular distribution could be consequently optimized for rational drug design.

References

Agrawal, S., 1991. In: Wickstrom, E. (Ed.), Prospects for Antisense Nucleic Acid Therapy of Cancer and AIDS.Wiley-Liss, NewYork, pp. 143–158.

Ahmad-Nejad, P., Hacker, H., Rutz, M., Bauer, S., Vabulas, R.M., Wagner, H., 2002. Bacterial CpG-DNA and lipopolysaccharides activate Toll like receptors at distinct cellular compartments. Eur. J. Immunol. 32, 1958– 1968.

Alliegro, M.C., 2001. Pigpen and endothelial cell differentiation. Cell Biol. Int. 7, 577– 584.

Anselmet, A., Mayat, E., Wietek, S., Layer, P.G., Payrastre, B., Massoulie, J., 2002. Non-antisense cellular responses to oligonucleotides. FEBS Lett. 510, 175– 180.

Arnott, D., O'Connell, K.L., King, K.L., Stults, J.T., 1998. An integrated approach to proteome analysis: identification of proteins associated with cardiac hypertrophy. Anal. Biochem. 258, 1– 18.

Blank, M., Weinschenk, T., Priemer, M., Schluesener, H., 2001. Systematic evolution of a DNA aptamer binding to rat brain tumor microvessels. selective targeting of endothelial regulatory protein pigpen. J. Biol. Chem. 276, 16464–16468.

Brukner, I., Tremblay, G.A., 2000. Cellular proteins prevent antisense phosphorothioate oligonucleotide (SdT18) to target sense RNA (rA18): development of a new in vitro assay. Biochemistry 39, 11463– 11466.

Carpentier, A.F., Xie, J., Mokhtari, K., Delattre, J.Y., 2000. Successful treatment of intracranial gliomas in rat by oligodeoxynucleotides containing CpG motifs. Clin. Cancer Res. 6, 2469– 2473.

Cho, Y.S., Kim, M.K., Cheadle, C., Neary, C., Becker, K.G., Cho-Chung, Y.S., 2001. Antisense DNAs as multisite genomic modulators identified by DNA microarray. Proc. Natl. Acad. Sci. U. S. A. 98, 9819– 9823.

Clarenc, J.P., Lebleu, B., Leonetti, J.P., 1993. Characterization of the nuclear binding sites of oligodeoxyribonucleotides and their analogs. J. Biol. Chem. 268, 5600– 5604.

Dalpke, A., Zimmermann, S., Heeg, K., 2001. CpG-oligonucleotides in vaccination: signaling and mechanisms of action. Immunobiology 204, 667–676.

Dalpke, A.H., Schafer, M.K., Frey, M., Zimmermann, S., Tebbe, J., Weihe, E., Heeg, K., 2002. Immunostimulatory CpG-DNA activates murine microglia. Immunology 168, 4854– 4863.

Dalpke, A.H., Zimmermann, S., Albrecht, I., Heeg, K., 2002. Phosphodiester CpG oligonucleotides as adjuvants: polyguanosine runs enhance cellular uptake and improve immunostimulative activity of phosphodiester CpG oligonucleotides in vitro and in vivo. Immunology 106, 102– 112.

Fisher, A.A., Ye, D., Sergueev, D.S., Fisher, M.H., Shaw, B.R., Juliano, R.L., 2002. Evaluating the specificity of antisense oligonucleotide conjugates. A DNA array analysis. J. Biol. Chem. 277, 22980–22984.

Ford, L.P., Wright, W.E., Shay, J.W., 2002. A model for heterogeneous nuclear ribonucleoproteins in telomere and telomerase regulation. Oncogene 21, 580–583.

Hamm, J., Lamond, A.I., 1998. Spliceosome assembly: the unwinding role of DEAD-box proteins. Curr. Biol. 8, R532–R534.

Hemmi, H., Takeuchi, O., Kawai, T., Kaisho, T., Sato, S., Sanjo, H., Matsumoto, M., Hoshino, K., Wagner, H., Takeda, K., Akira, S., 2000. A Toll-like receptor recognizes bacterial DNA. Nature 408, 740– 744.

Krieg, A.M., 2002. CpG motifs in bacterial DNA and their immune effects. Annu. Rev. Immunol. 20, 709– 760.

Krieg, A.M., Yi, A.K., Matson, S., Waldschmidt, T.J., Bishop, G.A.,Teasdale, R., Koretzky, G.A., Klinman, D.M., 1995. CpG motifs in bacterial DNA trigger direct B-cell activation. Nature 374, 546– 549.

LeGrow, A.B., Fielding, D.C., Pressley, T.A., 1999. Stimulation of Na,KATPase by hypothyroidism in the thyroid gland. J. Endocrinol. 160,453– 460.

Leonetti, J.P., Mechti, N., Degols, G., Gagnor, C., Lebleu, B., 1991. Intracellular distribution of microinjected antisense oligonucleotides. Proc. Natl. Acad. Sci. U. S. A. 88, 2702–2706.

Liang, H., Reich, C.F., Pisetsky, D.S., Lipsky, P.E., 2001. The role of surface Ig binding in the activation of human B cells by phosphorothioate oligodeoxynucleotides. Scand. J. Immunol. 54, 551–563.

Loke, S.L., Stein, C.A., Zhang, X.H., Mori, K., Nakanishi, M., Subasinghe, C., Cohen, J.S., Neckers, L.M., 1989. Characterization of oligonucleotide transport into living cells. Proc. Natl. Acad. Sci. U. S. A. 86, 3474– 3478.

Lorenz, P., Misteli, T., Baker, B.F., Bennett, C.F., Spector, D.L., 2000. Nucleocytoplasmic shuttling: a novel in vivo property of antisense phosphorothioate oligodeoxynucleotides. Nucleic Acids Res. 28, 582– 592.

Manzel, L., Strekowski, L., Ismail, F.M., Smith, J.C., Macfarlane, D.E., 1999. Antagonism of immunostimulatory CpG-oligodeoxynucleotides by 4-aminoquinolines and other weak bases: mechanistic studies. J. Pharmacol. Exp. Ther. 291, 1337–1347.

Medzhitov, R., 2001. Toll-like receptors and innate immunity. Nat. Rev., Immunol. 1, 135– 145.

Modlin, R.L., 2000. Immunology. A toll for DNA vaccines. Nature 408, 659– 660.

Perez, J.R., Li, Y., Stein, C.A., Majumder, S., van Oorschot, A., Narayanan, R., 1994. Sequence-independent induction of Sp1 transcription factor activity by phosphorothioate oligodeoxynucleotides. Proc. Natl. Acad. Sci. U. S. A. 91, 5957–5961.

Perkins, D.N., Pappin, D.J., Creasy, D.M., Cottrell, J.S., 1999. Probabilitybased protein identification by searching sequence databases using mass spectrometry data. Electrophoresis 20, 3551– 3567.

Rockwell, P., O'Connor, W.J., King, K., Goldstein, N.I., Zhang, L.M., 1997. Cell-surface perturbations of the epidermal growth factor and vascular endothelial growth factor receptors by phosphorothioate oligodeoxynucleotides. Proc. Natl. Acad. Sci. U. S. A. 94, 6523– 6528.

Rothenfusser, S., Tuma, E., Wagner, M., Endres, S., Hartmann, G., 2003. Recent advances in immunostimulatory CpG oligonucleotides. Curr. Opin. Mol. Ther. 5, 98– 106.

Schluesener, H.J., Seid, K., Deininger, M., Schwab, J., 2001. Transient in vivo activation of rat brain macrophages/microglial cells and astrocytes by immunostimulatory multiple CpG oligonucleotides. J. Neuroimmunol. 113, 89– 94.

Shevchenko, A., Wilm, M., Vorm, O., Mann, M., 1996. Mass spectrometric sequencing of proteins silver-stained polyacrylamide gels. Anal. Chem. 68, 850– 858.

Stein, C.A., Cheng, Y.C., 1993. Antisense oligonucleotides as therapeutic agents—is the bullet really magical? Science 261, 1004– 1012.

Stein, C.A., Tonkinson, J.L., Zhang, L.M., Yakubov, L., Gervasoni, J., Taub, R., Rotenberg, S.A., 1993. Dynamics of the internalization of phosphodiester oligodeoxynucleotides in HL60 cells. Biochemistry 32, 4855– 4861.

Takeshita, F., Leifer, C.A., Gursel, I., Ishii, K.J., Takeshita, S., Gursel, M., Klinman, D.M., 2001. Cutting edge: role of Toll-like receptor 9 in CpG DNA-induced activation of human cells. J. Immunol. 167, 3555– 3558.

Takeshita, S., Takeshita, F., Haddad, D.E., Janabi, N., Klinman, D.M., 2001. Activation of microglia and astrocytes by CpG oligodeoxynucleotides. NeuroReport 12, 3029– 3032.

Wang, W., Chen, H.J., Schwartz, A., Cannon, P.J., Stein, C.A., Rabbani, L.E., 1996. Sequence-independent inhibition of in vitro vascular smooth muscle cell proliferation, migration, and in vivo neointimal formation by phosphorothioate oligodeoxynucleotides. J. Clin. Invest. 98, 443– 450.

Wang, Z., Li, D.D., Liang, Y.Y., Wang, D.S., Cai, N.S., 2002. Activation of astrocytes by advanced glycation end products: cytokines induction and nitric oxide release. Acta Pharmacol. Sin. 23, 974– 980.

Wojcik, W.J., Swoveland, P., Zhang, X., Vanguri, P., 1996. Chronic intrathecal infusion of phosphorothioate or phosphodiester antisense oligonucleotides against cytokine responsive gene-2/IP-10 in experimental allergic encephalomyelitis of Lewis rat. J. Pharmacol. Exp. Ther. 278, 404– 410.

Yuen, A.R., Sikic, B.I., 2000. Clinical studies of antisense therapy in cancer. Front. Biosci. 5, D588–D59333.

Chapter 3.

The immunostimulatory activity of CpG oligonucleotides on microglial N9 cells is affected by a polyguanosine motif

Zhiren Zhang[a], Ketai Guo[b], Hermann J. Schluesener[a]

[a]Institute of Brain Research, University of Tuebingen, Calwer Street 3, Tuebingen D-72076, Germany

[b]Clinic for Thoracic, Cardiac and Vascular Surgery, University of Tuebingen, Calwer Str. 7-1, D-72076 Tuebingen, Germany

Journal of Neuroimmunology. 161, 68–77 (2005)

Abstract

Oligonucleotides (ODN) with hexameric motifs containing central unmethylated CpG dinucleotides are immunostimulatory. Also ODN with continuous guanosines (polyG motif) show a wide range of immunological activity. Depending on the position, the chemical property of the ODN backbone and the cell type, polyG motifs have either an enhancing or a suppressing effect on the immunostimulatory activity of the CpG-ODN. Microglial cells are central components of the innate immune system of the brain and are activated by CpG-ODN in vitro and in vivo. Here we present the analysis of the immunomodulatory effects of CpG-ODN carrying a polyG motif on the microglial cell line N9.

Our data show that N9 cells express Toll-like receptor 9 (TLR9) and are activated by CpG-ODN, which leads to expression of interleukin-12p40 (IL12p40), tumor necrosis factor-a (TNF-α) and inducible nitric oxide synthase (iNOS). A 3'-end polyG motif inhibits phosphothioate (PS) CpG-ODN immunostimulatory activity but enhances the immunostimulatory activity of phosphodiester (PE) CpG-ODN. Correspondingly, a 3'-end polyG motif improves the cellular uptake of PE CpG-ODN but does not change their cellular distribution pattern. Furthermore, PE CpG-ODN with a 3'-end polyG motif interact with a much higher number of cellular proteins than PE CpG-ODN. These data indicate that the 3'-end polyG motif could enhance the immunostimulatory activity of PE CpG-ODN in microglial N9 cells through increasing interaction with cellular proteins. Therefore PE CpG-ODN containing a 3'-end polyG motif resulting in increased immunostimulatory activity might be promising alternate analogues for studies in the central nervous system.

Keywords: Microglia; CpG oligonucleotides; Polyguanosine motif

1. Introduction

DNA has complex effects on the immune system. Unmethylated CpG dinucleotides within specific flanking bases (referred to as CpG motifs) are immunostimulatory and are at a much higher frequency present in bacterial than in vertebrate DNA. Bacterial DNA or synthetic ODN with CpG motifs are recognized as danger signal by the innate immune cells via TLR9 to trigger a Th1-polarized immune response. CpG-ODN can trigger B cells, plasmacytoid dendritic cells, monocytes, macrophages and natural killer cells to proliferate, mature, up-regulate several costimulatory molecules and to secrete a variety of Th1-promoting cytokines, chemokines and immunoglobulins. Based on their strong immunostimulatory activity, a number of CpG-ODN are at various stages of preclinical and clinical evaluation as adjuvant or therapeutic agents for cancer and allergic diseases (Krieg, 2002; Rothenfusser et al., 2003; Dalpke et al., 2001; Hemmi et al., 2000).

However the use of phosphodiester (PE) CpG-ODN is limited because of their rapid degradation in serum and inside cells, which results in weaker activity (Krieg et al., 1995). To overcome this problem, ODN with a phosphothioate (PS) backbone have been used to increase resistance to cellular nucleases and thus to prolong their action. However, PS ODN have non-specific effects. In vitro PS ODN cause transcription factor Sp1 activation, platelet aggregation, inhibition of cell proliferation and migration, perturbation of cell-surface receptor binding and induction of changes in cell morphology (Perez et al., 1994; Wang et al., 1996; Rockwell et al., 1997; Anselmet et al., 2002). In clinical studies, PS ODN caused thrombocytopenia, fatigue, fever, rashes, leukopenia and complement activation (Yuen and Sikic, 2000). In addition, PS ODN might be neurotoxic at therapeutic concentrations higher than 1 μM (Agrawal, 1991), causing paralysis and necrosis of the spinal cord (Wojcik et al., 1996). Moreover, PS CpG-ODN might exacerbate inflammatory tissue damage, cause autoimmune disease or increase sensitivity to toxic shock (Pisetsky, 1997; Deng et al., 1999; Segal et al., 2000; Cowdery et al., 1996; Sparwasser et al., 1997).

Single-stranded guanosine-rich ODN, which have the potency to form quadruplex structures that result in increased nuclease resistance and cellular uptake, are another type of ODN motifs that have a wide range of immunomodulatory activity (Dapic et al., 2003). In vitro, PS deoxyguanosine oligomers inhibit the production of interferon-γ by murine splenocytes upon induction by ConA or bacterial DNA (Halpern and Pisetsky, 1995). PE or PS deoxyguanosine oligomers can inhibit murine macrophage and dendritic cell activation (Zhu et al., 2002a,b). PS guanosine-rich ODN can directly lead to proliferation of macrophage progenitors from murine bone marrow cells (Lang et al., 1999). In addition, PS polyguanosines ODN display the ability to costimulate $CD8^+$ T cells (Lipford et al., 2000). They also block the downstream functions of interferon-γ, and the binding of interferon-g to its receptor (Balasubramanian et al., 1998; Lee et al., 1996). Furthermore, polyG motifs exert suppressive or stimulating effects on the immunostimulatory activity of CpG-ODN according to the cell type, the modification of the ODN backbone and their location within the ODN. The addition of a polyG motif to the 5' or 3'-end of the PE CpG-ODN improves their ability to activate NK cells (Kimura et al., 1994; Ballas et al., 1996) and to induce interferon-α production by dendritic cells (Krug et al., 2001). In PS CpG-ODN, a polyG motif can block their ability to activate NF-κB in B cells and in PE CpG-ODN it can reduce the level of B cell activation (Lenert et al., 2001). In macrophages and dendritic cells, only polyG located at the 3'-end of PE CpG-ODN can improve their immunostimulatory activity (Dalpke et al., 2002a; Lee et al., 2000).

Microglia, cells derived from the mononuclear-phagocyte lineage, are the brain's ubiquitous but normally inconspicuous immune effector cells. They constitute the brain's autochthonous source of macrophages and thus are part of its intrinsic immune system. Microglia respond rapidly to subtle, acute and chronic pathological stimuli and are prominently involved in many diseases. Microglial cells express TLR9 and can be activated by CpG-ODN in vitro and in vivo (Dalpke et al., 2002b; Takeshita et al., 2001; Schluesener et al., 2001; Carpentier et al., 2000). Up to now the effects of polyG motif on CpG-ODN have

not been studied in microglia. Here we describe the immunomodulatory effects of polyG motif on the immunostimulatory activity of CpG-ODN in microglial cells.

2. Materials and methods

2.1. Synthetic ODN

All ODN used in this study were synthesized by MWG-Biotech AG, Ebersberg, Germany. PS ODN, PE ODN, FITC labelled ODN, biotin labelled ODN and unlabelled ODN were used. Sequences of ODN are as follows: 1826: TCCATGACGTTCCTGACGTT, 1826GC: TCCATGAGCTTCCTGAGCTT, 1826-G6: TCCATGACGTTCCTGACGTTGGGGGG, 1826GC-G6: TCCATGAGCTTCCTGAGCTTGGGGGG, G6-1826: GGGGGGTCCATGACGTTCCTGACGTT, G6-1826GC: GGGGGGTCCATGAGCTTCCTGAGCTT.

2.2. Cell cultures

Murine N9 microglial cells were cultured in RPMI-1640 with 10% heat inactivated fetal calf serum (FCS) with penicillin and streptomycin at 100 U/mL (Gibco, Grand Island, NY) at 37°C in 5% CO_2.

2.3. RNA preparation and cDNA synthesis

Total RNA from cultured cells was prepared using the RNeasy Mini Kit (QIAGEN GmbH, Hilden, Germany) according to the manufacture's instruction. 1 µg RNA was reverse transcribed into cDNA using randomised primers.

2.4. RT-PCR

The expression of TLR9 and β-actin by N9 cells was determined using reverse transcription PCR with 1 µL cDNA. The following primers were used: β-actin

(sense, CCC TGT GCT GCT CAC CGA; antisense, ACA GTG TGG GTG ACC CCG TC), TLR9 (sense, GGG CCC ATT GTG ATG AAC C; antisense, GCT GCC ACA CTT CAC ACC AT). Cycle conditions were 94°C 3 min, then 30 cycles of: 94°C for 40 s, 55°C for 30 s and 72°C for 1 min. PCR products were analyzed on a 2% agarose gel using ethidium bromide staining.

2.5. Detecting cytokine induction by real-time RT-PCR

10^6 Cells/well were plated in 12-well cell culture plates and cultured for 24 h. Afterwards cells were stimulated with 1.5 μM of different unlabelled ODN and incubated for 12 h. Subsequently mRNA expression of IL12p40, iNOS and TNFα was quantified by real-time PCR in comparison with unstimulated cells using SYBR-Green as detection reagent and β-actin as reference standard. Following primers were used: IL12p40 (sense, CAG AAG CTA ACC CAT CTC CTG GTT TG; antisense, CCG GAG TAA TTT GGT GCT CCA CAC), TNF-α (sense,AAAATTCGAGTGACAAGCCTG TAG; antisense, CCC TTG AAG AGA ACC TGG GAG TAG) and iNOS (sense, CAG CTG GGC TGT ACA AAC CTT; antisense, CAT TGG AAG TGA AGC GTT TCG).

2.6. ODN uptake analysis

5 x 10^5 cells/well were seeded in 12-well cell culture plates and cultured for 24 h. Afterwards cells were washed with FCS-free RPMI 1640 and incubated with FCS-free RPMI 1640 at 37°C for 1 h to remove residual FCS. After incubation, medium was replaced by serum-free RPMI 1640 containing FITC labelled ODN at the indicated concentrations. Cells were cultured in the dark as long as indicated. After washing with PBA buffer (phosphate-buffered saline (PBS), 2% BSA, and 0.1% sodium azide) and PBS cells were detached from the plate with trypsin-EDTA. Cells were collected by centrifugation at 1000 rpm for 5 min and cell fluorescence intensity was quantified by flow cytometry. For the inhibition experiments, cells were preincubated with fucoidan (0.14 mg/mL) for 15 min at 37°C and then FITC labelled ODN were added for further incubation. The following steps were the same as described above.

2.7. ODN cellular distribution analysis using fluorescence microscopy

10^5 Cells in 0.4 mL RPMI1640 were seeded onto 6-well chamber slides (Lab-Tek Chamber Slide, Nalgene Nunc International Corp. Naperville, USA) and cultured for 24 h. After washing and incubating with FCS-free medium, cells were washed once with PBS and then incubated with FITC labelled ODN (0.5 μM) in FCS-free RPMI 1640 for 1 h at 37°C. Subsequently cells were fixed with 2% paraformaldehyde (PFA) for 15 min at room temperature and mounted in a mounting medium containing DAPI (VECTASHIELD Mounting Medium with DAPI, Vector Laboratories, Inc., Burlingame, USA). The cellular distribution of ODN was observed using fluorescence microscopy.

2.8. CpG-ODN mediated protein purification

N9 cells cultured in flasks were rinsed twice with ice-cold PBS and collected with a scraper. Cells were resuspended in solubilization buffer (50 mM Tris-HCl, 150 mM NaCl, 1% Triton X-100) and sonicated. Afterwards a protease inhibitor cocktail (Sigma, Munich, Germany) was added and cell lysates were incubated for 2 h on ice. Lysates were cleared by centrifugation at 14,000 g at 4°C for 5 min. An aliquot of the supernatants was used for protein purification.

One milligram (100 μL) of magnetic streptavidin microbeads (Dynal Biotech ASA, Oslo, Norway) was coated with 200 pmol of biotin labelled ODN by incubation in 1 mL selection buffer for 30 min at room temperature. The lysates were incubated with the ODN coated magnetic microbeads in the presence of a 100-fold excess of tRNA (20 nmol) as an unspecific competitor in selection buffer (total volume 1.5 mL, 0°C, 30 min). The protein-ODN-magnetic bead-complex was recovered in a magnet stand and washed five times (first wash: 1 mL of selection buffer with 150 mM NaCl; second through fifth wash: 200 μL of selection buffer with 100 mM NaCl with 2 nmol tRNA). Proteins were removed from CpG-ODN coated beads by heating in the loading buffer and analyzed by 10% polyacrylamide gel electrophoresis and detected by staining with Coomassie Blue (Blank et al., 2001).

3. Results

3.1. Microglial N9 cells express TLR9 and are activated by CpG-ODN

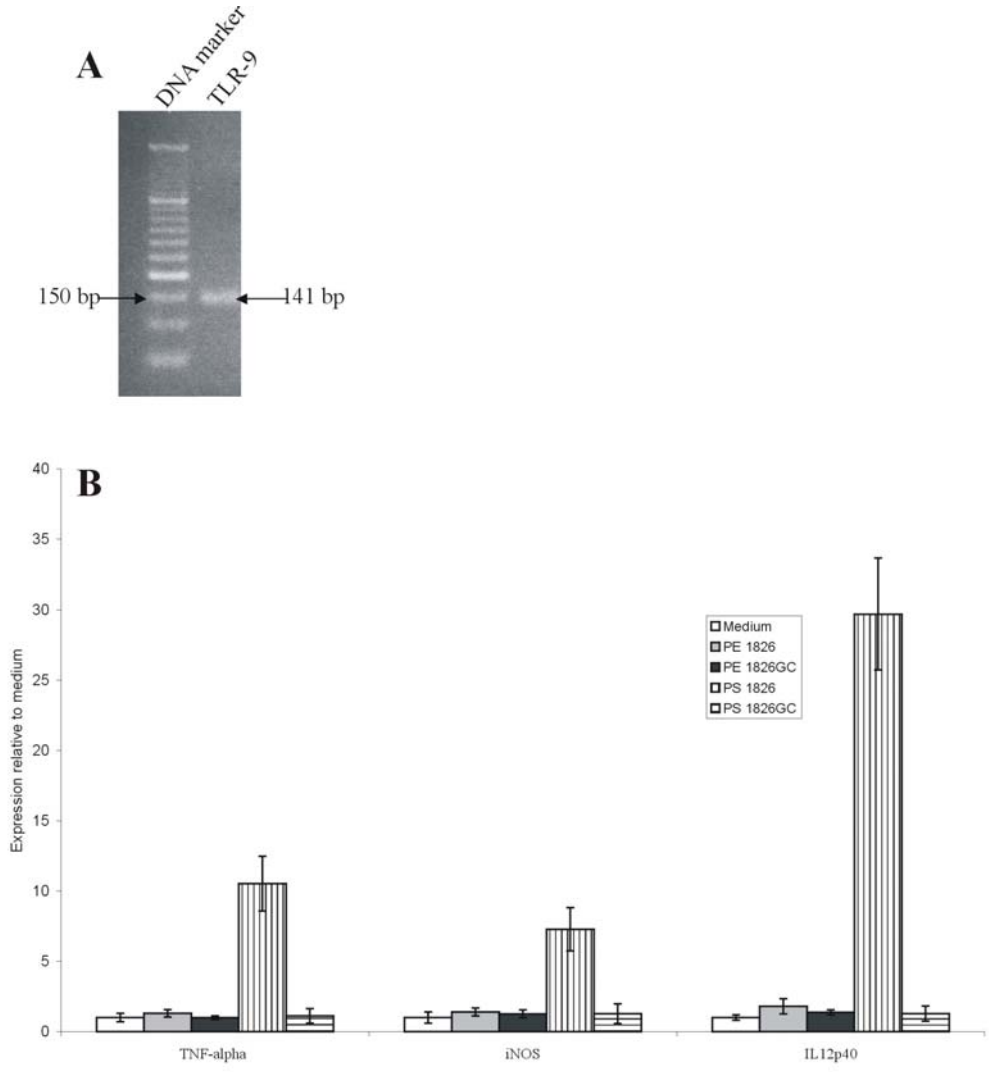


Fig. 1. Microglial N9 cells express TLR9 and produce TNF-α, iNOS and IL12p40 upon stimulation by CpG-ODN. (A) RT-PCR analysis shows that microglial N9 cells express TLR9. (B) N9 cells were stimulated for 12 h by various ODN (1.5 μM) and then expression of TNF-α, iNOS and IL12p40 was measured using real-time PCR. Data are expressed relative to incubation with medium alone and each bar represents the mean and SEM of triplicates.

To investigate the influence of a polyG motif on the immunostimulatory activity of CpG-ODN in microglial N9 cells, we first tested the expression of TLR9 in N9 cells using RT-PCR. As shown in Fig. 1A, microglial N9 cells expressed TLR9 at the mRNA level. Subsequently, a previously reported CpG-ODN (1826) was

applied to stimulate N9 cells and to examine the biological activity of TLR9. PS 1826 strongly induced the expression of proinflammatory cytokines, TNF-α and IL12p40, and anti-pathogen effector molecule, iNOS in N9 cells (Fig. 1B). This induction is CpG motif specific, since PS 1826GC did not have any immunostimulatory activity. PE 1826, however, only marginally induced N9 cells to express TNF-α, IL12p40 and iNOS, which correspond to previously reported data.

3.2. The extension of CpG-ODN by polyG motifs affects immunostimulatory activity on N9 cells

Subsequently the effects of polyG motifs on the immunostimulatory activity of PE and PS 1826 were probed in microglial N9 cells. As shown in Fig. 2, PE 1826-G6, which contained a 3'-end polyG motif, markedly enhanced TNF-α, IL12p40 and iNOS induction compared with PE 1826. The conversion of CpG dinucleotides to GpC dinucleotides (PE 1826-G6/PE 1826GC-G6) completely abrogated induction of TNF-α, IL12p40 and iNOS. This indicates that a polyG motif alone could not induce TNF-α, IL12p40 and iNOS expression. The introduction of a 5'-end polyG motif (PE G6-1826) only slightly enhanced TNF-α, IL12p40 and iNOS induction compared with PE 1826, which suggests that the location of the polyG motif is important for the immunomodulatory activity to PE CpG-ODN. These data indicate that the addition of a polyG motif at the 3'-end of PE CpG-ODN increases immunostimulatory activity in N9 cells.

Furthermore, the effects of a polyG motif on PS CpG-ODN were analyzed. Unexpectedly, extension of the 3'- or 5'-end (PS 1826-G6 or PS G6-1826) with polyG motif inhibited TNF-α, IL12p40 and iNOS induction compared with PS 1826, suggesting that the conjugation of a polyG motif at the 3' or 5' terminus inhibit the immunostimulatory potential of PS CpG-ODN (Fig. 2).

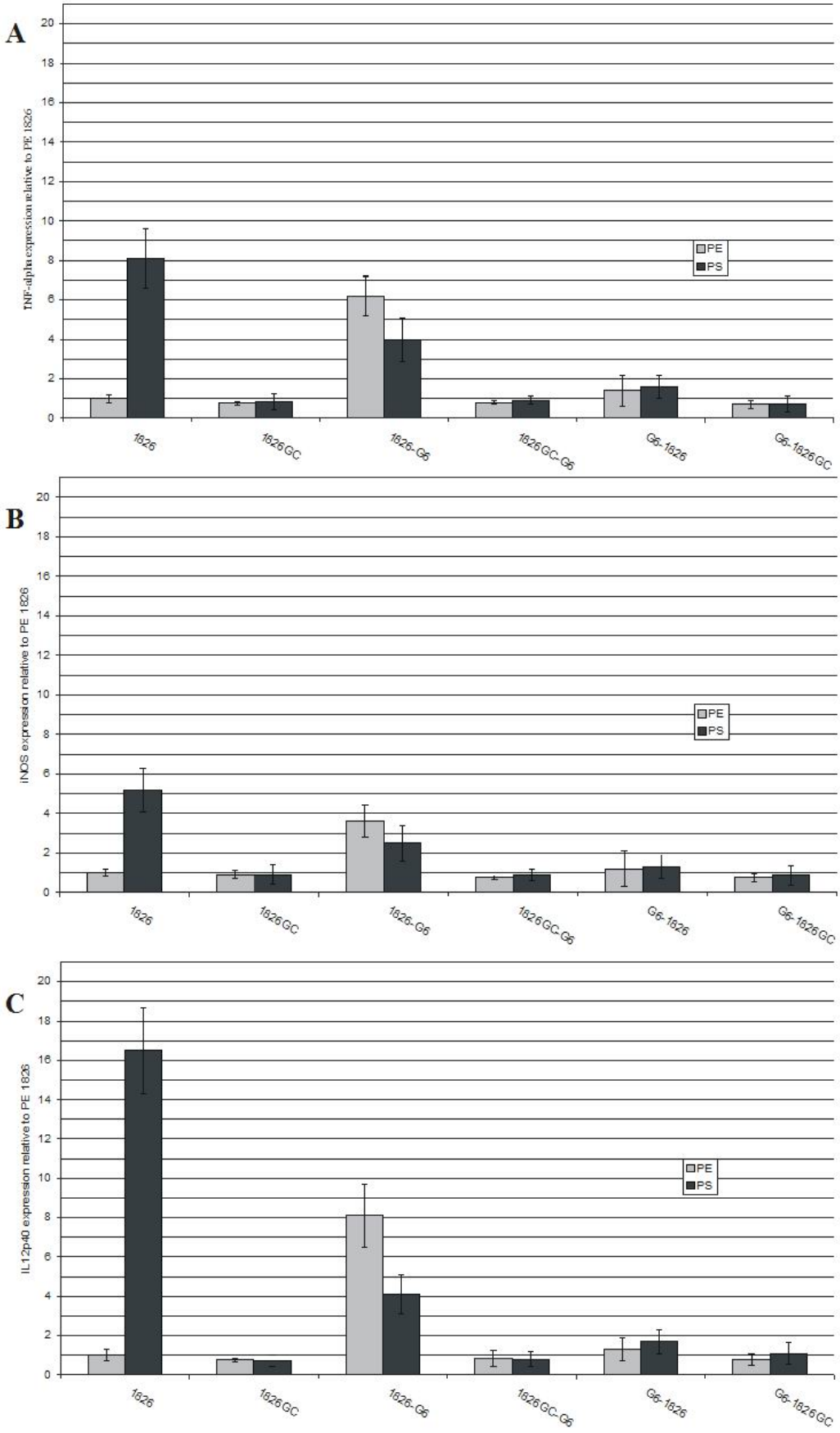

A
TNF-alpha expression relative to PE 1826
PE
PS
1826
1826 GC
1826-G6
1826 GC-G6
G6-1826
G6-1826 GC
B
iNOS expression relative to PE 1826
PE
PS
1826
1826 GC
1826-G6
1826 GC-G6
G6-1826
G6-1826 GC
C
IL12p40 expression relative to PE 1826
PE
PS
1826
1826 GC
1826-G6
1826 GC-G6
G6-1826
G6-1826 GC

Fig. 2. Influences of a polyG motif on the immunostimulatory activity of CpG-ODN in microglial N9 cells. N9 cells were incubated with various ODN (1.5 µM) for 12 h. Induction of TNF-α (A), iNOS (B) and IL12p40 (C) was measured using real-time PCR. Data are expressed relative to reference PE 1826 and each bar represents the mean and SEM of triplicates.

3.3. The extension of CpG-ODN with a polyG motif enhances CpG-ODN uptake but without affection cellular distribution

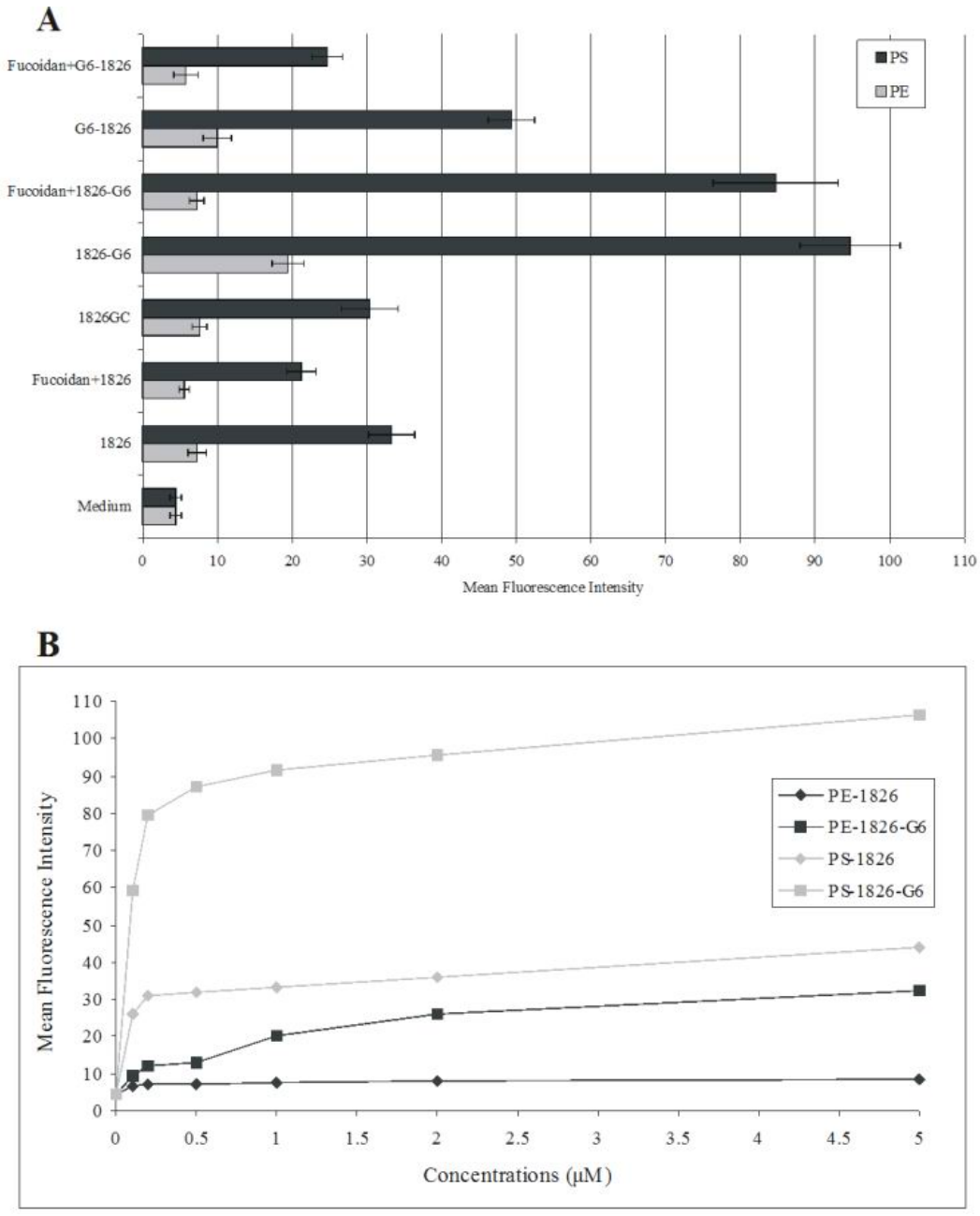


Fig. 3. Influences of a polyG motif on CpG-ODN uptake in microglial N9 cells. (A) N9 cells were incubated with various FITC labelled ODNs (1 µM) for 1 h at 37°C in serum-free RPMI 1640. Thereafter, cells were washed and mean fluorescence intensity was measured by flow cytometry. For inhibition, fucoidan (1.4 mg/mL) was added 15 min prior to addition of FITC-ODN. Each bar represents the mean and SEM of triplicates. (B) Dose–response curve for some of the ODN of panel (A) are given in detail.

Uptake is an essential prerequisite for the action of CpG-ODN.To study the mechanism of the effects of a polyG motif on CpG-ODN, a panel of PE or PS CpG-ODN, with or without polyG motif, was FITC labelled and their cellular uptake into N9 cells was analyzed. Cells were incubated with FITC labelled CpG-ODN (1 μM) for 1 h. Thereafter, cells were detached from the plate with trypsin-EDTA and dislodged cells were analyzed by flow cytometry.

As shown in Fig. 3A, uptake of the PS ODN was much higher than that of their PE counterparts with the same sequences (1826, 1826-G6 and G6-1826). This indicates that the PS phosphothioate modification highly increases cellular uptake. Moreover, uptake was independent of CpG motif (PE/PS 1826 compared with PE/PS 1826GC). PolyG motif either located at the 3' or the 5' terminus increased PE/PS 1826 uptake. Furthermore, cellular uptake of PE/PS ODN, with or without polyG motif, decreased when cells were co-incubated with fucoidan, a known ligand of scavenger receptor A. In addition, CpG-ODN uptake was dose-dependent (Fig. 3B).

After cell entry, CpG-ODN move into early endosomes and are subsequently transported to a lysosomal compartment. TLR9, which is localized to the endoplasmic reticulum, is recruited to the CpG-ODN containing compartment. The interaction of CpG-ODN with TLR9 activates a signal pathway and induces proinflammatory gene expression (Latz et al., 2004). Therefore we investigated whether the polyG motif could change the cellular distribution of CpG-ODN.

Fluorescence microscopy was used to study the intracellular distribution of FITC labelled CpG-ODN (nuclei were counterstained with DAPI). As shown in Fig. 4, PE and PS 1826 mainly localized in the cytoplasm and showed a spotted distribution after entry. PE/PS 1826-G6 as well as G6-1826 showed a similar spotted cytoplasmic distribution. Thus, in the present experimental setting polyG motifs did not change the cellular distribution of CpG-ODN.

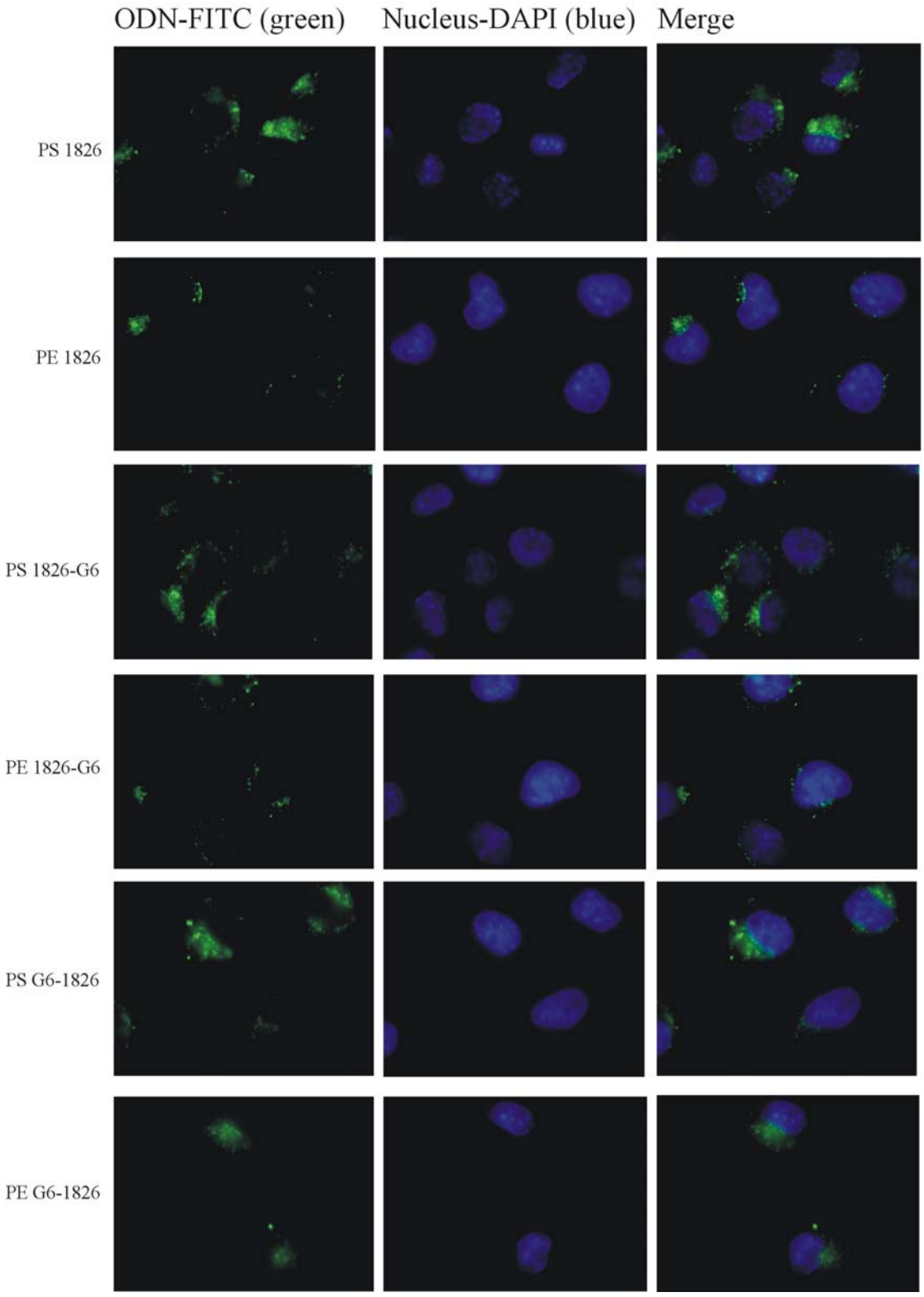


Fig. 4. PolyG motifs do not change the cellular distribution of CpG-ODN in microglial N9 cells. N9 cells were cultured in 6-wells chamber slides for 24 h and then incubated with 0.5 μM of various FITC labelled ODN for 1 h at 37°C. Subsequently cells were washed and fixed with PFA (2%) at room temperature for 15 min. Mounting medium with DAPI was used to show the cell nucleus. After covering with glass slides, cells were observed using a fluorescence microscope.

3.4. The extension of CpG-ODN with a 3'-end polyG motif enhances the number of cellular binding proteins

The affinity of ODN to cell membrane proteins positively correlates with their cellular uptake efficiency; therefore we tested whether a 3'-end polyG motif could increase the number of cellular binding proteins. PE 1826 and PE 1826-G6 were biotin labelled and were used to capture cellular proteins from microglial N9 cells. The captured proteins were analyzed by SDS-PAGE. As shown in Fig. 5, PE 1826-G6 interacted with more proteins than PE 1826.

Fig. 5. CpG-ODN containing a polyG motif bind more cellular proteins. Biotin labelled ODN were bound via streptavidin to magnetic microbeads. After incubation with cellular lysates and several washing steps, the proteins-CpG-ODN-biotin-streptavidin-magnetic beads-complex was separated. The bound proteins were then recovered from the complex by heating and analyzed by SDS-PAGE and finally detected by staining with Coomassie Blue.

4. Discussion

DNA has multiple and complex effects on the immune system. One of the most well-studied types of DNA is the CpG-ODN, which interacts with TLR9 and

induces a Th1- polarized immune response. Another type is the singlestranded guanosine-rich ODN, which forms a quadruplex structure under physiologic conditions that result in nuclease resistance and increase cellular uptake. Due to these advantages, CpG-ODN containing a polyG motif have been tested in several types of immune cells.

Microglia are the brain's immune effector cells. Several studies have shown that primary microglia as well as microglial cell line, such as BV-2, express TLR9 and can be stimulated by CpG-ODN to express different cytokines (Dalpke et al., 2002b; Takeshita et al., 2001; Bsibsi et al., 2002). These observations are corresponding with our result that microglial N9 cells express TLR9 and can be activated by CpG-ODN (Fig. 1).

In this study we investigated the effects of a polyG motif on the immunostimulatory activity of CpG-ODN in microglial N9 cells. In general, CpG-ODN containing a polyG motif showed immunomodulatory activity in microglial N9 cells but only polyG motifs located at the 3'-end of PE CpG-ODN increased the immunostimulatory activity. PolyG motifs located at the 3'-end of PS CpG-ODN or 5'-end of PE or PS CpG-ODN, however, suppressed the immunostimulatory activity in microglial N9 cells. PolyG alone has no immunostimulatory activity in microglial N9 cells either. Other groups have tested the immunomodulatory effects of PolyG motif on CpG-ODN in several other immune cells. Using macrophage cell line, RAW 264.7 and primary dendrite cells, Dalpke et al. and Lee et al. respectively prove only 3'-end polyG motifs increase immunostimulatory activity of PE CpG-ODN (Dalpke et al., 2002a; Lee et al., 2000).

The interaction of CpG-ODN with TLR9, which is localized to the endoplasmic reticulum, triggers a signal pathway and finally induces immunostimulatory gene expression (Latz et al., 2004). Therefore cellular uptake of CpG-ODN is considered to be the rate-limiting step for CpG-ODN activity. CpG-ODN that were linked to latex, magnetic or gold beads could not be taken up and lost their stimulatory activity (Manzel and Macfarlane, 1999; Sester et al., 2000). Being polyanionic chemicals, ODN are unlikely to enter cells by simple diffusion.

Receptor-mediated endocytosis is considered to be the major mechanism of ODN uptake in most cells (Juliano and Yoo, 2000). Several putative DNA binding/transport proteins have been isolated from cell membranes and Mac-1, a scavenger receptor and a cell membrane nucleic acid channel has been found to potentially mediate endocytosis of nucleic acids (Benimetskaya et al., 1997; Biessen et al., 1998; Hanss et al., 1998).

We observed that addition of a polyG motif at the 3'- or 5'-end enhanced cellular uptake of PE or PS CpG-ODN by N9 cells. But 3'-end polyG had greater efficiency in enhancing cellular uptake than 5'-end polyG. Uptake could be decreased by fucoidan, a ligand of scavenger receptor. Single-stranded guanosine-rich ODN, which have the ability to form quadruplex structures, were reported to be effective ligands of scavenger receptor A, one of the membrane receptors involved in ODN uptake (Kaur et al., 2003). Antisense ODN tethered to polyG sequences at their 3'-end were recognized by scavenger receptors on macrophages and were taken up more efficiently than those ODN that lacked the polyG sequences (Prasad et al., 1999). Furthermore, quadruplex structure formation of the polyG motif in CpG-ODN correlates with its ability to induce IL12 (Lee et al., 2000). The reports corroborating with our observation indicated that polyG motifs might enhance cellular uptake of ODN through quadruplex structure formation and the resulting interaction with the scavenger receptor. Furthermore, PE 1826-G6 binds a higher number of cellular proteins than PE 1826 (Fig. 5), this suggests that the polyG motif might also interact with other proteins to increase uptake. The location of the polyG motif within the ODN affects its ability to increase ODN uptake, 3'-end location proved to be better than 5'-end in our experiment. This might be due to quadruplex structure formation being dependent on the position of the polyG motif.

After cell entry CpG-ODN move into the early endosomes and are subsequently transported to a lysosomal compartment, where TLR9 and the adaptor molecule MyD88 are recruited and initiate a signal cascade. Thus the cellular distribution of CpG-ODN is important for its activity. We compared cellular distribution of CpG-ODN and CpG-ODN with a polyG motif. There was no significant difference between different CpG-ODN, indicating that the polyG motif does not

affect the cellular location of the CpG-ODN. The molecular recognition events between CpG-ODN and TLR9 are still not fully understood but a free 5'-end is important for CpG-ODN activity. Kandimalla et al. reported that the incorporation of ligands in the 5'-flanking sequences potentiates the immunostimulatory activity of CpG-ODN, while the incorporation of the same ligands in the 3'-flanking sequences has minimal effect on the immunostimulatory activity (Kandimalla et al., 2002). In addition, Klinman et al. observed that the immunostimulatory activity of CpG-ODN could be blocked by a 5'-end suppressing motif (Klinman et al., 2003). In our study, CpG-ODN with a 5'-end polyG motif were shown to be much less efficient at inducing TNF-α, iNOS and IL12p40 compared with CpG-ODN containing a 3'-end polyG motif. This can partly be explained by lower uptake, but might also partly be due to inhibitory effects of the 5'-end polyG motif.

Surprisingly, a 3'-end polyG motif increased PS CpG-ODN uptake but inhibited its immunostimulatory activity, which is totally different from that of PE CpG-ODN. The same inhibitory effects of 3'-end polyG motifs on PS CpG-ODN were also observed in macrophages and dendrite cells (Lee et al., 2000; Dalpke et al., 2002a,b). The reason for these contradictory observations is unknown but Dalpke et al. suggested a two-step model of CpG signalling that could explain the effects. In a first step CpG-ODN are taken up into an endosome. This step is CpG motif independent and enhanced by the polyG motif. In a second step CpG-ODN are transported to a lysosomal compartment where the CpG motifs are exposed with the help of certain enzymes and can then be recognized by TLR9. For this step, it is assumed that the PE polyG motif can be easily degraded and thereby allow exposure of the CpG motif, however, the PS polyG motif is difficult to degrade and thus the CpG motif is not presented.

CpG-ODN are strong Th1-polarized stimuli and thus potential therapeutic candidates. Complete or partial modification of the CpG-ODN backbone with phosphothioate is widely used to increase uptake and nuclease resistance. However PS ODN may cause certain kinds of unwanted effects and PS CpG-ODN might exacerbate inflammatory tissue damage, autoimmune disease or increase sensitivity to toxic shock, which limits the applications of PS CpG-

ODN. But PE CpG-ODN are easily digested by nucleases and therefore show low bioavailability, which is an obstacle for PE CpG-ODN application. Guanosine-rich ODN, which form a quadruplex structure that result in nuclease resistance and increase cellular uptake, might help to overcome this obstacle. Here we show that a 3'-end polyG motif enhances both the cellular uptake of PE CpG-ODN and their immunostimulatory activity in microglial N9 cells. Therefore these hybrid PE CpG-ODN might be promising alternative analogues for application of CpG-ODN in the central nervous system.

References

Agrawal, S., 1991. In: Wickstrom, E. (Ed.), Prospects for Antisense Nucleic Acid Therapy of Cancer and AIDS. Wiley-Liss, New York, pp. 143– 158.

Anselmet, A., Mayat, E., Wietek, S., Layer, P.G., Payrastre, B., Massoulie, J., 2002. Non-antisense cellular responses to oligonucleotides. FEBS Lett. 510, 175– 180.

Balasubramanian, V., Nguyen, L.T., Balasubramanian, S.V., Ramanathan, M., 1998. Interferon-gamma-inhibitory oligodeoxynucleotides alter the conformation of interferon-gamma. Mol. Pharmacol. 53, 926– 932.

Ballas, Z.K., Rasmussen, W.L., Krieg, A.M., 1996. Induction of NK activity in murine and human cells by CpG motifs in oligodeoxynucleotides and bacterial DNA. J. Immunol. 157, 1840–1845.

Benimetskaya, L., Loike, J.D., Khaled, Z., Loike, G., Silverstein, S.C., Cao, L., el Khoury, J., Cai, T.Q., Stein, C.A., 1997. Mac-1 (CD11b/CD18) is an oligodeoxynucleotide-binding protein. Nat. Med. 3, 414– 420.

Biessen, E.A., Vietsch, H., Kuiper, J., Bijsterbosch, M.K., Berkel, T.J., 1998. Liver uptake of phosphodiester oligodeoxynucleotides is mediated by scavenger receptors. Mol. Pharmacol. 53, 262– 269.

Blank, M., Weinschenk, T., Priemer, M., Schluesener, H., 2001. Systematic evolution of a DNA aptamer binding to rat brain tumor microvessels, selective targeting of endothelial regulatory protein pigpen. J. Biol. Chem. 276, 16464–16468.

Bsibsi, M., Ravid, R., Gveric, D., Noort, J.M., 2002. Broad expression of Toll-like receptors in the human central nervous system. J. Neuropathol. Exp. Neurol. 61, 1013–1021.

Carpentier, A.F., Xie, J., Mokhtari, K., Delattre, J.Y., 2000. Successful treatment of intracranial gliomas in rat by oligodeoxynucleotides containing CpG motifs. Clin. Cancer Res. 6, 2469– 2473.

Cowdery, J.S., Chace, J.H., Yi, A.K., Krieg, A.M., 1996. Bacterial DNA induces NK cells to produce IFN-gamma in vivo and increases the toxicity of lipopolysaccharides. J. Immunol. 156, 4570–4575.

Dalpke, A., Zimmermann, S., Heeg, K., 2001. CpG-oligonucleotides in vaccination: signaling and mechanisms of action. Immunobiology 204, 667–676.

Dalpke, A.H., Zimmermann, S., Albrecht, I., Heeg, K., 2002a. Phosphodiester CpG oligonucleotides as adjuvants: polyguanosine runs enhance cellular uptake and improve immunostimulative activity of phosphodiester CpG oligonucleotides in vitro and in vivo. Immunology 106, 102– 112.

Dalpke, A.H., Schafer, M.K., Frey, M., Zimmermann, S., Tebbe, J., Weihe, E., Heeg, K., 2002b. Immunostimulatory CpG-DNA activates murine microglia. J. Immunol. 168, 4854– 4863.

Dapic, V., Abdomerovic, V., Marrington, R., Peberdy, J., Rodger, A., Trent, J.O., Bates, P.J., 2003. Biophysical and biological properties of quadruplex oligodeoxyribonucleotides. Nucleic Acids Res. 31, 2097– 2107.

Deng, G.M., Nilsson, I.M., Verdrengh, M., Collins, L.V., Tarkowski, A., 1999. Intra-articularly localized bacterial DNA containing CpG motifs induces arthritis. Nat. Med. 5, 702– 705.

Halpern, M.D., Pisetsky, D.S., 1995. In vitro inhibition of murine IFN gamma production by phosphothioate deoxyguanosine oligomers. Immunopharmacology 29, 47–52.

Hanss, B., Leal-Pinto, E., Bruggeman, L.A., Copeland, T.D., Klotman, P.E., 1998. Identification and characterization of a cell membrane nucleic acid channel. Proc. Natl. Acad. Sci. U. S. A. 95, 1921–1926.

Hemmi, H., Takeuchi, O., Kawai, T., Kaisho, T., Sato, S., Sanjo, H., Matsumoto, M., Hoshino, K., Wagner, H., Takeda, K., Akira, S., 2000. A Toll-like receptor recognizes bacterial DNA. Nature 408, 740– 744.

Juliano, R.L., Yoo, H., 2000. Aspects of the transport and delivery of antisense oligonucleotides. Curr. Opin. Mol. Ther. 2, 297– 303.

Kandimalla, E.R., Bhagat, L., Yu, D., Cong, Y., Tang, J., Agrawal, S., 2002. Conjugation of ligands at the 5V-end of CpG DNA affects immunostimulatory activity. Bioconjug. Chem. 13, 966–974.

Kaur, H., Jaso-Friedmann, L., Evans, D.L., 2003. Identification of a scavenger receptor homologue on nonspecific cytotoxic cells and evidence for binding to oligodeoxyguanosine. Fish Shellfish Immunol. 15, 169– 181.

Kimura, Y., Sonehara, K., Kuramoto, E., Makino, T., Yamamoto, S., Yamamoto, T., Kataoka, T., Tokunaga, T., 1994. Binding of oligoguanylate to scavenger receptors is required for oligonucleotides to augment NK cell activity and induce IFN. J. Biochem. (Tokyo) 116, 991–994.

Klinman, D.M., Zeuner, R., Yamada, H., Gursel, M., Currie, D., Gursel, I., 2003. Regulation of CpG-induced immune activation by suppressive oligodeoxynucleotides. Ann. N.Y. Acad. Sci. 1002, 112– 123.

Krieg, A.M., 2002. CpG motifs in bacterial DNA and their immune effects. Annu. Rev. Immunol. 20, 709– 760.

Krieg, A.M., Yi, A.K., Matson, S., Waldschmidt, T.J., Bishop, G.A., Teasdale, R., Koretzky, G.A., Klinman, D.M., 1995. CpG motifs in bacterial DNA trigger direct B-cell activation. Nature 374, 546– 549.

Krug, A., Rothenfusser, S., Hornung, V., Jahrsdorfer, B., Blackwell, S., Ballas, Z.K., Endres, S., Krieg, A.M., Hartmann, G., 2001. Identification of CpG oligonucleotide sequences with high induction of IFN-alpha/beta in plasmacytoid dendritic cells. Eur. J. Immunol. 31, 2154–2163.

Lang, R., Hultner, L., Lipford, G.B., Wagner, H., Heeg, K., 1999. Guanosine-rich oligodeoxynucleotides induce proliferation of macrophage progenitors in cultures of murine bone marrow cells. Eur. J. Immunol. 29, 2496– 2506.

Latz, E., Schoenemeyer, A., Visintin, A., Fitzgerald, K.A., Monks, B.G., Knetter, C.F., Lien, E., Nilsen, N.J., Espevik, T., Golenbock, D.T., 2004. TLR9 signals after translocating from the ER to CpG DNA in the lysosome. Nat. Immunol. 5, 190–198.

Lee, P.P., Ramanathan, M., Hunt, C.A., Garovoy, M.R., 1996. An oligonucleotide blocks interferon-gamma signal transduction. Transplantation 62, 1297–1301.

Lee, S.W., Song, M.K., Baek, K.H., Park, Y., Kim, J.K., Lee, C.H., Cheong, H.K., Cheong, C., Sung, Y.C., 2000. Effects of a hexameric deoxyriboguanosine run conjugation into CpG oligodeoxynucleotides on their immunostimulatory potentials. J. Immunol. 165, 3631–3639.

Lenert, P., Stunz, L., Yi, A.K., Krieg, A.M., Ashman, R.F., 2001. CpG stimulation of primary mouse B cells is blocked by inhibitory oligodeoxyribonucleotides at a site proximal to NF-kappaB activation. Antisense Nucleic Acid Drug Dev. 11, 247– 256.

Lipford, G.B., Bendigs, S., Heeg, K., Wagner, H., 2000. Poly-guanosine motifs costimulate antigen-reactive CD8 T cells while bacterial CpG DNA affect T-cell

activation via antigen-presenting cell-derived cytokines. Immunology 101, 46–52.

Manzel, L., Macfarlane, D.E., 1999. Lack of immune stimulation by immobilized CpG-oligodeoxynucleotide. Antisense Nucleic Acid Drug Dev. 9, 459– 464.

Perez, J.R., Li, Y., Stein, C.A., Majumder, S., van Oorschot, A., Narayanan, R., 1994. Sequence-independent induction of Sp1 transcription factor activity by phosphorothioate oligodeoxynucleotides. Proc. Natl. Acad. Sci. U. S. A. 91, 5957– 5961.

Pisetsky, D.S., 1997. Immunostimulatory DNA: a clear and present danger? Nat. Med. 3, 829– 831.

Prasad, V., Hashim, S., Mukhopadhyay, A., Basu, S.K., Roy, R.P., 1999. Oligonucleotides tethered to a short polyguanylic acid stretch are targeted to macrophages: enhanced antiviral activity of a vesicular stomatitis virus-specific antisense oligonucleotide. Antimicrob. Agents Chemother. 43, 2689– 2696.

Rockwell, P., O'Connor, W.J., King, K., Goldstein, N.I., Zhang, L.M., 1997. Cell-surface perturbations of the epidermal growth factor and vascular endothelial growth factor receptors by phosphorothioate oligodeoxynucleotides. Proc. Natl. Acad. Sci. U. S. A. 94, 6523–6528.

Rothenfusser, S., Tuma, E., Wagner, M., Endres, S., Hartmann, G., 2003. Recent advances in immunostimulatory CpG oligonucleotides. Curr. Opin. Mol. Ther. 5, 98–106.

Schluesener, H.J., Seid, K., Deininger, M., Schwab, J., 2001. Transient in vivo activation of rat brain macrophages/microglial cells and astrocytes by immunostimulatory multiple CpG oligonucleotides. J. Neuroimmunol. 113, 89–94.

Segal, B.M., Chang, J.T., Shevach, E.M., 2000. CpG oligonucleotides are potent adjuvants for the activation of autoreactive encephalitogenic T cells in vivo. J. Immunol. 164, 5683– 5688.

Sester, D.P., Naik, S., Beasley, S.J., Hume, D.A., Stacey, K.J., 2000. Phosphorothioate backbone modification modulates macrophage activation by CpG DNA. J. Immunol. 165, 4165– 4173.

Sparwasser, T., Miethke, T., Lipford, G., Erdmann, A., Hacker, H., Heeg, K., Wagner, H., 1997. Macrophages sense pathogens via DNA motifs: induction of tumor necrosis factor-alpha-mediated shock. Eur. J. Immunol. 27, 1671–1679.

Takeshita, S., Takeshita, F., Haddad, D.E., Janabi, N., Klinman, D.M., 2001. Activation of microglia and astrocytes by CpG oligodeoxynucleotides. NeuroReport 12, 3029–3032.

Wang, W., Chen, H.J., Schwartz, A., Cannon, P.J., Stein, C.A., Rabbani, L.E., 1996. Sequence-independent inhibition of in vitro vascular smooth muscle cell proliferation, migration, and in vivo neointimal formation by phosphorothioate oligodeoxynucleotides. J. Clin. Invest. 98, 443– 450.

Wojcik, W.J., Swoveland, P., Zhang, X., Vanguri, P., 1996. Chronic intrathecal infusion of phosphorothioate or phosphodiester antisense oligonucleotides against cytokine responsive gene-2/IP-10 in experimental allergic encephalomyelitis of Lewis rat. J. Pharmacol. Exp. Ther. 278, 404– 410.

Yuen, A.R., Sikic, B.I., 2000. Clinical studies of antisense therapy in cancer. Front. Biosci. 5, D588– D59333.

Zhu, F.G., Reich, C.F., Pisetsky, D.S., 2002a. Inhibition of murine macrophage nitric oxide production by synthetic oligonucleotides. J. Leukoc. Biol. 71, 686–694.

Zhu, F.G., Reich, C.F., Pisetsky, D.S., 2002b. Inhibition of murine dendritic cell activation by synthetic phosphorothioate oligodeoxynucleotides. J. Leukoc. Biol. 72, 1154–1163.

Chapter 4.

Uptake, intracellular distribution, and novel binding proteins of immunostimulatory CpG oligodeoxynucleotides in glioblastoma cells

Zhiren Zhang[a], Toni Weinschenk[b], Hermann J. Schluesener[a]

[a]Institute of Brain Research, University of Tuebingen, Calwer Street 3, Tuebingen D-72076, Germany

[b]Institute for Cell Biology, Department of Immunology, University of Tuebingen, Auf der Morgenstelle 15, Tuebingen D-72076, Germany

Molecular and Cellular Biochemistry. 272, 35-46 (2005)

Abstract

Glioblastomas are the most malignant and most frequent brain tumors and exciting targets of gene and immunotherapy. Despite rapid development of experimental therapy little is known about the cellular behaviour of therapeutic oligodeoxynucleotides (ODNs). Here we designed uptake, cellular distribution and cellular binding proteins of immunostimulatory CpG-ODNs in glioblastoma cells by flow cytometry, fluorescence microscopy and mass spectrometry. Our data show that the phosphorothioate (PS) CpG-ODNs uptake in T98G and C6 cells is dose-, time-, temperature-dependent and independent of the CpG dinucleotides. Uptake can be inhibited by sodium azide, polyanions but not by chloroquine. After internalisation FITC labelled CpG-ODNs showed a spotted distribution in cytoplasm. Dozens of cellular binding proteins were identified using mass spectrometry. The binding of ODNs to proteins is dependent on modification and sequence but independent on CpG motif. ODNs bind to cellular proteins that are important for RNA processing and transport. Furthermore, three novel membrane proteins were identified, which might contribute to uptake of ODNs. ODNs binding to these proteins might interfere with the physiological function and thus might cause unwanted effects. Such binding also might influence the uptake efficiency or cellular distribution of therapeutic ODNs.

Key words: Glioblastoma, CpG-ODN, molecular therapy, drug design, mass spectrometry,

Introduction

Glioblastomas are one of the most malignant tumors. During the past decades, glioblastomas have retained their dismal prognosis despite the considerable progress in modern tumor therapy. Therefore, gene therapy and immunotherapy are expected to be an effective and possibly curative treatment in the case of glioblastomas (1, 2, 3).

Therapeutic genes can serve to directly kill or block growth of tumor cells, inhibit angiogenesis, stimulate immune responses to tumor antigens and block tumor invasion. Prerequisite to all these therapeutic applications is the internalisation of therapeutic oligonucleotides, access of the oligonucleotides to the target and their stability. Though much work has been done in glioblastoma gene therapy, the mechanism of ODN internalisation and the fate of cellular therapeutic ODNs inside tumor cells are still not fully understood.

Immunostimulatory CpG-ODNs also have been used for glioblastoma therapy and showed to be of great promise. Carpentier et al have successfully treated intracranial gliomas in rat by CpG-ODNs (4, 5) and similar results also have been obtained for neuroblastoma in mice (6, 7). Therefore we used CpG-ODNs to study the mechanism of ODN internalisation and the pattern of ODN distribution in glioblastoma cells.

Sequence-independent unspecific effects are another obstacle for successful gene therapy, particularly for the PS ODNs which have increased stability and therefore have been widely used (8). Unanticipated binding effects of ODNs to cellular proteins have been described from anti-sense therapeutics. As polyanions, ODNs have been shown to unspecifically bind to proteins, such as basic fibroblast growth factor and the epidermal growth factor (9). PS ODNs can activate the transcription factor Sp1, which plays a key role in transcription of multiple genes (10). In addition, PS ODNs might be neurotoxic at therapeutic concentrations higher than 1 μM (11) and may cause paralysis and necrosis of the spinal cord (12), probably because of the avid binding of PS ODNs to multiple cellular proteins (13). Here we used a previously developed *in vitro*

method to purify ODN binding proteins and subsequently identify these proteins using mass spectrometry.

Our results indicate for CpG-ODNs in particular and ODNs in general pattern of ODNs uptake, cellular distribution and interaction with cellular proteins. The data presented here suggest an active ODN internalisation in glioblastoma cells. After internalisation, ODN mainly localized to the cytoplasm. Several proteins binding to CpG-ODNs were identified. Such binding is CpG motif independent but sequence and modification dependent.

Materials and Methods

Synthetic ODNs

All ODNs were synthesised by MWG-Biotech AG, Ebersberg, Germany. Their sequences were as follows: CpG3: 5´- TCCATGACGTTCCTGATCGT-3`, CpG-inverse3: 5´- TCCATGAGCTTCCTGATGCT-3`, CpG4: 5´- TCGTCGTTTTGTCGTTTTGTCGT-3` and CpG-inverse4: 5´- TGCTGCTTTTGTGCTTTTGTGCT-3`. PS ODN, unmodified ODN, FITC labelled ODN, biotin labelled ODN and unlabelled ODN were used.

Cell Cultures

Human glioblastoma cell line T98G and rat glioblastoma cell line C6 were cultured in RPMI-1640 with 10% heat inactivated fetal calf serum (FCS) with penicillin and streptomycin at 100 U/ ml (Gibco, Grand Island, NY) at 37 °C in 5% CO_2.

Cytofluorometric Analysis of ODNs Uptake

5 x 10^5 T98G cells or C6 cells in 2 ml RPMI-1640 with 10% FCS were seeded into 12-well plates and cultured for 24 hours. Thereafter, medium was removed and cells were washed two times with FCS-free RPMI 1640 and incubated with

FCS-free RPMI-1640 at 37°C for 1 hour to remove residual FCS. After incubation, medium was replaced by serum-free RPMI 1640 containing FITC labelled ODNs with indicated concentration. Cells were cultured in the dark as long as indicated. Then cells were washed with PBA buffer (phosphate-buffered saline (PBS), 2% BSA, and 0.1% sodium azide) and PBS and then detached from dishes with trypsin-EDTA. After neutralizing the trypsin with medium containing FCS, cells were collected by spinning at 1000 rpm for 5 minutes. After washing with PBS, cellular fluorescence intensity was quantified by flow cytometry. For FACS analysis, the threshold was settled using the cells that incubated with FITC. The threshold was set to above the major cell population (14).

For inhibition experiments sodium azide (0.1%) (15), dextransulfate (0.14 mg/mL), polyvinylsulfate (0.14 mg/mL), fucoidan (0.14 mg/mL) (16), or chloroquine (2 µg/mL, 5 µg/mL and 10 µg/mL) (17) was added respectively 15 minutes prior to addition of ODNs.

Fluorescence Microscopy

10^5 cells in 0.4 ml normal RPMI-1640 were seeded onto 6-well chamber slides (Lab-Tek Chamber Slide, Nalge Nunc International Corp. Naperville, USA) and cultured at 37 °C, 5% CO_2 for 24 hours. Thereafter medium was removed and cells were washed once with RPMI-1640 and then cultured in RPMI-1640 at 37 °C for 1 hour to remove residual FCS. After incubation, cells were washed once with PBS and then FITC labelled ODNs (1 µM) in RPMI-1640 were added and incubated for 1 hour at 37 °C. After the medium had been removed, the cells were washed with PBS for two times and fixed with 2% Paraformaldehyde (PFA) for 15 minutes at room temperature. Cells were observed under fluorescence microscopy after they were washed with PBS and mounted with mounting medium containing DAPI (VECTASHIELD Mounting Medium with DAPI, Vector Labratories, Inc., Burlingame, USA).

CpG ODN-mediated Protein Purification

Glioblastoma cells cultured in flasks were collected with a scraper, sonicated and then incubated in solubilization buffer (50 mM Tris-HCl, 150 mM NaCl, 1% Triton X-100) containing a protease inhibitor cocktail (Sigma, Munich, Germany) for 2 hours on ice. Lysates were cleared by centrifugation at 14.000 g at 4 °C for 5 minutes. Aliquots of the supernatants were used for protein purification.

Magnetic streptavidin microbeads (Dynal Biotech ASA, Oslo, Norway) were coated with 200 pmol of biotin labelled CpG-ODNs by incubation in 1 ml selection buffer for 30 min at room temperature. As a control, 100 µl of magnetic streptavidin microbeads were coated with 200 pmol of biotin labelled CpG-inverse ODNs. Supernatants of cell lysates were incubated with CpG-ODNs coated magnetic beads in the presence of a 100-fold excess of tRNA (20 nmol) as an unspecific competitor in selection buffer. After washing of the beads, proteins were removed by heating in sample buffer, then analysed by 10% polyacrylamide gel electrophoresis and detected by staining with Coomassie Blue. Bands were analysed by tryptic digestion and mass spectrometry (18).

Protein Identification

In gel tryptic digestion was performed as described (19) and modified as outlined below. Briefly, the protein bands were excised from the gel, fully de-stained, and digested for 3 h with porcine trypsin (sequencing grade, modified; Promega, Mannheim, Germany) at a concentration of 67 ng/µl in 25 mM ammonium bicarbonate, pH 8.1, at 37 °C. Prior to peptide mass mapping and sequencing of tryptic fragments by tandem mass spectrometry, the peptide mixture were extracted from the gel by 1% formic acid followed by two changes of 50% methanol. The combined extracts were vacuum-dried until only 1-2 µl were left and the peptides was purified by ZipTip according to the manufacturers' instructions (Millipore, Bedford, MA, USA). MALDI-TOF analysis from the matrix α-cyano-4-hydroxycinnamic acid/nitrocellulose prepared on the target using the fast evaporation method (20) was performed on a Bruker Reflex III (Bruker Daltonik, Bremen, Germany) equipped with a N_2 337 nm laser,

gridless pulsed ion extraction and externally calibrated using synthetic peptides with known masses. The spectra were obtained in positive ionization mode at 23 kV.

Sequence verification of some fragments was performed by nanoelectrospray tandem mass spectrometry on a hybrid quadrupole orthogonal acceleration time of flight tandem mass spectrometer (Q-Tof, Micromass, Manchester, UK) equipped with a nanoflow electrospray ionization source. Gold/paladium-coated glass capillary nanoflow needles were obtained from PROXEON (Type Medium NanoES spray capillaries for the Micromass Q-Tof, Odense, Denmark). Database searches (NCBInr, non-redundant protein database) were done using the MASCOT software from Matrix Science (21).

Results

Uptake of CpG-ODN in Glioblastoma Cells

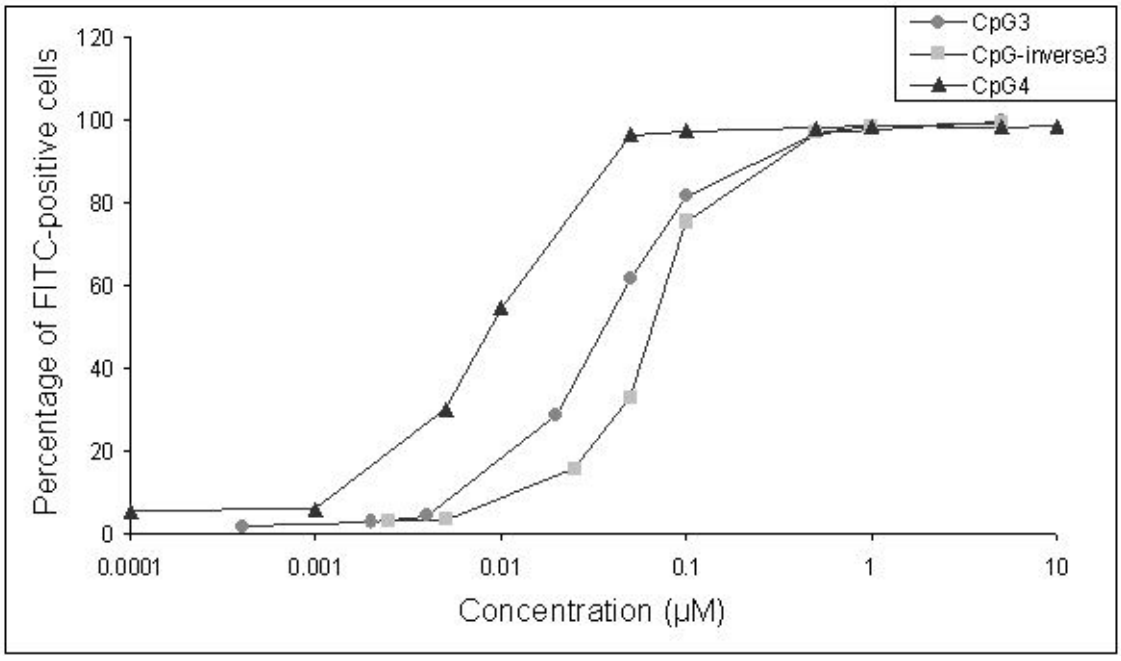


***Fig. 1*. Uptake of CpG-ODN and CpG-inverse ODN by glioblastoma cells is concentration dependent.** Cells were cultured in 12-well plates for 24 h and then washed with RPMI 1640 without FCS to remove residual FCS. Then FITC labelled ODNs were added at concentrations as indicated in FCS-free RPMI 1640 and incubated for 1 h at 37°C. Thereafter cells were washed with PBA to remove ODNs bound unspecifically to the cell surface and cells were detached with trypsin-EDTA and collected by spinning.

Fluorescence intensity of cells was measured by flow cytometry. PS CpG3 and CpG-inverse3 ODNs were studied in C6 cells and PS CpG4 ODNs were studied in T98G cells.

Several PS ODNs containing CpG dinucleotides or CpG-inverse dinucleotides were applied in this study. CpG3 containing the GACGTT sequence, which is the optimal motif for activating murine cells, was used for rat glioblastoma C6 cells. CpG4 containing the GTCGTT sequence, which is the optimal motif for activation of human cells was used for human glioblastoma T98G6 cells. Fluorescein isothiocyanate (FITC) labelled ODNs were used to study the uptake and the intracellular distribution, and biotin labelled ODNs were used for purification of binding proteins.

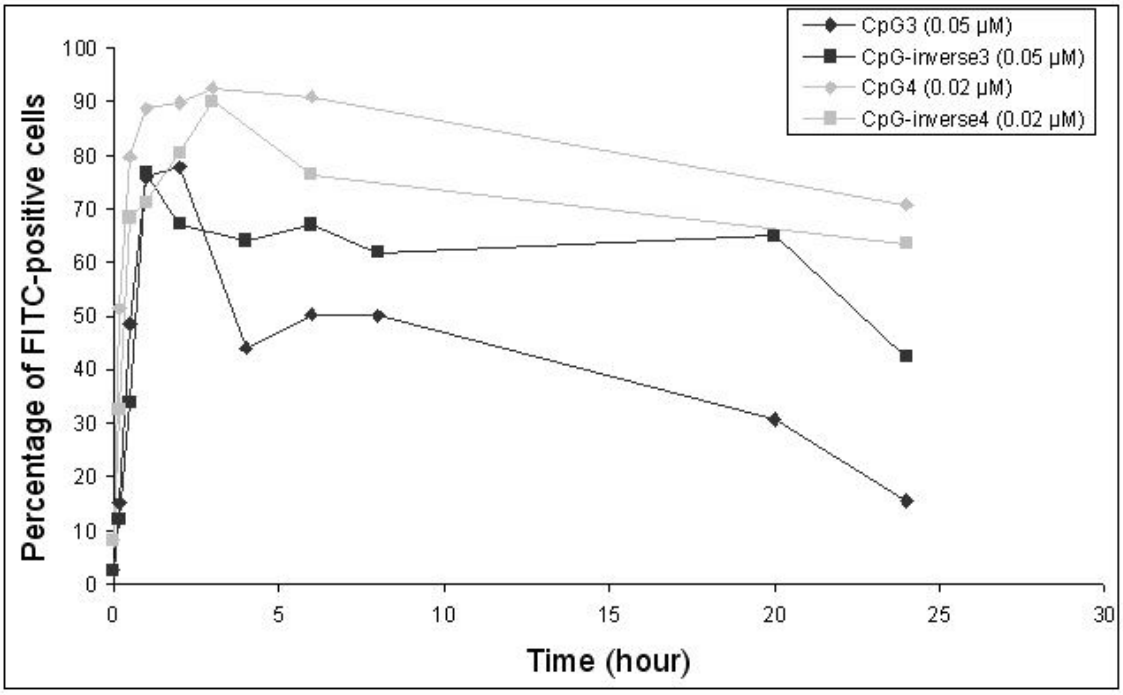


Fig. 2. **Uptake of CpG-ODN and CpG-inverse ODN by Glioblastoma Cells is Time Dependent.** Cells were cultured in 12-well plates for 24 hours and washed with RPMI 1640 without FCS to remove residual FCS. Then FITC labelled ODNs were added at concentrations as indicated in FCS-free RPMI 1640 and incubated for indicated time at 37 °C. Subsequently cells were washed with PBA to remove ODNs bound unspecifically to the cell surface and cells were detached with trypsin-EDTA and collected by spinning. Fluorescence intensity of the cells was measured by flow cytometry. PS CpG3 and CpG-inverse3 ODNs were studied in C6 cells and PS CpG4 and CpG-inverse4 ODNs were studied in T98G cells.

CpG-ODN uptake in human glioblastoma cell line T98G and rat glioblastoma cell line C6 is dose dependent and saturable (Figure 1). Glioblastoma cells were incubated with different concentration CpG-ODN or CpG-inverse ODN labelled with FITC for one hour. Then flow cytometry was used to detect cellular fluorescence intensity. The uptake dose-effect curve, which suggested a

saturable uptake, for CpG3 in C6 cells is similar with that of CpG4 in T98G cells. Uptake increases quickly in the concentration range from 1 nM to 100 nM. And uptake is independent of the CpG dinucleotides, as the CpG-inverse ODN shows the similar dose-effect curve.

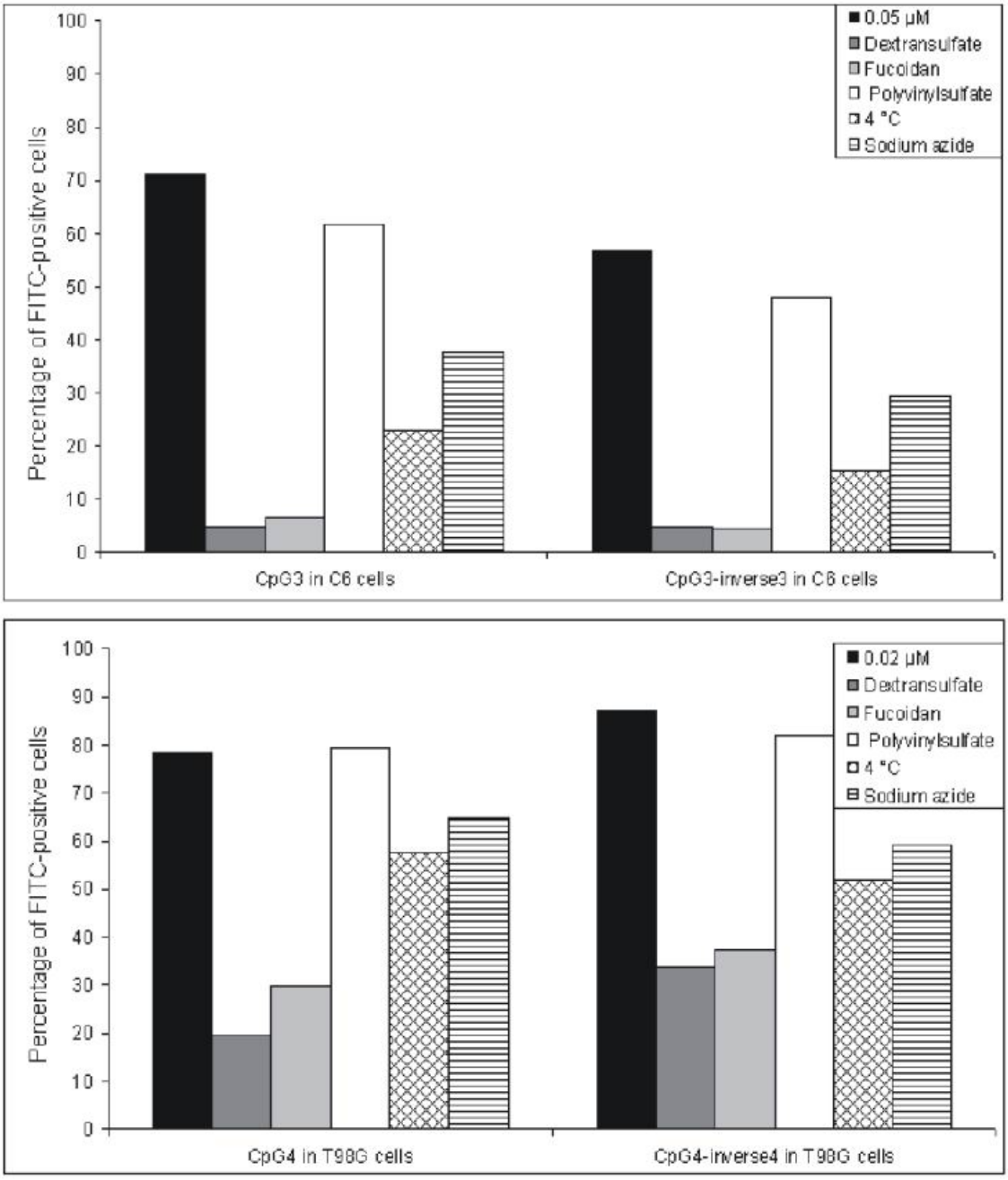


Fig. 3. **Uptake of CpG-ODN and CpG-inverse ODN by Glioblastoma Cells is Temperature Dependent and can be Inhibited by Dextransulfate, Fucoidan and Sodium Azide but not by Polyvinylsulfate.** Cells were cultured in 12-well plates for 24 hours and washed with RPMI 1640 without FCS to remove residual FCS. Then FITC labelled ODNs were added at concentrations as indicated in FCS-free RPMI 1640 and incubated for 1 hour at 37 or 4 °C. Thereafter, cells were washed with PBA to remove ODNs bound unspecifically to the cell surface and the cells were detached with trypsin-EDTA and collected by spinning. Fluorescence

intensity of the cells was measured by flow cytometry. For the inhibition experiments 0.1% sodium azide or fucoidan (140 µg/mL) or dextransulfate (140 µg/mL) or polyvinylsulfate (140 µg/mL) were added 15 minutes prior to addition of ODN, respectively. PS CpG3 and CpG-inverse3 ODNs were studied in C6 cells and PS CpG4 and CpG-inverse4 ODNs were studied in T98G cells.

CpG-ODN uptake in human glioblastoma cells T98G and rat glioblastoma cells C6 is time dependent (Figure 2). To investigate the time course of CpG-ODN uptake in glioblastoma cells, FITC-labelled CpG-ODNs and CpG-inverse ODNs were incubated with glioblastoma cells for up to 25 hours. The intracellular fluorescence intensity was measured at different time points by flow cytometry. Uptake is rapid and fluorescence can be detected as early as 10 minutes after addition of FITC-labelled ODNs. For the first 2 h (C6 cells) or 4 h (T98G cells), fluorescence intensity increased quickly, showing a high efficacy of uptake. Thereafter, fluorescence intensity begins to decrease slowly. Also the CpG and CpG-inverse ODNs showed a similar curve, which suggested a CpG dinucleotides independent uptake.

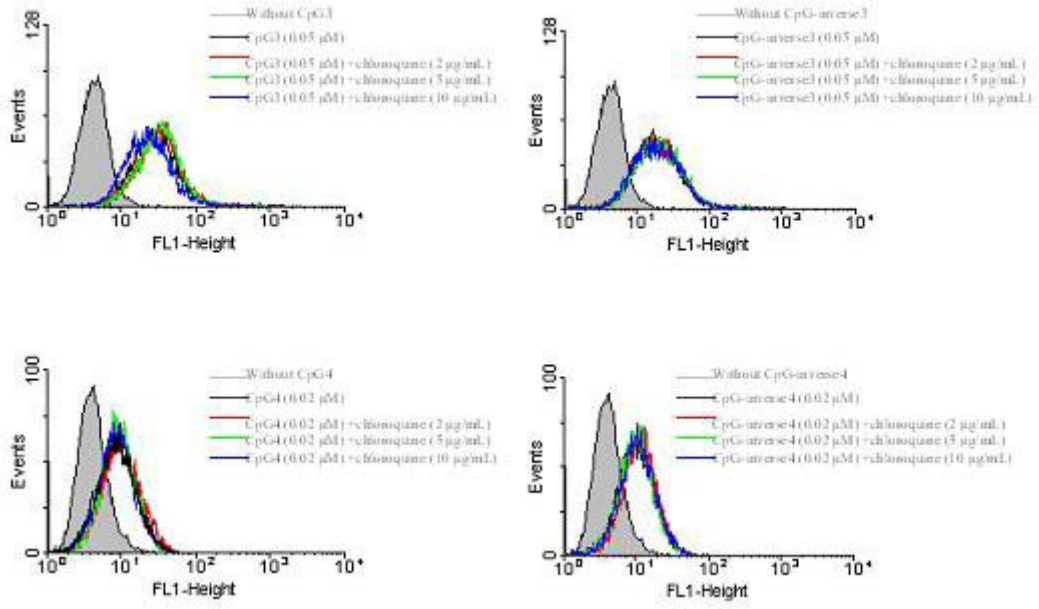


Fig. 4. **Uptake of CpG-ODN and CpG-inverse ODN by Glioblastoma Cells cannot be Inhibited by chloroquine.** Cells were cultured in 12-well plates for 24 hours and washed with RPMI 1640 to remove residual FCS. Chloroquine (2 µg/mL, 5 µg/mL or 10 µg/mL) was added 15 minutes prior to addition of FITC-labelled ODNs. After addition of the ODNs, the cells were incubated for 1 hour at 37°C. Thereafter cells were washed with PBA to remove ODNs bound unspecifically to the cell surface and cells were detached with trypsin-EDTA and collected by spinning. Fluorescence intensity of the cells was measured by flow cytometry. PS CpG3 and CpG-inverse3 ODNs were studied in C6 cells and PS CpG4 and CpG-inverse4 ODNs were studied in T98G cells.

CpG-ODNs uptake in human glioblastoma cells T98G and rat glioblastoma cells C6 is energy dependent (Figure 3). Fluorescence intensity was reduced if cells

were incubated with FITC labelled CpG-ODNs at 4°C or treated with the inhibitor sodium azide (0.1%). Sodium azide is an inhibitor of cellular respiration and thus inhibits active transport. These results strongly support an energy-dependent uptake mechanism.

CpG-ODN uptake in human glioblastoma cell T98G and rat glioblastoma cell C6 can be inhibited by certain polyanions (Figure 3). Previous studies have indicated that a variety of polyanions can influence the response of cells to stimulatory ODNs, which suggested that polyanions with certain structure could interact with receptors for stimulatory ODNs. To investigate this, uptake of FITC-labelled CpG or CpG-inverse ODNs by glioblastoma cells in the presence or absence of a variety of polyanions were determined. As shown in Figure 3, dextransulfate and fucoidan inhibited uptake both in T98G and C6 cells, but not polyvinysulfate.

CpG-ODN uptake in human glioblastoma cells T98G and rat glioblastoma cells C6 could not be inhibited by chloroquine, which was reported to abolish most of the stimulatory effects of CpG-ODNs at concentrations as low as 5 μM (Figure 4). Data in Figure 4 showed that chloroquine could not inhibit CpG nor CpG-inverse ODN uptake in neither T98G nor C6 cells at concentrations up to 15 μg/mL (30 μM). These findings support the hypothesis that chloroquine abolishing CpG-ODN activity doesn't act through inhibition of ODN uptake.

Intracellular Distribution of CpG-ODNs in Glioblastoma Cells

To investigate the intracellular distribution of these CpG-ODNs, FITC-labelled CpG-ODNs were incubated with cells and nuclei were counterstained with DAPI. As shown in Figure 5, after internalisation, CpG-ODNs mainly localized to certain cellular compartments and showed a spotted distribution patterns in the cytoplasm, which strongly suggested a receptor mediated endocytosis mechanism. Up to 1 hour later, no CpG-ODNs appeared in the nuclei. CpG and CpG-inverse ODNs shared the same intracellular distribution pattern in glioblastoma cells, which suggests a common uptake mechanism.

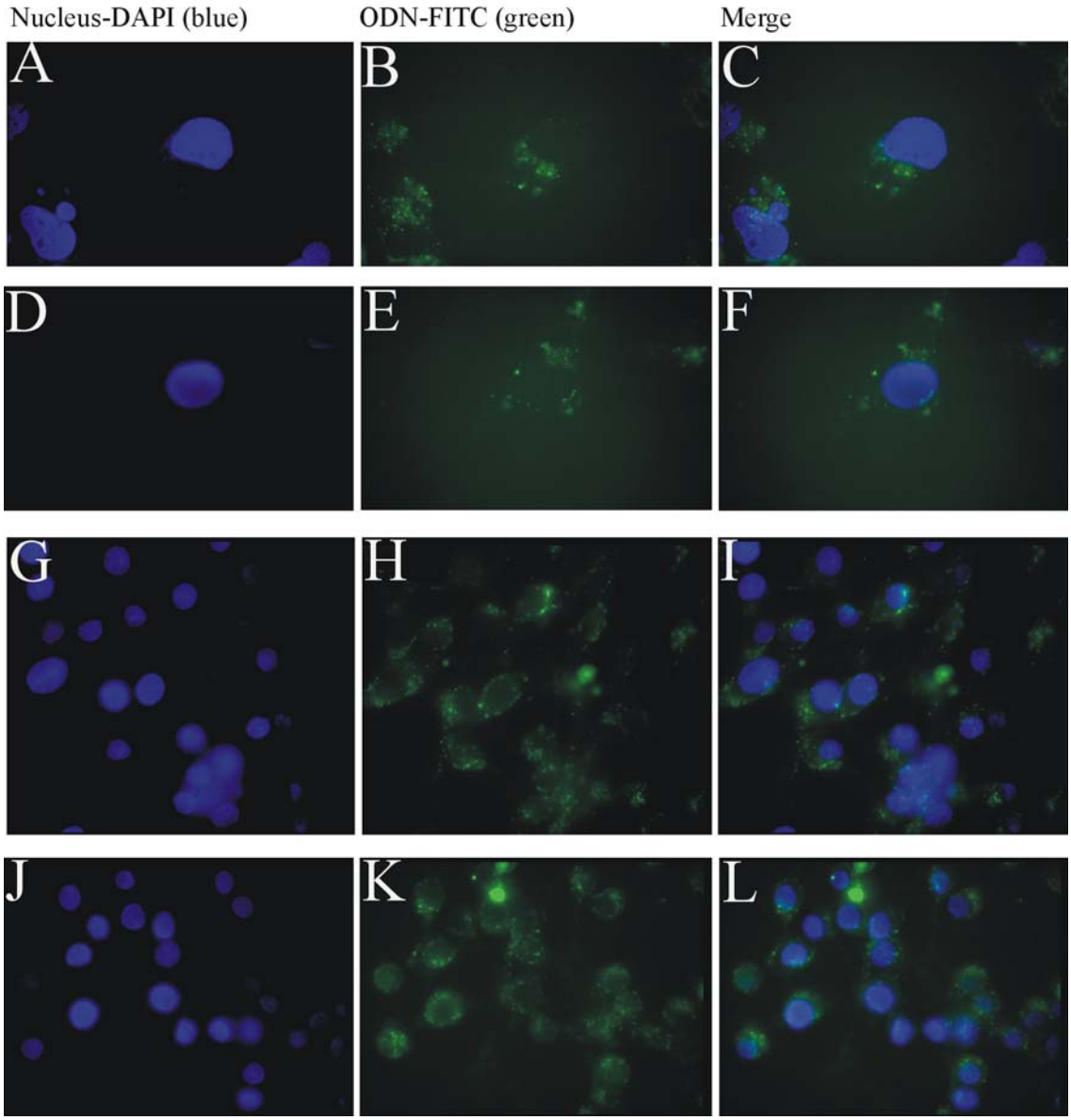


Fig. 5. Intracellular Localization of PS CpG-ODNs and CpG-inverse ODNs in Glioblastoma Cells. Cells were cultured on 6-well chamber slides with 1 μM ODN (labelled with FITC) for 1 hour. Cells were washed with PBS three times and fixed with PFA (2%) at room temperature for 15 minutes. Then cells were mounted with mounting medium with DAPI and observed under fluorescence microscope. A, B and C refer to the intracellular distribution of PS CpG4 ODNs in T98G cells; D, E and F refer to the intracellular distribution of PS CpG-inverse4 ODNs in T98G cells; G, H and I refer to the intracellular distribution of PS CpG3 ODNs in C6 cells and J, K and L refer to the intracellular distribution of PS CpG-inverse3 ODNs in C6 cells.

Purification and Identification of Proteins Binding to CpG-ODNs

Biotin labelled CpG-ODNs were used to purify proteins and mass spectrometry was used to identify these proteins. Results in Figure 6 showed that cellular proteins binding to CpG-ODNs were not unique to the CpG dinucleotides all these proteins bound to CpG or CpG-inverse ODNs. And cellular proteins

binding to ODNs were backbone-dependent, because PS ODNs and unmodified ODNs had different binding proteins. In C6 cells, PS ODNs had many more binding proteins than the unmodified ODNs but in T98G cells PS ODNs and unmodified ODNs had almost the same binding proteins. Finally, cellular binding proteins were sequence dependent, because the unmodified CpG3 and CpG4 had different binding proteins in C6 cells but PS CpG3 and CpG4 had the same binding proteins in C6 and T98G cells, respectively. The pattern of binding proteins for ODNs was complex but clearly CpG dinucleotides independent and specific for backbones and sequences. Some of the purified proteins were identified by mass spectrometry. The identified proteins and their functions are shown in table 1.

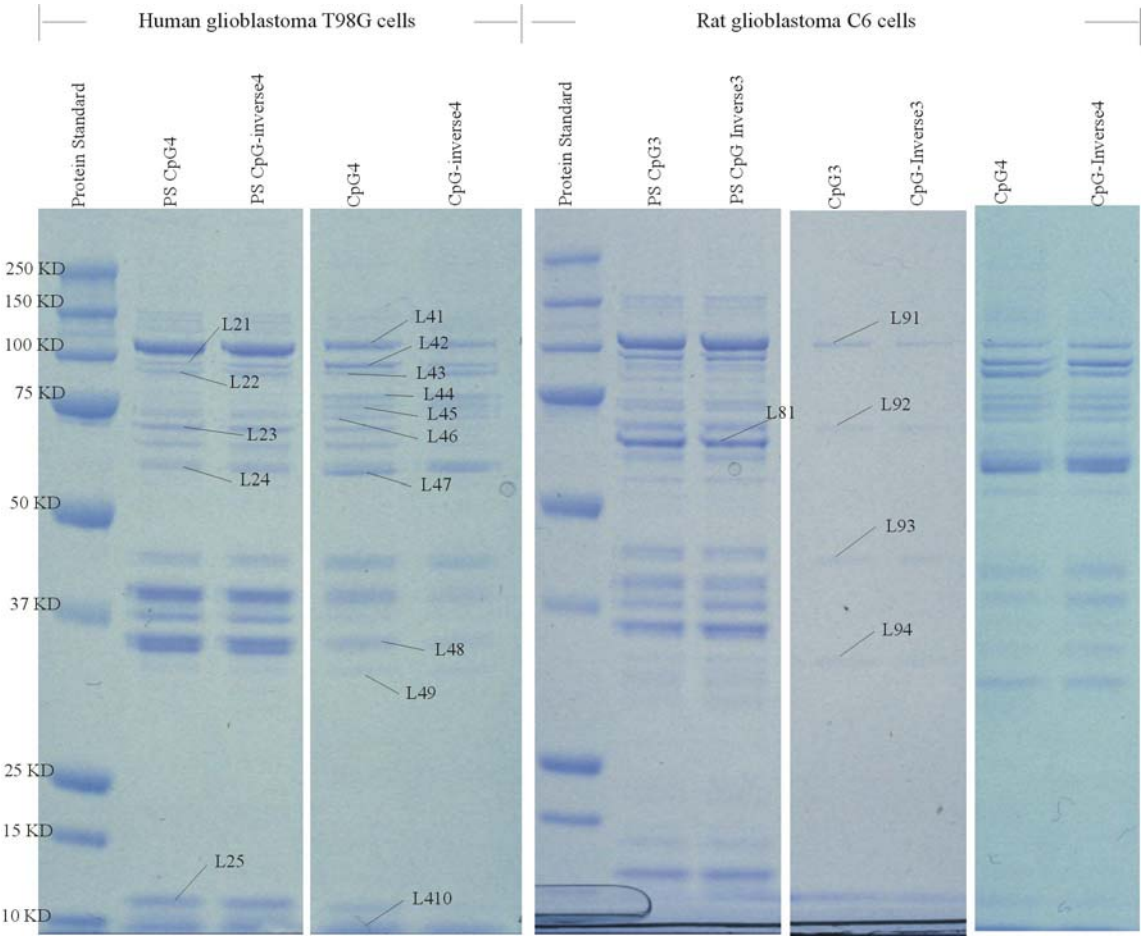


Fig. 6. **Cellular Binding Proteins for CpG-ODNs in Glioblastoma Cells.** Biotin labelled ODNs were used for purification of cellular binding proteins as described in the experimental procedures. PS and unmodified ODNs were used. Unmodified CpG4 and CpG-inverse4 were also used in rat glioblatoma cells to observe whether the same ODNs have different binding proteins in different cells.

Table 1. Proteins binding to CpG-ODN[1]

Band	Symbol	Name	Functions/Localization	Comments
		Human glioblastoma cell line T98G		
		Lane 2 (PS CpG4-ODN mediated protein purification)		
L21	SFPQ	Splicing factor proline/glutamine rich (polypyrimidine tract binding protein associated)	Pre-mRNA splicing factor activity; RNA binding; DNA binding; RNA splicing; nuclear mRNA splicing via spliceosome; *nucleus (28)	Two proteins were identified in this band
	ILT7	Leukocyte immunoglobulin-like receptor, subfamily A (without TM domain),	Receptor activity; immune response; *integral to membrane (29)	Low reliability, score was not significat
L22	M11S1	Membrane component, chromosome 11, surface marker 1	*Integral to plasma membrane; candidates for apical to basolateral transcytosis (30)	3 peptides have been sequenced in this band
L23	TAF15	TAF15 RNA polymerase II, TATA box binding protein (TBP)-associated factor	Single-stranded DNA binding; RNA polymerase II transcription factor activity; single-stranded RNA binding; cell growth and/or maintenance; transcription factor TFIID complex; *nucleus (31)	
L24	G3BP (interim)	Ras-GTPase-activating protein SH3-domain-binding protein	ATP dependent DNA helicase activity; ATP dependent RNA helicase activity; protein transporter activity; protein binding; RNA binding; protein-nucleus import; transport; RAS protein signal transduction; *cytoplasm or nucleus (32)	Two proteins were identified in this band
	G3BP2	Ras-GTPase activating protein SH3 domain-binding protein 2	Protein transporter activity; RNA binding; cytoplasmic sequestering of NF-kappaB; protein-nucleus import; transport; RAS protein signal transduction; *cytoplasm or nucleus; receptor signaling complex scaffold activity (33)	
L25	HIST2H2B (E, or C, or D..)	Histone 2, H2b (e, or c, or d..)	DNA binding; nucleosome assembly; chromosome organization and biogenesis (sensu Eukarya); DNA-dependent regulation of transcription; *nucleus (34)	Not clear which member of the family
	H3F3A	H3 histone, family 3A	Maintenance of the chromatin structure (35)	Could also be H3F3B etc
		Lane 4 (Unmodified CpG4-ODN mediated protein purification)		
L41	HNRPU	Heterogeneous nuclear ribonucleoprotein U (scaffold attachment factor A)	Nucleoplasmic phosphoprotein; binds pre-mRNA in vivo and binds both RNA and DNA in vitro; a component of hnRNP particles and an element in the higher-order organization of chromatin (36)	
L42	SFPQ	Splicing factor proline/glutamine rich (polypyrimidine tract binding protein associated)	Pre-mRNA splicing factor activity; RNA binding; DNA binding; RNA splicing; nuclear mRNA splicing via spliceosome; *nucleus (28)	
L43	M11S1	Membrane component, chromosome 11, surface marker 1	*Integral to plasma membrane; candidates for apical to basolateral transcytosis (30)	4 peptides have been sequenced
L44	HNRPM	Heterogeneous nuclear ribonucleoprotein M	Transmembrane receptor activity; *integral to plasma membrane; pre-mRNA splicing; heart-shock response (37)	
L45	FUBP1	Far upstream element (FUSE) binding protein 1	DNA binding; DNA-dependent regulation of transcription; *nucleus (38)	
L46	RBM14	RNA binding motif protein 14	Nucleic acid binding (39)	
L47	TAF15	TAF15 RNA polymerase II, TATA box binding protein (TBP)-associated factor, 68kDa	Same as above.	
L48	NONO	Non-POU domain containing, octamer-binding	Double and single-stranded DNA and RNA binding; RNA processing; transcription regulation; DNA unwinding and DNA pairing (40)	
L49	RBMX	RNA binding motif protein, X-linked	RNA binding; DNA binding; DNA-dependent regulation of transcription, biological process unknown; heterogeneous nuclear ribonucleoprotein complex (41)	
L410	hnRNPA3 (interim)	Heterogeneous nuclear ribonucleoprotein A3	RNA binding; cytoplasmic RNA trafficking (42)	
		Rat glioblastoma cell line C6		
		Lane 8 (PS CpG inverse3-ODN mediated protein purification)		
L81	LOC317385	Similar to pigpen	RNA binding; may function in the growth and differentiation of endothelial cells during angiogenesis (43)	Low reliability, score was not significat
		Lane 9 (Unmodified CpG3-ODN mediated protein purification)		
L91	LOC315362	Similar to cell division cycle 2 homolog (S. pombe)-like 2; cell division cycle 2-like 2	Regulation of transcription/splicing and apoptotic signaling (44)	Low reliability, score was not significat
L92	LOC287524	Similar to replication protein A1	DNA replication, recombination, repair and also in regulation of transcription (45)	

L93	Hnrpd (interim)	RNA binding protein p45AUF1	RNA binding; mRNA stability (46)
L94	LOC364672	Similar to heterogeneous nuclear ribonucleoprotein A0; hnRNA binding protein	RNA binding; post-transcriptional regulation (47)

[1]: If not depicted otherwise, protein identification is based on the peptide mass fingerprint and significance is judged by the probability based Mowse score. In some cases, tryptic peptide fragments have been sequenced by MSMS experiments. The scores of ILT7, TAF15, LOC317385, and LOC315362 were little below the significance level.

[*]: Cellular localization of protein.

Discussion

In the present study, uptake and cellular distribution of immunostimulatory CpG-ODNs in human and rat glioblastoma cells was studied. Further, cellular binding proteins of PS and unmodified CpG and CpG-inverse ODNs were purified and identified.

In brain, because of the blood-brain barrier (BBB), direct microinjection is the most widely used method for ODN-based therapy strategies. Naked ODN is mainly used for injection because of the neurotoxicity of transfection reagents. Since ODNs are negatively charged macromolecules, it is impossible for them to diffuse through cell membranes freely (22, 23). To elicit therapeutic effects ODNs need to interact with their cellular targets. Therefore, uptake efficiency is an important rate-limiting step for ODN-based therapy.

Due to the development of antisense technology, ODN uptake has been studied and receptor-mediated endocytosis is considered to be the main mechanism for foreign ODN uptake (24, 25, 26, 51, 52, 53). But CpG-ODN uptake has not been fully studied. Our results here showed that ODN-uptake in glioblastoma cells is dose, time, temperature and energy dependent and can be inhibited by molecules with similar characteristics, i.e. negatively charged macromolecules. These results indicated a receptor-mediated endocytosis mechanism which was considered to be the major mechanism for ODNs uptake from previous reports. The spotted cellular distribution of internalised ODNs was also a clue for receptor-mediated endocytosis mechanism (24, 25, 26).

Interestingly, uptake and intracellular distribution of CpG-ODNs in glioblastomal cells were independent of the CpG dinucleotides. In our study, CpG-ODNs and CpG-inverse ODNs showed similar uptake characteristics and cellular distribution patterns. Though ionic interaction between ODNs and cell-surface proteins usually are responsible for binding, the structures of the ODNs may also contribute to the binding specificity. For example, ODNs with continuous guanosine can form the quadruplex structure and thus influence binding properties (27). Our data showed that the CpG dinucleotides contributed little or not at all to uptake, which indicated that there were no receptors specific for CpG-ODN-uptake on glioblastoma cells or it was impossible for CpG dinucleotides to form structures which may contribute to the binding specificity. So our results for CpG-ODNs in particular and ODNs in general may also apply to other different ODNs.

Observations from antisense RNA research revealed non-antisense effects of ODNs, particularly of PS ODNs. Such interactions were less dependent of the sequence but rather dependent of the ODN modifications and would cause non-antisense effects, which might interfere with the evaluation of the therapeutic effects or even would cause serious side effects in ODN-based therapy (9, 10, 11, 12, 13). Therefore it is important to learn more about these interactions.

In the present study ODN-mediated purification of binding proteins and mass spectrometry was used to study such interactions. Our results showed that these interactions were independent of CpG dinucleotides but dependent of the ODN backbones and ODN sequences. The binding of proteins to PS ODNs was sequence independent in contrast to the binding to unmodified ODNs, which are sequence dependent.

Several of the purified binding proteins were identified by mass spectrometry. Known functions of these identified proteins are shown in Table 1. Most of these identified proteins were RNA- or DNA-binding proteins and had RNA or DNA binding domains. These proteins play important roles in cellular RNA transport, maturation, splicing, stabilization, transcription, translation, chromosome organization, biogenesis and structure maintenance. The protein G3BP and

G3BP2, which play an important role in cellular signal transduction, were also identified. Interestingly, three plasma membrane proteins, ILT7, M11S1 and HNRPM, were shown to bind to CpG-ODNs.

These identified RNA and DNA binding proteins are important for chromosome organization, transcription regulation and RNA processing and the identified cellular signal adaptors are also necessary for normal cellular functions. Therefore foreign ODNs binding to these proteins might cause unwanted effects. And such effects could be complex and hard to evaluate in that such binding is sequence dependent and it is hard to define the affected gene since such binding might interfere transcription regulation, RNA processing and cellular signal pathways. Corresponding to our results, data from microarray experiment to study the specificity of antisense therapy also revealed global gene expression changes (48). Further interactions between foreign ODNs and RNA or DNA binding proteins might also influence the cellular localization of foreign ODNs, which might ultimately impact their therapeutic effects. RNA or DNA binding proteins identified here have different cellular localizations or even shuttle between cytoplasm and nuclei. So different foreign ODNs might bind to different cellular proteins and finally localize to different cellular compartments. Accumulation of foreign ODNs in the nucleus was observed by several groups, and nucleoplasmic shuttling of foreign ODNs was also reported (49, 50). These different localization patterns of foreign ODNs might be in accordance with the different localization of their binding proteins.

Receptor-mediated endocytosis is considered to be the main mechanism for foreign ODN uptake. Several cell-surface proteins have been shown to play a role in the binding and uptake of ODNs, but until now only three receptor proteins, Mac-1, scavenger receptor and a protein channel, have been isolated and characterized (51, 52, 53). Presently three plasma membrane proteins, M11S1, ILT7 and HNRPM, were identified as ODN binding proteins *in vitro*. M11S1 is attached to the plasma membrane by a GPI anchor and is a transcytosed protein. In Caco-2 cells, this membrane protein is present in both the apical and basolateral plasma membrane and transcytosed in both directions. ILT7, which belongs to the leukocyte-expressed receptors of the

immunoglobulin (Ig) superfamily, has a charged arginine amino acid in the transmembrane domain and a short cytoplasmic domain that is postulated to associate with the FcεRγ chain to transduce stimulatory signals. HNRPM are involved in pre-mRNA splicing, early stress-induced splicing arrest and a receptor for carcinoembryonic antigen in Kupffer cells. Figure 6 shows that these three membrane proteins have different binding preferences. In our study, M11S1 might bind to PS CpG3, CpG4, their inverse control, unmodified CpG4 and its inverse control; HNRPM might only bind to unmodified CpG4 and its inverse control and ILT7 might only bind to PS CpG4 and its inverse control. Though until now there are no reports about these three proteins contributing to ODNs uptake but our data presented here indicate these membrane proteins might contribute to ODN uptake, especially the protein HNRPM which has a RNA-binding motif. But whether these three membrane proteins truly contribute to foreign ODNs uptake is not sure and needs further investigation.

Generally, data presented here showed that CpG-ODN uptake in T98G and C6 cell lines was dose-, time-, temperature dependent and could be inhibited by sodium azide, polyanions but not by chloroquine. Though CpG-ODNs were taken-up by glioblastomal cells this process was not selective for the CpG dinucleotides, and could be considered more as a transport process for ODNs in general. Dozens of ODN-binding proteins were identified. Most of them were RNA-binding proteins, which are important for transcription regulation and RNA processing, and three plasma membrane proteins, which might contribute to foreign ODN uptake, were also identified.

The interaction between foreign ODNs and RNA-binding proteins bears the intrinsic problem of interference with an essential cellular pathway of cellular RNA processing and foreign ODN localization. The binding of foreign therapeutic ODNs to cellular binding proteins should be taken into account when rational ODN-based therapeutic reagents are designed and when the mechanisms and sites of action are considered. Further, such binding might influence the uptake efficiency or cellular distribution of therapeutic ODNs. However, considering endogeneous cellular binding proteins, ODN uptake and cellular distribution could consequently be optimised for rational drug design.

References

1. Nieder C, Nestle U: A review of current and future treatment strategies for malignant astrocytomas in adults. Strahlenther Onkol 176:251-258, 2000

2. Giese A, Westphal M: Treatment of malignant glioma: a problem beyond the margins of resection. J Cancer Res Clin Oncol 127:217-225, 2001

3. Lam PY, Breakefield XO: Potential of gene therapy for brain tumors. Hum Mol Genet 10:777-787, 2000

4. Carpentier AF, Xie J, Mokhtari K, Delattre JY: Successful treatment of intracranial gliomas in rat by oligodeoxynucleotides containing CpG motifs. Clin Cancer Res 6: 2469-2473, 2000

5. Auf G, Carpentier AF, Chen L, Le Clanche C, Delattre JY: Implication of macrophages in tumor rejection induced by CpG-oligodeoxynucleotides without antigen. Clin Cancer Res 7:3540-3543, 2001

6. Carpentier AF, Chen L, Maltonti F, Delattre JY: Oligodeoxynucleotides containing CpG motifs can induce rejection of a neuroblastoma in mice. Cancer Res 59:5429-5432, 1999

7. Sandler AD, Chihara H, Kobayashi G, Zhu X, Miller MA, Scott DL, Krieg AM: CpG oligonucleotides enhance the tumor antigen-specific immune response of a granulocyte macrophage colony-stimulating factor-based vaccine strategy in neuroblastoma. Cancer Res 63:394-399, 2003

8. Micklefield J: Backbone modification of nucleic acids: synthesis, structure and therapeutic applications. Curr Med Chem 8:1157-1179, 2001

9. Guvakova MA, Yakubov LA, Vlodavsky I, Tonkinson JL, Stein CA: Phosphorothioate oligodeoxynucleotides bind to basic fibroblast growth factor, inhibit its binding to cell surface receptors, and remove it from low affinity binding sites on extracellular matrix. J Biol Chem 270:2620-2627, 1995

10. Perez JR, Li Y, Stein CA, Majumder S, van Oorschot A, Narayanan R: Sequence-independent induction of Sp1 transcription factor activity by phosphorothioate oligodeoxynucleotides. Proc Natl Acad Sci USA 91:5957-5961, 1994

11. Agrawal S, eds. Prospects for Antisense Nucleic Acid Therapy of Cancer and AIDS, New York: Wiley-Liss, p143, 1991

12. Wojcik WJ, Swoveland P, Zhang X, Vanguri P: Chronic intrathecal infusion of phosphorothioate or phosphodiester antisense oligonucleotides against

cytokine responsive gene-2/IP-10 in experimental allergic encephalomyelitis of lewis rat. J Pharmacol Exp Ther 278:404-410, 1996

13. Rockwell P, O'Connor WJ, King K, Goldstein NI, Zhang LM: Cell-surface perturbations of the epidermal growth factor and vascular endothelial growth factor receptors by phosphorothioate oligodeoxynucleotides. Proc Natl Acad Sci USA 94:6523-6528, 1997

14. Dalpke AH, Zimmermann S, Albrecht I, Heeg K: Phosphodiester CpG oligonucleotides as adjuvants: polyguanosine runs enhance cellular uptake and improve immunostimulative activity of phosphodiester CpG oligonucleotides in vitro and in vivo. Immunology 106:102-112, 2002

15. LeGrow AB, Fielding DC, Pressley TA: Stimulation of Na,K-ATPase by hypothyroidism in the thyroid gland. J Endocrinol 160:453-460, 1999

16. Liang H, Reich CF, Pisetsky DS, Lipsky PE: The role of surface ig binding in the activation of human B cells by phosphorothioate oligodeoxynucleotides. Scand J Immunol 54:551-563, 2001

17. Manzel L, Strekowski L, Ismail FM, Smith JC, Macfarlane DE: Antagonism of immunostimulatory CpG-oligodeoxynucleotides by 4-aminoquinolines and other weak bases: mechanistic studies. J Pharmacol Exp Ther 291:1337-1347, 1999

18. Blank M, Weinschenk T, Priemer M, Schluesener H: Systematic evolution of a DNA aptamer binding to rat brain tumor microvessels. selective targeting of endothelial regulatory protein pigpen. J Biol Chem 276:16464-16468, 2001

19. Shevchenko A, Wilm M, Vorm O, Mann M: Mass spectrometric sequencing of proteins silver-stained polyacrylamide gels. Anal Chem 68:850-858, 1996

20. Arnott D, O'Connell KL, King KL, Stults JT: An integrated approach to proteome analysis: identification of proteins associated with cardiac hypertrophy. Anal Biochem 258:1-18, 1998

21. Perkins DN, Pappin DJ, Creasy DM, Cottrell JS: Probability-based protein identification by searching sequence databases using mass spectrometry data. Electrophoresis 20:3551-3567, 1999

22. Weyerbrock A, Oldfield EH: Gene transfer technologies for malignant gliomas. Curr Opin Oncol 11:168-173, 1999

23. Engelhard HH: Antisense Oligodeoxynucleotide Technology: Potential Use for the Treatment of Malignant Brain Tumors. Cancer Control 5:163-170, 1998

24. Krieg AM, Yi AK, Matson S, Waldschmidt TJ, Bishop GA, Teasdale R, Koretzky GA, Klinman DM: CpG motifs in bacterial DNA trigger direct B-cell activation. Nature 374:546-549, 1995

25. Loke SL, Stein CA, Zhang XH, Mori K, Nakanishi M, Subasinghe C, Cohen JS, Neckers LM: Characterization of oligonucleotide transport into living cells. Proc Natl Acad Sci USA 86:3474-3478, 1989

26. Stein CA, Tonkinson JL, Zhang LM, Yakubov L, Gervasoni J, Taub R, Rotenberg SA: Dynamics of the internalization of phosphodiester oligodeoxynucleotides in HL60 cells. Biochemistry 32:4855-4861, 1993

27. Shen W, Waldschmidt M, Zhao X, Ratliff T, Krieg AM: Antitumor mechanisms of oligodeoxynucleotides with CpG and polyG motifs in murine prostate cancer cells: decrease of NF-kappaB and AP-1 binding activities and induction of apoptosis. Antisense Nucleic Acid Drug Dev12:155-164, 2002

28. Patton JG, Porro EB, Galceran J, Tempst P, Nadal-Ginard B: Cloning and characterization of PSF, a novel pre-mRNA splicing factor. Genes Dev 7:393-406, 1993

29. Wende H, Volz A, Ziegler A: Extensive gene duplications and a large inversion characterize the human leukocyte receptor cluster. Immunogenetics 51:703-713, 2000

30. Ellis JA, Luzio JP: Identification and characterization of a novel protein (p137) which transcytoses bidirectionally in Caco-2 cells. J Biol Chem 270:20717-20723, 1995

31. Hoffmann A, Roeder RG: Cloning and characterization of human TAF20/15. Multiple interactions suggest a central role in TFIID complex formation. J Biol Chem 271:18194-18202, 1996

32. Parker F, Maurier F, Delumeau I, Duchesne M, Faucher D, Debussche L, Dugue A, Schweighoffer F, Tocque B: A Ras-GTPase-activating protein SH3-domain-binding protein. Mol Cell Biol 16:2561-2569, 1996

33. Prigent M, Barlat I, Langen H, Dargemont C: IkappaBalpha and IkappaBalpha /NF-kappa B complexes are retained in the cytoplasm through interaction with a novel partner, RasGAP SH3-binding protein 2. J Biol Chem 275:36441-3649, 2000

34. Albig W, Doenecke D: The human histone gene cluster at the D6S105 locus. Hum Genet 101:284-294, 1997

35. Lin X, Wells DE: Localization of the human H3F3A histone gene to 1q41, outside of the normal histone gene clusters. Genomics 46:526-528, 1997

36. Kiledjian M, Dreyfuss G: Primary structure and binding activity of the hnRNP U protein: binding RNA through RGG box. EMBO J 11:2655-2664, 1992

37. Bajenova O, Stolper E, Gapon S, Sundina N, Zimmer R, Thomas P: Surface expression of heterogeneous nuclear RNA binding protein M4 on Kupffer cell

relates to its function as a carcinoembryonic antigen receptor. Exp Cell Res 291:228-241, 2003

38. Duncan R, Bazar L, Michelotti G, Tomonaga T, Krutzsch H, Avigan M, Levens DA: Sequence-specific, single-strand binding protein activates the far upstream element of c-myc and defines a new DNA-binding motif. Genes Dev 8:465-480, 1994

39. Misra P, Owuor ED, Li W, Yu S, Qi C, Meyer K, Zhu YJ, Rao MS, Kong AN, Reddy JK: Phosphorylation of transcriptional coactivator peroxisome proliferator-activated receptor (PPAR)-binding protein (PBP). Stimulation of transcriptional regulation by mitogen-activated protein kinase. J Biol Chem 277:48745-8754, 2002

40. Shav-Tal Y, Zipori D: PSF and p54(nrb)/NonO--multi-functional nuclear proteins. FEBS Lett 531:109-114, 2002

41. Lingenfelter PA, Delbridge ML, Thomas S, Hoekstra HE, Mitchell MJ, Graves JA, Disteche CM: Expression and conservation of processed copies of the RBMX gene. Mamm Genome 12:538-545, 2001

42. Ma AS, Moran-Jones K, Shan J, Munro TP, Snee MJ, Hoek KS, Smith R: Heterogeneous nuclear ribonucleoprotein A3, a novel RNA trafficking response element-binding protein. J Biol Chem 277:18010-18020, 2002

43. Alliegro MC: Pigpen and endothelial cell differentiation. Cell Biol Int 25:577-584, 2001

44. Gururajan R, Lahti JM, Grenet J, Easton J, Gruber I, Ambros PF, Kidd VJ: Duplication of a genomic region containing the Cdc2L1-2 and MMP21-22 genes on human chromosome 1p36.3 and their linkage to D1Z2. Genome Res 8:929-939, 1998

45. Esther van der Knaap, Sandrine Jagoueix, Hans Kende: Expression of an ortholog of replication protein A1 (RPA1) is induced by gibberellin in deepwater rice. Proc Natl Acad Sci USA 94: 9979-9983, 1997

46. Dempsey LA, Hanakahi LA, Maizels N: A specific isoform of hnRNP D interacts with DNA in the LR1 heterodimer: canonical RNA binding motifs in a sequence-specific duplex DNA binding protein. J Biol Chem 273:29224-29229, 1998

47. Myer VE, Steitz JA: Isolation and characterization of a novel, low abundance hnRNP protein: A0. RNA 1:171-182, 1995

48. Fisher AA, Ye D, Sergueev DS, Fisher MH, Shaw BR, Juliano RL: Evaluating the specificity of antisense oligonucleotide conjugates. A DNA array analysis. J Biol Chem 277:22980-22984, 2002

49. Lorenz P, Baker BF, Bennett CF, Spector DL: Phosphorothioate antisense oligonucleotides induce the formation of nuclear bodies. Mol Biol Cell 9:1007-1023, 1998

50. Lorenz P, Misteli T, Baker BF, Bennett CF, Spector DL: Nucleocytoplasmic shuttling: a novel in vivo property of antisense phosphorothioate oligodeoxynucleotides. Nucleic Acids Res 28:582-592, 2000

51. Benimetskaya L, Loike JD, Khaled Z, Loike G, Silverstein SC, Cao L, el Khoury J, Cai TQ, Stein CA: Mac-1 (CD11b/CD18) is an oligodeoxynucleotide-binding protein. Nat Med. 3:414-420, 1997

52. Biessen EA, Vietsch H, Kuiper J, Bijsterbosch MK, Berkel TJ: Liver uptake of phosphodiester oligodeoxynucleotides is mediated by scavenger receptors. Mol Pharmacol. 53:262-269, 1998

53. Hanss B, Leal-Pinto E, Bruggeman LA, Copeland TD, Klotman PE: Identification and characterization of a cell membrane nucleic acid channel. Proc. Natl. Acad. Sci. USA. 95:1921-1926, 1998

Chapter 5.

siRNA binding proteins of microglial cells: PKR is an unanticipated ligand

Zhiren Zhang[a], Toni Weinschenk[b], Hermann J. Schluesener[a]

[a]Institute of Brain Research, University of Tuebingen, Calwer Street 3, Tuebingen D-72076, Germany

[b]Institute for Cell Biology, Department of Immunology, University of Tuebingen, Auf der Morgenstelle 15, Tuebingen D-72076, Germany

Journal of Cellular Biochemistry. 97, 1217-1229 (2006)

Abstract

Small interfering RNA (siRNA), double-stranded RNA (dsRNA) 21- to 23-nucleotides (nt) long with two nt 3' overhangs, has been shown to mediate powerful sequence-specific gene silence in mammalian cells through RNA interference (RNAi). Due to its high efficiency and high specificity siRNA has been used as a powerful post genomic tool and a potent therapeutic candidate. However, there is still a lot to learn about the mobility of siRNA inside cells and the cellular factors that might interfere with the specificity and activity of siRNA. Microglia are the brain's effectors cells of the innate immune system and suitable targets in the development of novel therapeutic strategies. Here we show the cellular uptake and intracellular distribution of siRNA in murine microglial N9 cells. siRNA was internalised by microglial N9 cells without transfection reagent and mainly localized to the endosomes However no significant gene silencing effects was observed. Its cellular uptake and cellular distribution pattern was similar with that of a same length single stranded DNA (ssDNA). Further, cellular binding proteins of siRNA were purified and identified by mass spectrometry. Negative control siRNA and siRNA targeted to β-actin were used in this part of experiment. Most of the siRNA binding proteins for negative control siRNA and siRNA targeted to β-actin were dsRNA-binding proteins, such as dsRNA-dependent protein kinase R (PKR). Furthermore, both control siRNA and siRNA targeted to β-actin activated PKR in N9 cells, which suggest that siRNA might cause off-target effects through activation of PKR.

Keywords: Microglia; siRNA; RNAi; PKR; uptake; cellular distribution; unspecific effects

1. Introduction

siRNA has been shown to mediate powerful sequence-specific gene silence in mammalian cells through RNAi. RNAi is an evolutionary conserved post-transcriptional gene silencing mechanism that proceeds through a two-step process. In the first step, long dsRNAs are recognised and digested by an RNase III enzyme, Dicer, to generate siRNA. Subsequently, these siRNAs, which act as a guide to ensure specific interaction with the target transcript, are incorporated into the RNA-induced silencing complex, which cleaves the corresponding transcript. This dsRNA mediated gene silencing was first named RNAi by Fire and co-workers. Since then RNAi has been discovered in many different biological systems, ranging from *Caenorhabditis elegans* to mammalian cells (Hannon et al. 2002; Tijsterman et al. 2002; Zamore et al. 2001).

In mammals, long dsRNA results into global non-sequence specific changes of gene expression through two major pathways. The first involves the activation of the PKR (Clemens et al. 1997) and the second involves the activation of a sequence-nonspecific RNase, RNaseL (Player et al. 1998). Activation of either of these pathways will result in global gene expression changes, which obscures any gene-specific knockdown. To specifically silence a target gene in mammalian cells, chemically synthesized or *in vitro* transcribed 21- to 23-nt-long siRNA, which were reported that they wouldn't cause any non-specific effects, were used (Caplen et al. 2001; Elbashir et al. 2001; Castanotto et al. 2002). To obtain stable transfection in cells or animals, DNA expression vector-based siRNAs were developed (Sui et al. 2002; Brummelkamp et al. 2002). Such specific gene silencing methods provide a powerful tool to elucidate gene function, to identify drug targets and to develop specific therapeutics.

Because of its powerful gene knockdown activity and high specificity, siRNA has been widely studied from different point of views in mammalian cells. A variety of cell lines from different species have been shown to be good recipients for siRNA (McManus et al. 2002). Several groups used siRNA as a functional genomics tool (Fraser et al. 2000; Paddison et al. 2002). Also siRNA has been experimentally applied in therapies against cancer or virus

infections and in treatment of some genetic diseases (Scherr et al. 2003; Capodici et al. 2002; Xia et al. 2002).

Despite the wide range of applications there is still a lot to learn about cellular uptake of siRNA, the distribution of siRNA inside cells and the cellular factors that can interfere with the specificity and action of siRNA.

A vital assumption for siRNA-mediated RNAi as genomic tool is that siRNA only specifically knocks-down target gene. siRNA-mediated RNAi was shown to have a high specificity and little attention has been given to its potential unspecific effects (Miller et al. 2003). Lessons from antisense research showed that unspecific effects of oligonucleotide-based reagents might result from mismatched pairing, from specific nucleotide group such as the CpG motif, from cellular responses to foreign oligonucleotides and from unspecific binding to cellular proteins. Recently, several groups reported observations about unspecific effects of siRNA in mammalian cells. Unspecific effects due to mismatched pairing and cellular responses to siRNA were proven in mammalian cells (Saxena et al. 2003; Scacheri et al. 2004; Sledz et al. 2003) and studies of siRNA cellular binding proteins are urgently needed. Here siRNA-mediated protein purification and identification by mass spectrometry were used to identify cellular siRNA binding proteins *in vitro*.

Microglial cells are the brain's effectors cells of the innate immune system and play a key role in all major central nervous system pathologies ranging from acute events such as bacterial infections to neuro-inflammatory and degenerative diseases such as Alzheimer's disease (Aldskogius et al. 2001). siRNA mediated gene silencing was also applied in microglial cells (Gan et al. 2003; Giri et al. 2003).

Here we compared the cellular uptake and cellular distribution of siRNA with a same length ssDNA. Cellular binding proteins for siRNA were purified and identified in microglial N9 cells.

2. Material and methods

Synthetic siRNA and ssDNA

Sequences of negative control siRNA were as follows: 5'-UUCUCCGAACGUGUCACGUdTdT-3' (sense strand), 5'-ACGUGACACGUUCGGAGAAdTdT-3' (antisense strand). 5' end fluorescein isothiocyanate (FITC) labelled, or Alexa Fluor 555 labelled and 5' end biotin labeled negative control siRNA were used and were obtained from QIAGEN GmbH, Hilden, Germany. Sequences of siRNA targeted to β-actin were as follows: 5'-GAUGAGAUUGGCAUGGCUUdTdT-3' (sense strand), 5'-AAGCCAUGCCAAUCUCAUCdTdT-3' (antisense strand) and was obtained from Ambion (Europe) Ltd, Huntingdon, United Kingdom. 5' end biotin labeled β-actin siRNA was from QIAGEN GmbH, Hilden, Germany. Sequences of applied ssDNA were 5´-TCCATGAGCTTCCTGATGCT-3` and 5' end FITC labeled ssDNA were synthesised by MWG-Biotech AG, Ebersberg, Germany.

Cell cultures

Murine N9 microglial cells [Ferrari et al. 1996] and human cerebromicrovasular endothelial cells (HCEC) [Esco et al. 2002] were cultured in RPMI-1640 with 10% heat inactivated fetal calt serum (FCS) with penicillin and streptomycin at 100 U/ mL (Gibco, Grand Island, NY) at 37 °C in 5% CO_2.

Cytofluorometric analysis of siRNA uptake

10^6 microglial cells in 10 ml RPMI 1640 with 10% FCS were seeded into a 10 cm^2 Petri dish and cultured for 24 hours. Afterwards, medium was removed and cells were washed twice with FCS-free RPMI 1640 and then incubated with FCS-free RPMI 1640 at 37 °C for 1 hour to remove residual FCS. After incubation, medium was replaced by serum-free RPMI 1640 containing FITC-siRNA (10 µM) or FITC-ssDNA (10 µM). Cells were cultured in the dark for 1 hour. Then, incubation was terminated and cells were incubated with PBA buffer (Phosphate-buffered saline (PBS), 2% bovine serum albumin (BSA),

0.1% sodium azide) at 4 °C for 10 minutes to remove siRNA bound to the cell surface. Thereafter, cells were washed twice with PBS and detached from dishes with trypsin-EDTA. After neutralizing trypsin with medium containing FCS, cells were collected by centrifugation at 1000 rpm for 5 minutes. After washing, cellular fluorescence intensity was quantified by flow cytometry.

Fluorescence microscopy

After flow cytometric analysis, cells were collected by spinning at 1000 rpm for 5 minutes. Thereafter, supernatant was removed and two drops of mounting medium containing DAPI (VECTASHIELD Mounting Medium with DAPI, Vector Labratories, Inc., Burlingame, USA) were added. After vortex, the cell suspension was dropped onto slides glass and cellular distribution of siRNA was observed by fluorescence microscopy.

To further define the sub-cellular distribution of siRNA co-localization of siRNA with endosome was studied using immunocytochemistry. Briefly, 10^5 cells in 1 mL RPMI1640 were seeded onto 4-well chamber slides (Lab-Tek Chamber Slide, Nalgene Nunc International Corp. Naperville, USA) and cultured at 37 °C, 5% CO_2 for 24 hours. Afterwards, cells were washed with FCS-free RPMI1640 and incubated with Alexa Fluor 555 labelled negative control siRNA (1 μM) for 3 days. After wash twice with PBS cells were fixed with 4% formaldehyde (PFA) for 5 minutes at room temperature. Subsequently, cells were permeabilized and blocked by incubation in 0.1% TritonX-100 containing 3% BSA for 10 minutes. Early endosome marker Rab4 (Abcam Ltd., Cambridge, UK; 1:500 diluted with 1% BSA in PBS) was added and incubated with cells at room temperature for 90 minutes. After washing, cells were incubated with FITC labelled secondary antibody (Abcam Ltd., Cambridge, UK; 1:200 diluted with 1% BSA in PBS) at room temperature for 90 minutes. After washing, cells were mounted in a mounting medium containing DAPI (VECTASHIELD Mounting Medium with DAPI, Vector Laboratories, Inc., Burlingame, USA), and then observed by fluorescence microscopy.

siRNA mediated gene silencing

The transfection reagent mediated siRNA delivery was performed according to the *Silencer*™ siRNA Transfection Kit Instruction Manual (Ambion Europe Ltd, Huntingdon, United Kingdom). Briefly, 2×10^4 N9 cells were seeded into 4-well chamber slides (Lab-Tek Chamber Slide, Nalgene Nunc International Corp. Naperville, USA) and cultured at 37 °C, 5% CO_2 for 24 hours. Then cells were washed with FCS and antibiotic-free medium and cultured in 200 µL FCS and antibiotic-free medium. siRNA targeted to β-actin or negative control (final concentration: 25 nM) and Ambion siPORT Lipid complex was added into medium and co-cultured with cells for 4 hours. Afterwards 1 mL medium with 10% FCS was added and cells were cultured for further 72 hours. Subsequently, cellular β-actin protein expression was analysed using standard immunofluorescence methods.

For siRNA delivery without transfection reagent most steps were performed as above described except that cells were cultured in six-well plates and siRNA was added directly to the cell suspension (final concentration: 500 nM). Cellular β-actin expression was analysed using real-time PCR because of the relative low gene-silencing efficiency of naked siRNA. Total RNA from cultured cells was prepared using the RNeasy Mini Kit (QIAGEN GmbH, Hilden, Germany) according to the manufacture's instruction. 1 µg RNA was reverse transcribed into cDNA using randomised primers. Subsequently mRNA expression of β-actin was quantified by real-time PCR using SYBR-Green as detection reagent and 18s rRNA as reference standard. Following primers were used: β-actin (sense, CCC TGT GCT GCT CAC CGA; antisense, ACA GTG TGG GTG ACC CCG TC), and 18s rRNA (sense, ACA TCC AAG GAA GGC AGC AG; antisense, TTT TCG TCA CTA CCT CCC CA).

siRNA mediated protein purification

Cells cultured in flasks were rinsed two times with ice-cold PBS and then 20 mL ice-cold PBS containing PMSF was added and cells were collected with a scraper. 1.5×10^8 cells were sonicated and incubated in solubilization buffer (50 mM Tris-HCl, 150 mM NaCl, 1% Triton X-100) containing a protease

inhibitor cocktail (Sigma, Munich, Germany) for 2 hours on ice. Lysates were cleared by centrifugation at 14.000 g at 4 °C for 5 minutes. An aliquot of the supernatants was used for protein purification.

Magnetic streptavidin microbeads (Dynal Biotech ASA, Oslo, Norway) were treated according to the handbook to deactivate RNase activity. 1 mg (100 µl) of treated magnetic streptavidin microbeads were coated with 200 pmol of biotin labelled siRNA by incubation in 1 ml selection buffer with RNasin® Ribonuclease Inhibitor (Promega, Mannheim, Germany) (30 min, room temperature). The lysates were incubated with the siRNA coated magnetic microbeads in the presence of a 100-fold excess of tRNA (20 nmol) as an unspecific competitor in selection buffer containing RNasin® Ribonuclease Inhibitor and DDT (total volume 1.5 ml, 0 °C, 15 min). Magnetic streptavidin microbeads without siRNA were also incubated with cell lysate and served as control. The protein-siRNA-magnetic bead-complex was recovered in a magnetic device and washed four times. Proteins were removed from siRNA coated beads by heating in loading buffer and analyzed by 10% polyacrylamide gel electrophoresis and detected by staining with Coomassie Blue [Zhang et al. 2005a]. Bands were analysed by in-gel tryptic digestion and mass spectrometry.

Protein identification

In-gel tryptic digestion was performed as described [Shevchenko et al. 1996] and modified as outlined below. Briefly, the protein band was excised from the gel, fully de-stained, and digested for 3 h with porcine trypsine (sequencing grade, modified; Promega, Mannheim, Germany) at a concentration of 67 ng/µl in 25 mM ammonium bicarbonate, pH 8.1, at 37 °C. Prior to peptide mass mapping and sequencing of tryptic fragments by tandem mass spectrometry, the peptide mixture was extracted from the gel by 1% formic acid followed by two changes of 50% methanol. The combined extracts were vacuum-dried until only 1-2 µl were left and the peptides were purified by ZipTip according to the manufacturers' instructions (Millipore, Bedford, MA, USA). MALDI-TOF analysis from the matrix α-cyano-4-hydroxycinnamic

acid/nitrocellulose prepared on the target using the fast evaporation method [Arnott et al. 1998] was performed on a Bruker Reflex III (Bruker Daltonik, Bremen, Germany) equipped with a N_2 337 nm laser, gridless pulsed ion extraction and externally calibrated using synthetic peptides with known masses. The spectra were obtained in positive ionization mode at 23 kV.

Sequence verification of some fragments was performed by nanoelectrospray tandem mass spectrometry on a hybrid quadrupole orthogonal acceleration time of flight tandem mass spectrometer (Q-Tof, Micromass, Manchester, UK) equipped with a nanoflow electrospray ionization source. Gold/palladium-coated glass capillary nanoflow needles were obtained from PROXEO (Type Medium NanoES spray capillaries for the Micromass Q-Tof, Odense, Denmark). Database searches (NCBInr, non-redundant protein database) were done using the MASCOT software from Matrix Science [Perkins et al. 1999].

PKR activation assays

Negative control siRNA and siRNA targeted to β-actin, whose sequence were showed above, were used here. PKR activation was analysed using western blotting. Briefly, 3 x 10^5 N9 cells per well were seeded into 12-well cell culture plates and cultured overnight. Subsequently, negative control siRNA (50 nM), β-actin siRNA (50 nM) or poly rI:rC (Sigma, Munich, Germany) were transfected into cells respectively using Ambion siPORT Lipid. Transfection reagent alone was used as negative control and poly rI:rC (50 ng/mL) was served as positive control [Sledz et al. 2003]. The transfection process was performed according to the *Silencer*™ siRNA Transfection Kit Instruction Manual. Total protein lysates were collected 48 hours post-transfection. 50 μg total protein were separated on 10% SDS-polyacrylamide gel and transferred to PVDF membrane. Immunostaining was performed using ECL (Amersham Pharmacia Biotech). The antibody against PKR (Cell Signaling Technology) and antibody against phospho-PKR (BioSource Europe) were used at a 1:1000 dilution and peroxidase-linked anti-rabbit secondary antibody (Amersham Pharmacia Biotech) was used at a 1:3000 dilution.

3. Results

Uptake, cellular distribution and gene-silencing effects of naked siRNA in microglial cells

To study the characteristics of siRNA uptake and cellular distribution independent of gene knockdown effects, we used a FITC-labelled siRNA, which has no homologous sequence in mammalians (negative control siRNA). In antisense technology, oligonucleotide uptake and cellular distribution have been well studied. So here siRNA uptake and cellular distribution were compared with a 20-nt long ssDNA whose uptake and cellular distribution has been described in N9 cells previously [Zhang et al. 2005b].

Without transfection reagent, negative control siRNA or ssDNA was incubated with N9 cells. As shown in Figure 1, after incubation with FITC-labelled siRNA the fluorescence intensity of N9 cells increased, which indicated that siRNA can be imported by N9 cells without transfection reagent. Similar phenomena were observed using the ssDNA and the fluorescence intensity enhancement for siRNA and ssDNA was comparable, which suggested that they have similar uptake efficiency in N9 cells.

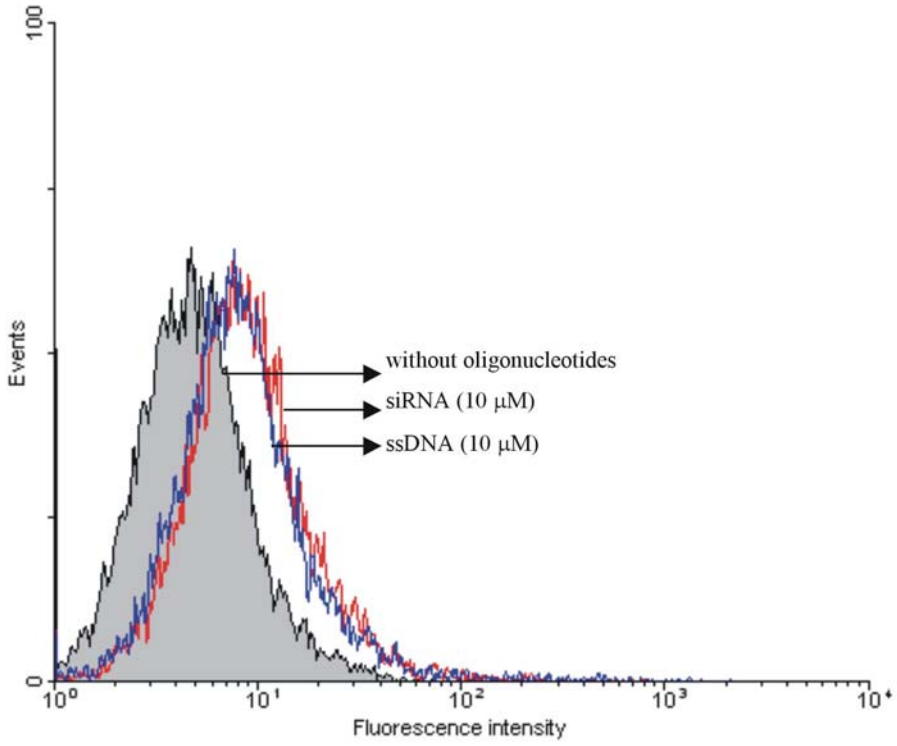


***Fig. 1.* siRNA and ssDNA uptake by microglial N9 cells.** FITC-labelled negative control siRNA (10 μM) or ssDNA (10 μM) were incubated with N9 cells at 37 °C for 1 hour. The fluorescence intensity of N9 cells was measured by flow cytometry.

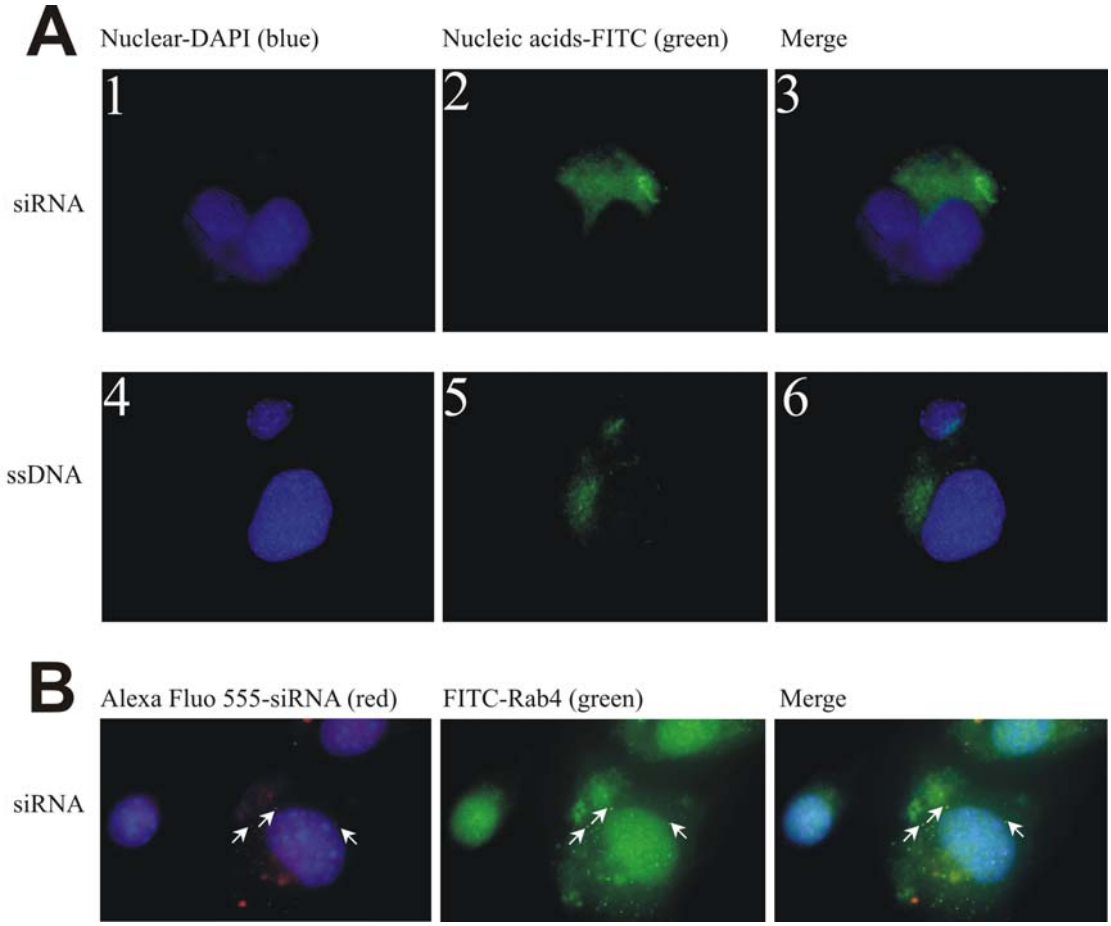


***Fig. 2.* Cellular distribution of naked negative control siRNA in microglial N9 cells.** (A): Cellular localization of naked siRNA and ssDNA in unfixed N9 cells. Negative control siRNA and ssDNA were FITC labelled. After FACS analysis described above N9 cells were observed by fluorescence microscopy. The upper panel showed the negative control siRNA cellular distribution and the lower panel showed the ssDNA cellular distribution. (B): Co-localization of naked siRNA with endosome. Alexa Fluor 555 labelled negative control siRNA (1 μM) was incubated with cultured N9 cells for 3 days. After incubation the endosomes were stained by early endosome marker Rab4, which was visualized with FITC labeled secondary antibody, and nucleus were stained by DAPI. Double-labeled siRNA are marked with arrows.

Cellular distribution of FITC-labelled siRNA was studied using fluorescence microscopy. As shown in Figure 2A, after 1h of incubation, siRNA was unequally distributed in the cytoplasm of unfixed N9 cells. Nuclear distribution was not observed at this point of time. For the ssDNA, after 1h incubation, ssDNA also unevenly localized to the cytoplasm but not to the nucleus in N9 cells (Figure 2A), which was similar to previous observation [Zhang et al. 2005b]. These observations further support that siRNA can be internalised by N9 cells without transfection reagent. As naked siRNA showed a spotted distribution in N9 cells, which indicated an endosomal localization, the sub-

cellular distribution of siRNA was further characterized by double staining with the endosome marker Rab4. As shown in Figure 2B co-localization of siRNA (red) and early endosome (green) was observed. Much less siRNA fluorescence was seen in fixed cells (Figure 2B) as compared to living cells (Figure 2A), which may be due to the fixation, permeabilization and washing processes.

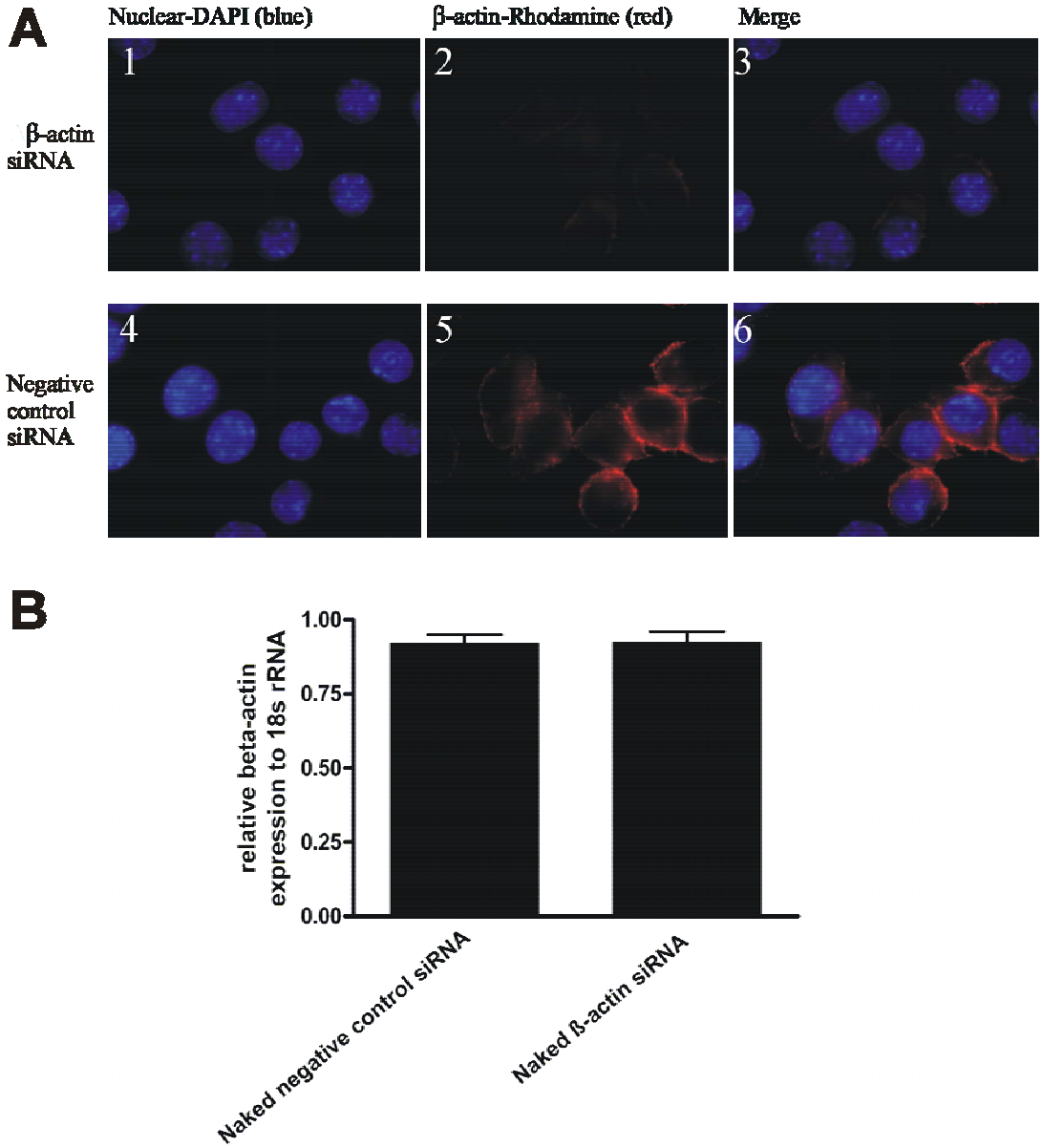


Fig. 3. **Gene silencing effects of siRNA targeted to β-actin in microglial N9 cells with (A) or without (B) transfection reagents.** (A): N9 cells were transfected with β-actin siRNA (1, 2 and 3, 25 nM) or negative control siRNA (4, 5 and 6, 25 nM) according to the SilencerTM siRNA Transfection Kit Instruction Manual. β-actin expression was assayed using immunofluorescence methods with an anti-β-actin antibody. Result represented one of three separate experiments. (B): β-actin siRNA (500 nM) or negative control siRNA (500 nM) were added directly to cell suspension. β-actin expression was assayed

using real-time PCR. Data are expressed relative to the expression of intern reference 18s rRNA. Each bar represents the mean and SEM of triplicates.

To evaluate the gene-silencing effects of naked siRNA, siRNA targeted to β-actin were added directly to cell suspension at high concentrations. The gene silencing effects of this β-actin siRNA was verified in N9 cells using siPORT Lipid transfection (Figure 3A). As shown in Figure 3A, at relative low concentrations (25 nM) β-actin siRNA can knockdown β-actin protein expression in N9 cells as the β-actin fluorescence intensity of N9 cells treated with siRNA targeted to β-actin was much lower than that of N9 cells treated with negative control siRNA. However without transfection reagent the same siRNA targeted to β-actin didn't significantly reduce β-actin RNA expression even at much higher concentrations (500 nM) (Figure 3B).

Cellular binding proteins of siRNA in microglial cells

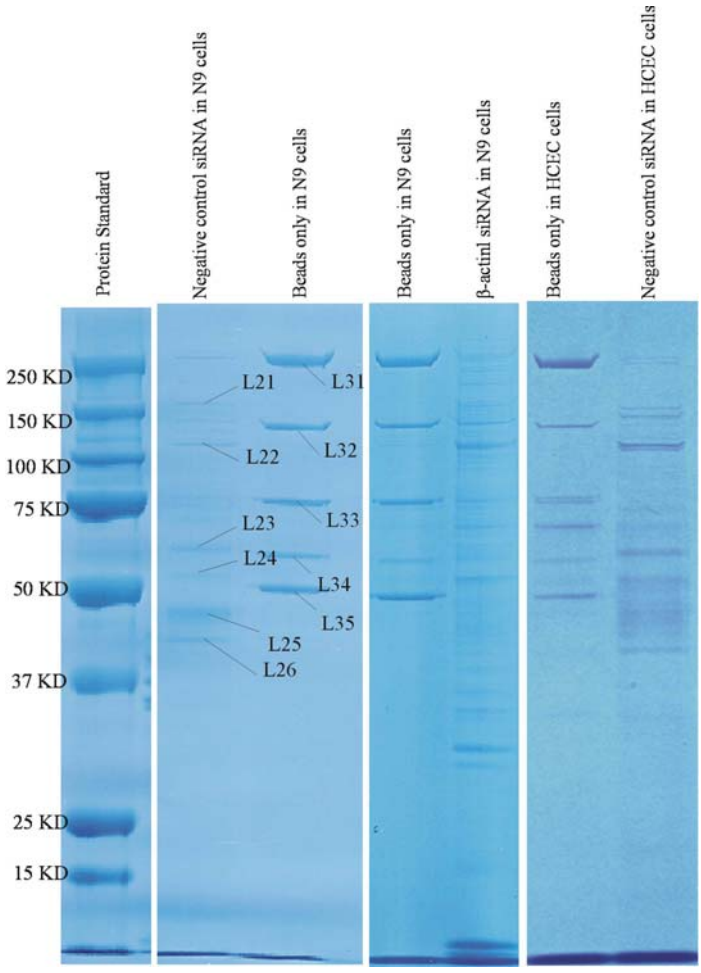


Fig. 4. **Cellular binding proteins for siRNA.** Biotin-labeled siRNAs were used for cellular binding protein purification. Negative control siRNA and β-actin siRNA were applied. Two cell lines, murine microglial N9 cells and human cerebromicrovasular endothelial HCEC cells, were used. Magnetic streptavidin microbeads alone was served as negative control. The indicated bands were identified as: L21: DHX9, L22: ADAR, L23: PKR and Staul, L24: unnamed protein product, L25: Rahl, L26: Tial1, L31: Acac, L32: Pcx, L33: Pcca and Mccc1, L34: Pccb and 4930552N12Rik and L35: Dbt.

The same negative control siRNA was biotinylated and used to study N9 cellular proteins that bind to siRNA. Biotinylated siRNA was adsorbed to magnetic streptavidin microbeads and magnetic streptavidin microbeads alone served as control. After protein purification, siRNA was released from magnetic microbeads, analysed on 4% low-melting gel and shown to remain integrity (data not shown).

As shown in Figure 4, siRNA bound to a number of proteins but magnetic streptavidin microbeads also bound to several proteins. Proteins bound to siRNA or magnetic streptavidin microbeads were identified by mass spectrometry. It is obvious, that siRNA binding proteins were different from that of magnetic microbeads binding proteins (Table 1), which are biotinylated proteins binding to streptavidin.

Table 1. siRNA mediated protein purification in N9 cells

Band	Symbol[1]	Name	Functions	Comments
Lane 3 (siRNA mediated protein purification)				
L21	Dhx9	DEAH (Asp-Glu-Ala-His) box polypeptide 9	Double-stranded RNA binding; ATP binding; RNA dependent and ATP driven helicase activity; pre-mRNA conformational rearrangement (Persengiev et al. 2004)	
L22	Adar	Double-stranded RNA - specific adenosine deaminase	Double-stranded RNA binding; interferon-inducible RNA-editing enzyme implicated in the site-selective deamination of adenosine to insone in double-stranded RNA (Saunders et al. 2003)	
L23	Prkr	Protein kinase, interferon-inducible double stranded RNA dependent	Double-stranded RNA and structured single-stranded RNA binding protein; serine/threonine kinase activity; interferon induction activity and eIF2α inhibition activity (Clemens et al. 1997)	Two proteins were identified in this band.
	Stau1	Staufen (RNA binding protein) homolog 1 (Drosophila)	Double-stranded RNA binding; tubulin binding; mRNA intracellular transport (Chi et al. 2003)	
L24	Ugt1a10	UDP glycosyltransferase 1 family, polypeptide A10		

L25	Rnh1	Ribonuclease/angiogenin inhibitor 1	Ribonucleolytic activity and angiogenic activity inhibition (Semizarov et al. 2003)	
L26	Tial1	Tial1 cytotoxic granule-associated RNA binding protein-like 1	RNA binding; nucleolytic activity; induction of apoptosis (Ferrari et al. 1996)	
Lane 4 (Only streptavidin beads Without oligonucleotides)				
L31	Acac	Acetyl-Coenzyme A carboxylase		Biotinylated protein
L32	Pcx	Pyruvate carboxylase		Biotinylated protein
L33	Pcca	Propionyl-Coenzyme A carboxylase, alpha polypeptide		Biotinylated protein
	Mccc1	Methylcrotonoyl-Coenzyme A carboxylase 1 (alpha)		Biotinylated protein
L34	Pccb	Propionyl Coenzyme A carboxylase, beta polypeptide		Biotinylated protein
	Mccc2	Methylcrotonyl-Coenzyme A carboxylase 2 (beta)		Biotinylated protein
L35	Dbt	Dihydrolipoamide branched chain transacylase E2		Biotinylated protein

[1] Official Gene Symbol according to LocusLink (http://www.ncbi.nlm.nih.gov/LocusLink/)

As further control, we used a different cell line, HCEC cells, which were derived from human microvessel endothelial cells, to compare whether the same siRNA has different binding proteins in different cell types. As shown in Figure 4, the binding proteins of the negative control siRNA in N9 and HCEC cells were not completely identical.

To further compare whether different sequence siRNA have different binding protein in the same cell type, a siRNA targeted to the mammalian cellular β-actin transcript was used to purify binding proteins. The gene silencing effects of this β-actin siRNA was verified in N9 cells (Figure 3A). As shown in Figure 3A, this β-actin siRNA can knockdown β-actin protein expression in N9 cells as the β-actin fluorescence intensity in N9 cells treated with this siRNA targeted to β-actin was much lower than that in N9 cells treated with negative control siRNA. Subsequently, cellular binding proteins of this β-actin siRNA in N9 cells were studied with the same method as described above. As shown in Figure 4, β-actin siRNA also has a number of cellular binding proteins in N9 cells and the binding proteins in the SDS-PAGE gel seemed to be not completely identical with that of the negative control siRNA in N9 cells (Figure 4).

Part of the purified siRNA binding proteins was identified using mass spectrometry sequencing. Names and functions of the identified siRNA binding proteins are shown in Table 1. Among those, DHX9, ADAR, PKR and Stau1 belong to the dsRNA binding protein family, which share a common evolutionarily conserved motif specifically facilitating interaction with dsRNA. Protein Tial1 also has RNA binding activity. All these proteins have important physiological roles.

PKR activation by siRNA in microglial cells

PKR is a regulator of global expression pattern and it is believed that siRNA did not trigger PKR activation. Interestingly, PKR was identified to bind siRNA in N9 cells. Further, we want to see whether siRNA can activate PKR. Activation of PKR results in PKR dimerization and autophosphorylation at threonine 446 and threonine 451 in the activation loop. Antibodies specific for PKR phosphorylated at threonine 451 were used to detect activated PKR and antibodies against total PKR were used to normalize protein loading. Both negative control siRNA and siRNA targeted to mammalian β-actin gene were used in this experiment. siRNA were introduced into cells by transfection reagent. Transfection reagent alone was used as negative control and poly rI:rC was used as positive control [Sledz et al. 2003]. Experiments were repeated three times and similar results were observed. Representative data are shown in Figure 5. Both, siRNA and poly rI:rC significantly induced PKR activation in N9 cells but not the transfection reagent alone. Furthermore, siRNA targeted to β-actin decreased β-actin expression in N9 cells as well (Figure 3A).

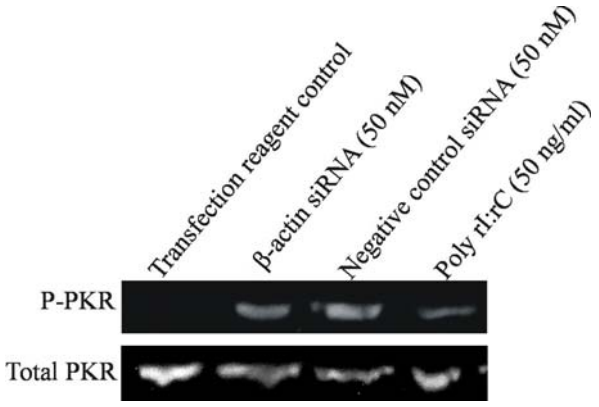


Fig. 5. **Western analysis of PKR activation by siRNA in microglial N9 cells.** N9 cells were transfected with negative control siRNA (50 nM), β-actin siRNA (50 nM) or poly rI:rC (50 ng/mL) using transfection reagent and transfection reagent alone was served as negative control. PKR activation was assayed using western analysis. The upper panel used an antibody specific to activated PKR and the lower panel used an antibody specific to whole PKR. Experiments were performed in triplicates.

4. Discussion

RNAi technology is an efficient high-throughput method to investigate gene function and is also a potential method to develop highly specific dsRNA based gene-silencing therapeutics. Due to these advantages, RNAi technology has been widely used to elucidate mammalian gene functions and to treat certain kind of experimental diseases [Dykxhoorn et al. 2003; Trulzsch et al. 2004]. In microglial cells, siRNA mediated RNAi is also used as a powerful tool to elucidate molecular functions [Gan et al. 2003; Giri et al. 2003]. But the cellular uptake efficiency and cellular localization of siRNA still hasn't been fully elucidated. Though the specificity of siRNA mediated RNAi has been reported by many groups, recently some studies gave clues to further non-specific effects caused by siRNA in mammalian cells [Saxena et al. 2003; Scacheri et al. 2004; Sledz et al. 2003; Jackson et al. 2003].

Here our study showed that naked siRNA (without transfection reagent) could be internalised by microglial N9 cells, and unevenly localized to the cytoplasm but could not silence gene expression even at high concentrations (500 nM). Its cellular uptake and cellular distribution was similar to that of ssDNA. siRNA had a number of cellular binding proteins, most of which belong to the dsRNA binding protein family. siRNA can not only bind PKR in vitro but also activate PKR in vivo in N9 cells.

In vivo transfection efficiency is one of the major problems for oligonucleotide-based therapeutic strategies, including siRNA applications. In the brain, because of the blood-brain barrier (BBB), direct microinjection is the most widely used method for oligonucleotide-based therapeutic strategies. Naked oligonucleotide is mainly used for injection because of the neurotoxicity of transfection reagents. *In vivo,* viral vector based RNAi is used [Sui et al. 2002; Brummelkamp et al. 2002]. Naked siRNA can also be efficiently delivered to

peripheral organs in adult animals and inhibit specific gene expression with the hydrodynamic transfection methods [Lewis et al. 2002; Makimura et al. 2002]. Though gene knockdown in brain using vector-mediated RNAi has been reported, one observation indicated that naked siRNA applied directly to rat brain might not induce RNAi because of low efficiency [Hommel et al. 2003; Isacson et al. 2003]. Therefore it is important to investigate naked siRNA uptake and cellular distribution in brain derived cells. Here we studied the characteristics of naked siRNA uptake and cellular distribution in N9 microglial cells. Naked siRNA can be internalised by N9 cells and mainly localized to the cytoplasm after entry, which is similar with that of ssDNA. Further double-staining experiments showed that naked siRNA mainly restrict in endosomes. Due to the development of antisense technology, ssDNA uptake and cellular distribution has been fully studied. The similar uptake efficiency and cellular distribution of siRNA with ssDNA indicated they might share similar uptake mechanism.

Although naked siRNA can be internalised by N9 cells, no significant gene silencing effects was detected after naked siRNA treatment. siRNA targeted to β-actin significantly reduced β-actin expression at low concentrations (25 nM) using transfection reagent however the same siRNA didn't silence β-actin expression even at high concentrations (500 nM) without transfection reagent. In accordance with our observations, Lingor et al., reported that naked siRNA (205 nM) could not silence endogenous or reporter genes expression (Lingor et al., 2004).

Interestingly, a number of cellular siRNA binding proteins were identified *in vitro*. Two biotin labelled siRNAs, control siRNA and β-actin siRNA, were used for purification of binding proteins. Though sequences of these two siRNA are very different they still share a number of binding proteins. And the negative control siRNA also shares a number of binding proteins in murine microglia N9 cells and human endothelial HCEC cells.

Further, some binding proteins were identified by mass spectrometry sequencing. As shown in Table 1, except protein Rnh1and an unnamed protein, the other five proteins all have RNA binding activity. Among these five RNA-binding proteins, DHX9, ADAR, PKR and Stau1 are membranes of the

dsRNA binding protein family, which share a common evolutionarily conserved motif specifically facilitating interaction with dsRNA. This interaction is independent of nucleotide sequence arrangement. All these identified binding proteins play important physiological roles *in vivo* and their interaction with siRNA might interfere with their normal cellular functions. In particular, DHX9 has helicase and dsRNA binding activity. In theory, siRNA might bind to DHX9 within cells and might be resolved by the helicase activity through unwinding the two strands. However the relevant enzyme for dsRNA degradation within cells is ADAR. ADAR catalyses the conversion of adenosines to inosines. The resulting dsRNAs containing I-U pairs are unstable and easy to unwind [Sui et al. 2002; Wagner et al. 1998; Kumar et al. 1998; Saunders et al. 2003]. DHX9 and ADAR might be involved in cellular siRNA degradation. Stau1 has dsRNA binding and mRNA intracellular transport activity [Belanger et al. 2003]. Within cells, siRNA might also be transported and Stau1 might play a role in siRNA cellular localization.

Notably, the direct interaction of siRNA with PKR was observed. PKR has two dsRNA binding domains (DRBDs) and a serine/threonine kinase domain. The DRBDs have been reported to interact with as little as 11 bp dsRNA and such interaction appears to be independent of any specific RNA nucleotide motif or sequence. PKR's interaction with dsRNA causes PKR to form homodimers and to autophosphorylate on serine/threonine residues. After autophosphorylation, PKR is able to catalyse the phosphorylation of target substrates, the most well characterized being the eIF2α subunit and IκB. Phosphorylated eIF2α sequesters eIF2B, a rate-limiting component of translation, leading to inhibition of protein synthesis. Phosphorylated IκB is released from NF-κB, which then translocates to the nucleus and activates transcription of genes containing NF-κB binding gene, including the interferon-β (INF- β) gene. Subsequently, a specific class of genes, the IFN-stimulated genes, are activated for transcription [Kumar et al. 1998; Sui et al. 2002].

It has been reported, that activation of PKR is dependent on length of the dsRNA. And only dsRNA longer than 80 base pairs activate PKR [Manche et al. 1992], but that the smaller siRNA avoids this triggering of non-specific responses to dsRNA, such as PKR activation [Caplen et al. 2001; Elbashir et

al. 2001]. But our data here demonstrate that siRNA cannot only bind to PKR in vitro, but also causes activation of PKR in vivo. Our observations are in agreement with previous studies, which reported that siRNA globally up-regulates interferon-stimulated genes through PKR activation [Sledz et al. 2003]. Further, expression profiling performed in cultured mammalian cells treated by siRNA also revealed non-specific, concentration-dependent global changes of gene expression [Persengiev et al. 2004]. However, our result should not rule out the many known selective effects of siRNA [Chi et al. 2003; Semizarov et al. 2003] as we also have demonstrated the selective effect of anti β-actin siRNA on the actin cytoskeltion of microglial cells.

Briefly data presented here demonstrate that naked siRNA can be directly taken up by N9 cells and mainly localized to the endosomes. The uptake efficiency and cellular distribution of siRNA is similar with that of ssDNA. siRNA has a number of cellular binding proteins. Most of these binding proteins belong to the dsRNA binding protein family. Most notable is the direct interaction of siRNA with PKR. Furthermore, siRNA can not only bind PKR in vitro but also activate PKR in vivo in N9 cells. siRNAs have, in addition to their specific effects, broad effects beyond the selective silencing of target genes. This is very important, as high specificity is of critical importance for siRNA either serving as a genomic tool or a therapeutic candidate.

References

Arnott, D., O'Connell, K.L., King, K.L., Stults, J.T., 1998. An integrated approach to proteome analysis: identification of proteins associated with cardiac hypertrophy. Anal. Biochem. 258, 1-18.

Belanger, G., Stocksley, M.A., Vandromme, M., Schaeffer, L., Furic, L., DesGroseillers, L., Jasmin, B.J., 2003. Localization of the RNA-binding proteins Staufen1 and Staufen2 at the mammalian neuromuscular junction. J. Neurochem. 86, 669-677.

Blank, M., Weinschenk, T., Priemer, M., Schluesener, H., 2001. Systematic evolution of a DNA aptamer binding to rat brain tumor microvessels: selective targeting of endothelial regulatory protein pigpen. J. Biol. Chem. 276, 16464-16468.

Brummelkamp, T.R., Bernards, R., Agami, R., 2002. A system for stable expression of short interfering RNAs in mammalian cells. Science 296, 550-553.

Caplen, N.J., Parrish, S., Imani, F., Fire, A., Morgan, R.A., 2001. Specific inhibition of gene expression by small double-stranded RNAs in invertebrate and vertebrate systems. Proc. Natl. Acad. Sci. USA. 98, 9742-9747.

Capodici, J., Kariko, K., Weissman, D., 2002. Inhibition of HIV-1 infection by small interfering RNA-mediated RNA interference. J. Immunol. 169, 5196-5201.

Castanotto, D., Li, H., Rossi, J.J., 2002. Functional siRNA expression from transfected PCR products. RNA 8, 1454-1460.

Chi, J.T., Chang, H.Y., Wang, N.N., Chang, D.S., Dunphy, N., Brown, P.O., 2003. Genomewide view of gene silencing by small interfering RNAs. Proc. Natl. Acad. Sci. USA. 100, 6343-6346.

Clemens, M.J., Elia, A., 1997. The double-stranded RNA-dependent protein kinase PKR: structure and function. J. Interferon Cytokine Res. 17, 503-524.

Dykxhoorn, D.M., Novina, C.D., Sharp, P., 2003. Killing the messenger: short RNAs that silence gene expression. Nat. Rev. Mol. Cell Biol. 4, 457-467.

Elbashir, S.M., Harborth, J., Lendeckel, W., Yalcin, A., Weber, K., Tuschl, T., 2001. Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. Nature 411, 494-498.

Esco, M.A., Hazlett, L.D., Kurpakus-Wheater, M., 2002. Pseudomonas aeruginosa binds to extracellular matrix deposited by human corneal epithelial cells. Invest. Ophthalmol. Vis. Sci. 43, 3654-3659.

Ferrari, D., Villalba, M., Chiozzi, P., Falzoni, S., Ricciardi-Castagnoli, P., Di-Virgilio, F., 1996. Mouse microglial cells express a plasma membrane pore gated by extracellular ATP. J. Immunol. 156, 1531-1539.

Fraser, A.G., Kamath, R.S., Zipperlen, P., Martinez-Campos, M., Sohrmann, M., Ahringer, J., 2000. Functional genomic analysis of C. elegans chromosome I by systematic RNA interference. Nature 408, 325-330.

Gan, L., Ye, S., Chu, A., Anton, K.E., Yi, S., Vincent, V.A., Von Schack, D., Chin, D., Murray, J., Lohr, S., Patthy, L., Gonzalez-Zulueta, M., Nikolich, K., Urfer, R., 2003. Identification of cathepsin B as a mediator of neuronal death induced by abeta-activated microglial cells using a functional genomics approach. J. Biol. Chem. 279, 5565-5572.

Giri, R.K., Selvaraj, S.K., Kalra, V.K., 2003. Amyloid peptide-induced cytokine and chemokine expression in THP-1 monocytes is blocked by small inhibitory RNA duplexes for early growth response-1 messenger RNA. J. Immunol. 170, 5281-5294.

Hannon, G.J., 2002. RNA interference. Nature 418, 244-251.

Hommel, J.D., Sears, R.M., Georgescu, D., Simmons, D.L., DiLeone, R.J., 2003. Local gene knockdown in the brain using viral-mediated RNA interference. Nat. Med. 9, 1539-1544.

Isacson, R., Kull, B., Salmi, P., Wahlestedt, C., 2003. Lack of efficacy of 'naked' small interfering RNA applied directly to rat brain. Acta. Physiol. Scand. 179, 173-177.

Jackson, A.L., Bartz, S.R., Schelter, J., Kobayashi, S.V., Burchard, J., Mao, M., Li, B., Cavet, G., Linsley, P.S., 2003. Expression profiling reveals off-target gene regulation by RNAi. Nat. Biotechnol. 21, 635-637.

Kumar, M., Carmichael, G.G., 1998. Antisense RNA: function and fate of duplex RNA in cells of higher eukaryotes. Microbiol. Mol. Biol. Rev. 62, 1415-1434.

Lewis, D.L., Hagstrom, J.E., Loomis, A.G., Wolff, J.A., Herweijer, H., 2002. Efficient delivery of siRNA for inhibition of gene expression in postnatal mice. Nat. Genet 32, 107-108.

Makimura, H., Mizuno, T.M., Mastaitis, J.W., Agami, R., Mobbs, C.V., 2002. Reducing hypothalamic AGRP by RNA interference increases metabolic rate and decreases body weight without influencing food intake. BMC Neurosci. 3, 18-23.

Manche, L., Green, S.R., Schmedt, C., Mathews, M.B., 1992. Interactions between double-stranded RNA regulators and the protein kinase DAI. Mol. Cell Biol. 12, 5238-5248.

McManus, M.T., Sharp, P.A., 2002. Gene silencing in mammals by small interfering RNAs. Nat. Rev. Genet. 3, 737-747.

Miller, V.M., Xia, H., Marrs, G.L., Gouvion, C.M., Lee, G., Davidson, B.L., Paulson, H.L., 2003. Allele-specific silencing of dominant disease genes. Proc. Natl. Acad. Sci. USA 100, 7195-7200.

Paddison, P.J., Hannon, G.J., 2002. RNA interference: the new somatic cell genetics? Cancer Cell 2, 17-23.

Perkins, D.N., Pappin, D.J., Creasy, D.M., Cottrell, J.S., 1999. Probability-based protein identification by searching sequence databases using mass spectrometry data. Electrophoresis 20, 3551-3567.

Persengiev, S.P., Zhu, X., Green, M.R., 2004. Nonspecific, concentration-dependent stimulation and repression of mammalian gene expression by small interfering RNAs (siRNAs). RNA 10, 12-18.

Player, M.R., Torrence, P.F., 1998. The 2-5A system: modulation of viral and cellular processes through acceleration of RNA degradation. Pharmacol. Ther. 78, 55-113.

Saunders, L.R., Barber, G.N., 2003. The dsRNA binding protein family: critical roles, diverse cellular functions. FASEB J. 17, 961- 983.

Saxena, S., Jonsson, Z.O., Dutta, A., 2003. Small RNAs with imperfect match to endogenous mRNA repress translation. Implications for off-target activity of small inhibitory RNA in mammalian cells. J. Biol. Chem. 278, 44312-44319.

Scacheri, P.C., Rozenblatt-Rosen, O., Caplen, N.J., Wolfsberg, T.G., Umayam, L., Lee, J.C., Hughes, C.M., Shanmugam, K.S., Bhattacharjee, A., Meyerson, M., Collins, F.S., 2004. Short interfering RNAs can induce unexpected and divergent changes in the levels of untargeted proteins in mammalian cells. Proc. Natl. Acad. Sci. USA 101, 1892-1897.

Scherr, M., Battmer, K., Winkler, T., Heidenreich, O., Ganser, A., Eder, M., 2003. Specific inhibition of bcr-abl gene expression by small interfering RNA. Blood 101, 1566-1569.

Schneider, R., Schneider-Scherzer, E., Thurnher, M., Auer, B., Schweiger, M., 1988. The primary structure of human ribonuclease/angiogenin inhibitor (RAI) discloses a novel highly diversified protein superfamily with a common repetitive module. EMBO J. 7, 4151-4156.

Semizarov, D., Frost, L., Sarthy, A., Kroeger, P., Halbert, D.N., Fesik, S.W., 2003. Specificity of short interfering RNA determined through gene expression signatures. Proc. Natl. Acad. Sci. USA. 100, 6347-6352.

Shevchenko, A., Wilm, M., Vorm, O., Mann, M., 1996. Mass spectrometric sequencing of proteins silver-stained polyacrylamide gels. Anal. Chem. 68, 850-858.

Sledz, C.A., Holko, M., de Veer, M.J., Silverman, R.H., Williams, B.R., 2003. Activation of the interferon system by short-interfering RNAs. Nat. Cell Biol. 5, 834-839.

Aldskogius, H., 2001. Regulation of microglia - potential new drug targets in the CNS. Expert Opin. Ther. Targets 5, 655-668.

Sui, G., Soohoo, C., Affar, E.B., Gay, F., Shi, Y., Forrester, W.C., Shi, Y., 2002. A DNA vector-based RNAi technology to suppress gene expression in mammalian cells. Proc. Natl. Acad. Sci. USA 99, 5515-5520.

Tian, Q., Streuli, M., Saito, H., Schlossman, S.F., Anderson, P., 1991. A polyadenylate binding protein localized to the granules of cytolytic lymphocytes induces DNA fragmentation in target cells. Cell 67, 629-639.

Tijsterman, M., Ketting, R.F., Plasterk, R.H., 2002. The genetics of RNA silencing. Annu. Rev. Genet. 36, 489-519.

Trulzsch, B., Wood, M., 2004. Applications of nucleic acid technology in the CNS. J. of Neurochemistry 88, 257-265.

Wagner, J.D., Jankowsky, E., Company, M., Pyle, A.M., Abelson, J.N., 1998. The DEAH-box protein PRP22 is an ATPase that mediates ATP-dependent mRNA release from the spliceosome and unwinds RNA duplexes. EMBO J. 17, 2926-2937.

Xia, H., Mao, Q., Paulson, H.L., Davidson, B.L., 2002. siRNA-mediated gene silencing in vitro and in vivo. Nat. Biotechnol. 20, 1006-1010.

Zamore, P.D., 2001. RNA interference: listening to the sound of silence. Nat. Struct. Biol. 8, 746-750.

Zhang Z, Guo K, Schluesener HJ. 2005a. The immunostimulatory activity of CpG oligonucleotides on microglial N9 cells is affected by a polyguanosine motif. J Neuroimmunol. 161:68-77.

Zhang Z, Weinschenk T, Schluesener HJ. 2005b. Uptake, intracellular distribution, and novel binding proteins of immunostimulatory CpG oligodeoxynucleotides in microglial cells. J Neuroimmunol 160:32-40.

Chapter 6.

Microglia Activation in Rat Spinal Cord by Systemic Injection of TLR3 and TLR7/8 Agonists

Zhiren Zhang, Katrin Trautmann, and Hermann J. Schluesener

Institute of Brain Research, University of Tuebingen, Calwer Street 3, Tuebingen D-72076, Germany

Journal of Neuroimmunology 164, 154-160 (2005)

Abstract

Here we describe activation of microglia in the rat spinal cord by systemic injections of toll-like receptor agonist polyinosine-polycytidylic acid (poly (I:C), a TLR3 ligand) and R848 (a TLR7/8 ligand). A significant but transient increase of ED-1^+ spinal cord microglia was observed 4 days after a single intraperitoneal (i.p.) injection. Immunostainings by different microglial markers, AIF-1, EMAPII, OX6, $P2X_4$ receptor ($P2X_4R$) indicated that microglia were not fully activated and tracing of cell proliferation by 5-bromo-2´-deoxyuridine revealed only a small fraction of proliferating cells were microglia (less than 5%).

Thus, these stimulators of the innate immune system have, after peripheral administration, clearly effects on the innate immune system of the spinal cord. This should be considered in the design of clinical trials, as both TLR ligands have been used in patients. As injections of TLR ligands can be used to modulate immune activity in the spinal cord, such agents might be tools to modulate local regenerative processes in the spinal cord.

Key words: Polyinosine-polycytidylic acid, R848, Microglia, Spinal cord

1. Introduction

Poly (I:C) is a synthetic double-stranded RNA (dsRNA) that activates the innate immune response through TLR3 (Alexopoulou et al., 2001). Poly (I:C) is one if the most potent type I interferon (IFN) inducers and can activate monocytes to produce tumor necrosis factor-α, interleukin-6, interleukin-1β and interleukin-12 and promotes dendritic cell maturation (Akiyama et al., 1985; Manetti et al, 1995). It has already been used in clinical trials in cancer (Robinson et al., 1976; Salazar et al., 1996), chronic fatigue (Strayer et al., 1994) and human immunedeficiency virus infection (Carter et al., 1987; Gillespie et al., 1994). Further, it is also often used as a model of viral infection.

R848 (resiquimod; S-28463) is a guanine nucleotide analog and activates innate immune system selectively via TLR7/8 (Heil et al., 2004; Diebold et al., 2004). Similar to poly (I:C), R848 induces production of interferon-α, interleukin-12, tumor necrosis factor-α and other cytokines (Dockrell and Kinghorn, 2001; Buates and Matlashewski, 2001). It has been used in clinical trials of human papillomavirus infection and is considered in the therapy of cancers and as vaccine adjuvant (Sauder et al., 2003).

An innate immune response was often considered to engage peripheral lymphoid organs but not the central nervous system (CNS). In fact during systemic bacterial/viral infection, the nervous system and the immune system together mount a co-ordinated response to danger signals. These two systems communicate through intricate chemical messengers that are able to breach their independent and sequestered anatomical locations (Nguyen et al., 2002; Steinman, 2004). Regeneration induction in the spinal cord is essential for the recovery from spinal cord injury. Studies have revealed the local immunological/inflammatory process play an important role in regulating regeneration in the spinal cord (Popovich, 2002) and activated microglial cells were shown to promote the regeneration in the injured spinal cord (Prewitt et al., 1997). Our previous studies also observed microglia activation after spinal cord injury and, further, inflammation suppression after spinal cord injury

inhibits regeneration (Schwab et al., 2004; Schwab et al., 2000). It is important to find reagent that may influence local inflammatory process. Poly (I:C) and R848 are synthetic TLR ligands, which trigger host innate immunity through activation of TLR, and are potential therapeutics. Further, peripheral administration of poly (I:C) causes “sickness behaviour”, which is owed to the effects of its induced cytokines on CNS (Fortier et al., 2004; Katafuchi et al., 2003; Kent et al., 1992; Toien and Mercer, 1995). So it is interesting to know whether systemic administration of these reagents can affect the local inflammatory state in the spinal cord.

Microglia are the resident mononuclear phagocyte populations in the CNS. Under physiological conditions, these cells are quiescent and scattered throughout the CNS. The most characteristic feature of microglial cells is their rapid activation in response to signals from the inside as well as from the outside (Kreutzberg, 1996). Activated microglia change their ramified morphology into an amoeboid shape and up-regulate certain immunologically relevant molecules (Ayoub and Salm, 2003). In spinal cord, microglia are activated in response to a variety of peripheral stimuli, resulting from degeneration of central terminals of dying sensory neurons or through the release of substances by incoming sensory afferents or pain-responsive neurons in the dorsal horn (Watkins and Maier, 2002). Microglial cells play a key role in regulating the communication among neuronal and other types of glial cells and are very sensitive to changes in the CNS microenvironment and rapidly become activated in virtually all conditions that affect normal neuronal function (Nakamura, 2002; Piehl and Lidman, 2001). Here we have analyzed, whether a systemic injection of poly (I:C) or R848 results into stimulation of the spinal cord microglial cells.

2. Material and Methods

2.1. Animals

6-8 weeks old male Lewis rats (170-200 g, Charles River, Sulzfeld, Germany) were housed under standard laboratory conditions. Food and water were

available *ad libitum*. All procedures were performed in accordance with the published International Health Guidelines under a protocol approved by the University of Tuebingen Institutional Animal Care and Use Committee and the Administration District Official Committee.

Poly (I:C) (from InVivogen, San Diego, CA; 5 mg/kg rat) or R848 (from InVivogen, San Diego, CA; 1 mg/kg rat) were injected i.p. as a single bolus (3 rats per group). Control group (3 rats) received injection of the same volume of phosphate buffered saline (PBS). Rats survived for different times after injections according to the protocol of tissue sampling. For tracing of cell proliferation, rats were given i.p. injections of BrdU (from Serva, Heidelberg, Germany; 50 mg/kg rat), twice a day for two days before sacrifice. Then, rats were deeply anesthethized with diethylether and perfused intracardially with 4% paraformaldehyde (PFA) in PBS. Spinal cords were removed and post-fixed in 4% PFA overnight at 4°C. Spinal cords were divided into 8 mm segments and embedded in paraffin, serially sectioned (3 µm) and mounted on silan covered slides.

2.2. Immunhistochemistry

After deparaffination, slices of spinal cord sections were boiled (in a 600 W microwave oven) for 15 min in citrate buffer (2.1 g sodium citrate/L, pH 6). Endogenous peroxidase was inhibited with 1% H_2O_2 in methanol for 15 minutes. Slices were incubated with 10% normal pig serum (Biochrom, Berlin, Germany) to block non-specific binding of immunoglobulins and were then exposed to the following mouse monoclonal antibodies: ED-1 (1:100; anti-rat, Serotec, Oxford, Great Britain), allograft inflammatory factor 1 (AIF-1; described previously: Schluesener et al., 1998), OX-6 to class MHCII (1:100; Serotec, Oxford, Great Britain), EMAPII (1:100; BMA, Augst, Switzerland), or the rabbit antibodies, $P2X_4R$ (1:200; Alomone Labs, Jerusalem, Israel) respectively to detect microglia activation, and the mouse anti-BrdU antibodies (BD Primagen, 1:100) to detect proliferating cells.

Antibody binding to tissue sections was visualized with a biotinylated swine anti-rabbit (DAKO, Hamburg, Germany) or rabbit anti-mouse IgG $F(ab)_2$

antibody fragment. Subsequently sections were incubated with a Streptavidin-Avidin-Biotin complex (DAKO, Hamburg, Germany), followed by development with diaminobenzidine (DAB) substrate (Fluka, Neu-Ulm, Germany). Finally, sections were counterstained with hematoxylin.

2.3. Double Staining

In double labelling experiments, spinal cord slices were pretreated as described above and then incubated with the appropriate monoclonal antibody. Visualization was achieved by adding secondary antibody (biotinylated rabbit anti-mouse IgG) at a dilution of 1:400 in TBS-BSA for 30 min and alkaline phosphatase conjugated ABC complex diluted 1:400 in Tris-BSA for 30 min. Consecutively, immunostaining was developed with Fast Blue BB salt chromogen-substrate solution. Then slices were once more irradiated in a microwave for 15 min in citrate buffer and were immunolabeled as described above, but by omission of counterstaining with hematoxylin.

BrdU-incorporation by activated microglia was detected by double labeling with antibodies directed against ED-1 and BrdU (see above).

2.4. Evaluation and Statistical Analysis

Spinal cord tissue sections were arranged in groups of eight to twelve sections of different levels of spinal cord placed on each slide. After immunostaining, sections were examined under light microscopy. The grey or white matter area of spinal cord was quantified by using AxioVision LE Rel. 4.1 (Carl Zeiss Vision GmbH). Results are given as arithmetic means of positive cells per square millimetre (mm^2) and standard errors of means (SEM). Statistical analysis was performed by one-way ANOVA followed by Dunnett´s Multiple Comparison test (Graph Pad Prism 4.0 software). For all statistical analyses, significance levels were set at $p < 0.05$.

3. Results

3.1. Microglia activation in the spinal cord after Poly (I:C) or R848 injection

To asses the effects of poly (I:C) and R848 on microglia in the rat spinal cord, these TLR-ligands were i.p. injected and microglial activation quantified by immunostaining with the lysosomal marker ED-1. Only positive cells with the visible nucleus at the focal plane were counted. Upon activation, microglial cells transform from the ramified morphology to a rounded (amoeboid) macrophage-like morphology (Ayoub and Salm, 2003). As shown in Figure 1D, in our study typical microglia with ED-1 staining exhibited a hypertrophic morphology with thick processes which could easily be distinguished from the elongated perivascular cells (PVC) situated adjacent to the basement membrane of medium to small vessels (Figure 1E).

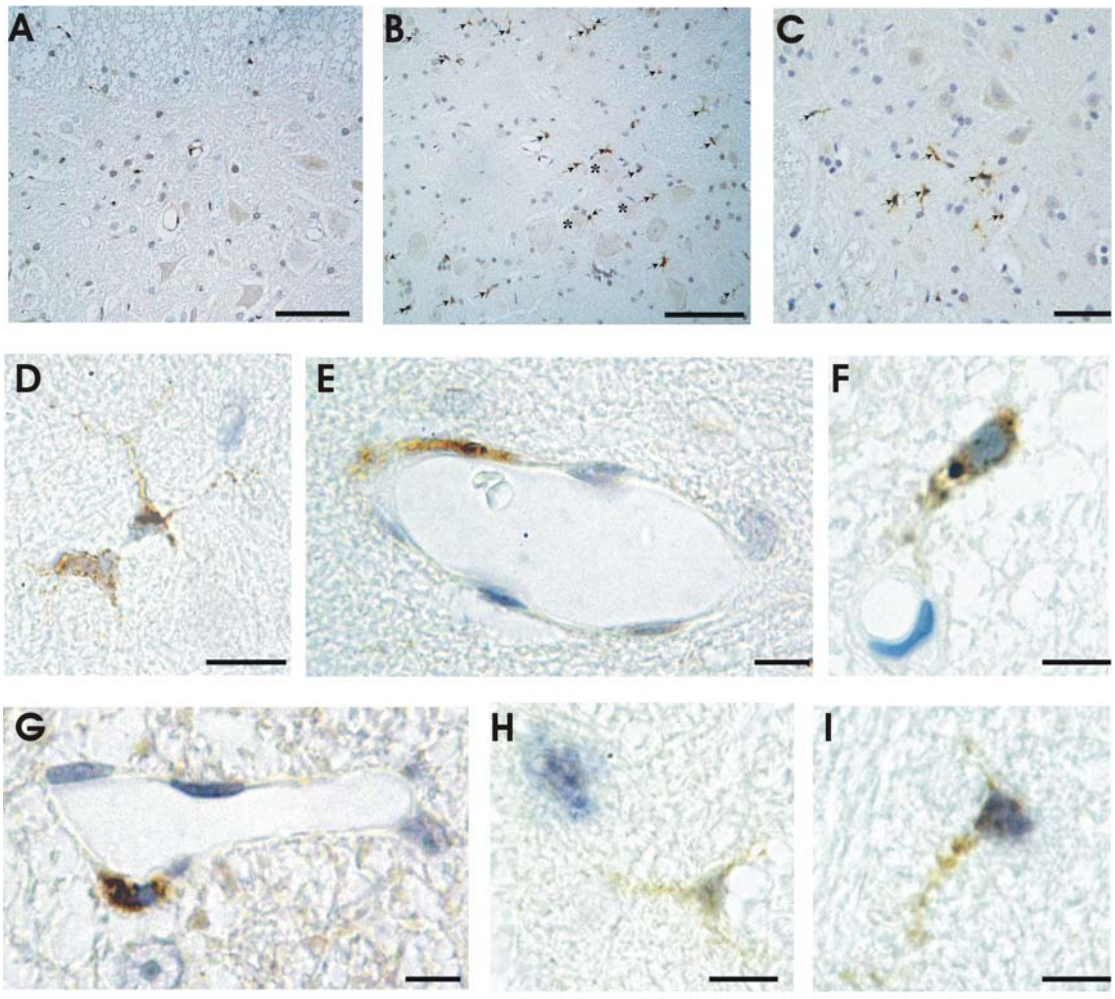
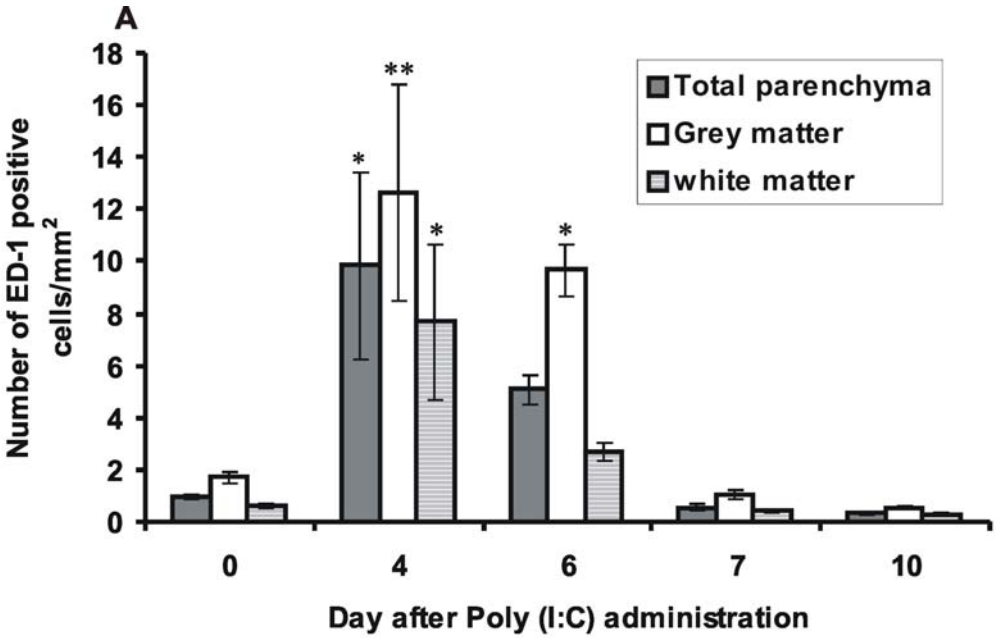


Fig. 1. **Microglial immunostaining in rat spinal cord 4 days after peripheral administration of poly (I:C) or R848 by antibodies to ED-1 (A, B, C, D, E), EMAPII (F), $P2X_4R$ (G) and double-staining by antibodies to ED-1 and BrdU (H, I).** A, B, C: ED-1 immunostaining of rat spinal cord 4 day after ip injection of PBS (A), poly (I:C) (B) or R848 (C). Brown, arrow indicated cells are microglia. Activated microglial cells were observed in the vicinity of large motoneurons (asterisk, motoneurons). D: 4 days after peripheral administration of poly (I:C) or R848, rat spinal cord microglia exhibited a hypertrophic morphology characterized by short, thick process with ED-1 immunostaining. E: ED-1^+ PVC could easily distinguish from the ED-1^+ microglia. F: A few EMAPII$^+$ (brown) microglial cells could be observed in the parenchyma of spinal cord 4 days after ip injection of poly (I:C) or R848. G: In the spinal cord of control, poly (I:C) or R848 treated rats, $P2X_4R$ (brown) expression was only observed on the PVCs. H, I: ED-1 and BrdU double lanelling of rats spinal cord 4 days after poly (I:C) or R848 administration. Rats were given 4 times ip injection of BrdU (twice a day, 50 mg/kg rat) before sacrifice. Most proliferative cells were not ED-1^+ cells (H; BrdU, blue; ED-1, brown). Co-localization of BrdU and ED-1 could be observed but only on a small part of cells (I; blue, BrdU; brown, ED-1). Scale bars: A, B, C = 100 μm, D, E, F, G, H, I = 10 μm.

For poly (I:C) treated group, significant ED-1^+ microglia was observed 4 days post injection in the whole parenchyma of spinal cord (Figure 1A and B, average density: control group, 0.95 ± 0.05 cells/mm^2; poly (I:C) treatment group, 9.84 ± 3.59 cells/mm^2 and $p < 0.05$). The density of ED-1^+ microglial cells started to decrease by day 6 post injection (5.1 ± 0.56 cells/mm^2) and then declined to the baseline level by day 7 (0.55 ± 0.13 cells/mm^2). Density of ED-1^+ microglia was higher in grey matter than in white matter over the whole time course (Figure 2A). At day 4, ED-1^+ microglia density in gery and white matter was 12.65 ± 4.16 cells/mm^2 ($p < 0.01$, compared with control group) and 7.69 ± 2.98 cells/mm^2 ($p < 0.05$, compared with control group), respectively. In the ventral grey matter, ED-1^+ microglial cells could be observed in the vicinity of large motoneurons (Fig. 1B). The number of ED-1^+ microglial cell was higher in lumbar than in cervical or thoracic regions (Figure 3A, 3B, 3C). Four days after poly (I:C) injection, the density of ED-1^+ microglial cells in different regions was 9.2 ± 3.13 cells/mm^2 (cervical), 8.19 ± 3.51 cells/mm^2 (thoracic) and 11.74 ± 3.99 cells/mm^2 (lumbar) in the whole parenchyma, 10.45 ± 3.03 cells/mm^2 (cervical), 9.67 ± 2.65 cells/mm^2 (thoracic) and 16.36 ± 5.97 cells/mm^2 (lumbar) in the grey matter, 6.54 ± 2.25 cells/mm^2 (cervical), 8.34 ± 4.63 cells/mm^2 (thoracic) and 9.05 ± 3.23 cells/mm^2 (lumbar) in the white matter. In the R848 treated group, the number of ED-1^+ microglial cells was higher in the thoracic than in the cervical or the lumbar (Figure 3D, 3E, 3F), which is different from the poly (I:C) treated group.

A similar ED-1^+ microglia pattern was observed in the R848 treatment group. The maximal ED-1^+ microglia density was observed 4 days after R848

administration (Figure 1A and C, 3.42 ± 0.55 cells/mm^2, p < 0.01) and remained significantly elevated until day 5 post injection (2.36 ± 0.27 cells/mm^2, p < 0.05) and then declined to baseline level by day 6 (Figure 2B). Similar to the poly (I:C) treated group, density of ED-1$^+$ microglia in the R848 treated group was higher in grey matter than in white matter over the time course studied (Figure 2B). On day 4 post injection, ED-1$^+$ microglial cell density in grey and white matter was 3.59 ± 0.62 cells/mm^2 (p < 0.05) and 2.48 ± 0.55 cells/mm^2 (p < 0.01), separately. ED-1$^+$ microglial cells also could be observed in the vicinity of large motoneurons in the ventral grey matter.



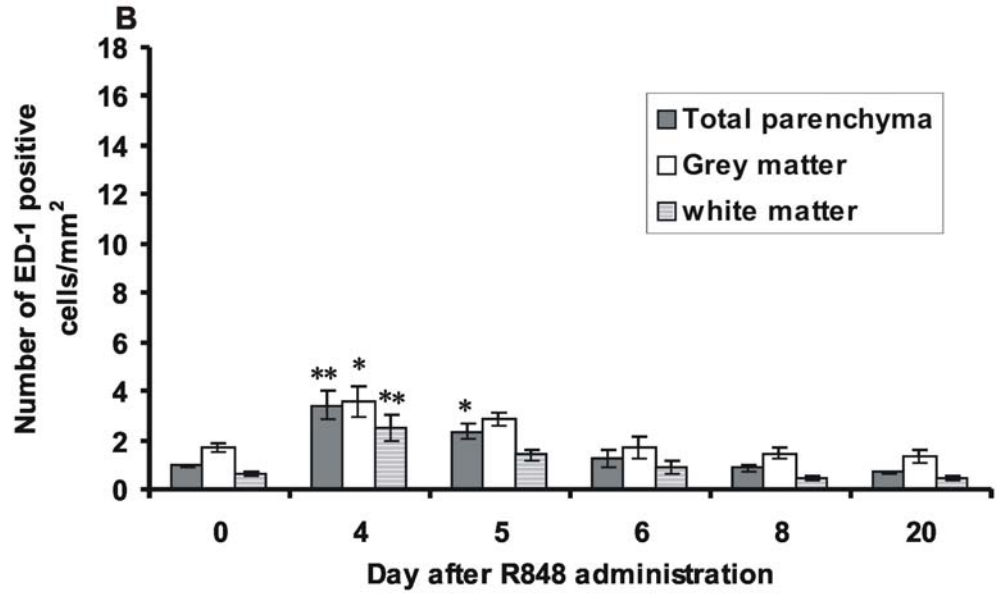


***Fig. 2.* ED-1$^+$ microglial cell distribution in the whole parenchyma, grey matter and white matter after poly (I:C) or R848 administration.** Upper panel represents the poly (I:C) treated groups (A) and the lower panel represents the R848 treated groups (B). Error bars represent means ± SEM, n=3. *$p < 0.05$, ** $p < 0.01$ vs. respective controls (Dunnett's test after one-way ANOVA).

Furthermore, microglia activation pattern in spinal cord of poly (I:C) or R848 treated rats was analyzed using a panel of other antibodies. Allograft inflammatory factor-1 (AIF-1) was implicated in the initiation of the early microglial response preceding the phagocytic state (Schwab et al., 2001). AIF-1 expression was not observed in poly (I:C) or R848 treated rat spinal cords at any time studied. Definition of microglia activation was further refined using monoclonal antibodies against OX6 or endothelial monocyte activating polypeptide II (EMAPII). OX6 is a marker for the major histocompatibility complex class II molecules (MHCII) and EMAPII is a proinflammatory cytokine connected with microglial/macrophage activation in autoimmune inflammation of the CNS (Schluesener et al., 1997). OX6 expression in spinal cord was not observed at any time in the spinal cords of poly (I:C) or R848 treated rats. A few EMAPII$^+$ cells were only observed on days 4 after poly (I:C) (0.55 ± 0.05 cells/mm^2) or R848 (0.58 ± 0.09 cells/mm^2) injection (Figure 1F), but data are not significantly different from the control group ($p > 0.05$).

The expression of $P2X_4R$, a recently discovered ATP-gated ion channel expressed by activated microglia/macrophage, involved in pain signaling after peripheral nerve injury, was also analyzed. But $P2X_4R$ expression was only observed on the PVCs (Figure 1G) but not on microglial cells at any time studied, which is similar to the control group.

3.2. Microglial cell proliferation in the spinal cord after Poly (I:C) or R848 peripheral administration

Microglia cell proliferation was analyzed in spinal cords of rats, which had received BrdU-injections in addition to TLR-ligands to trace proliferating cells. Proliferative cells in the spinal cord were further double labeled with ED-1 and BrdU. Double-labeled cells were analyzed on day 4 post poly (I:C) or R848 injection (Figure 1H, I) but only a small fraction of BrdU$^+$ cells were ED-1$^+$ microglia (less than 5%) in both poly (I:C) and R848 treated rats.

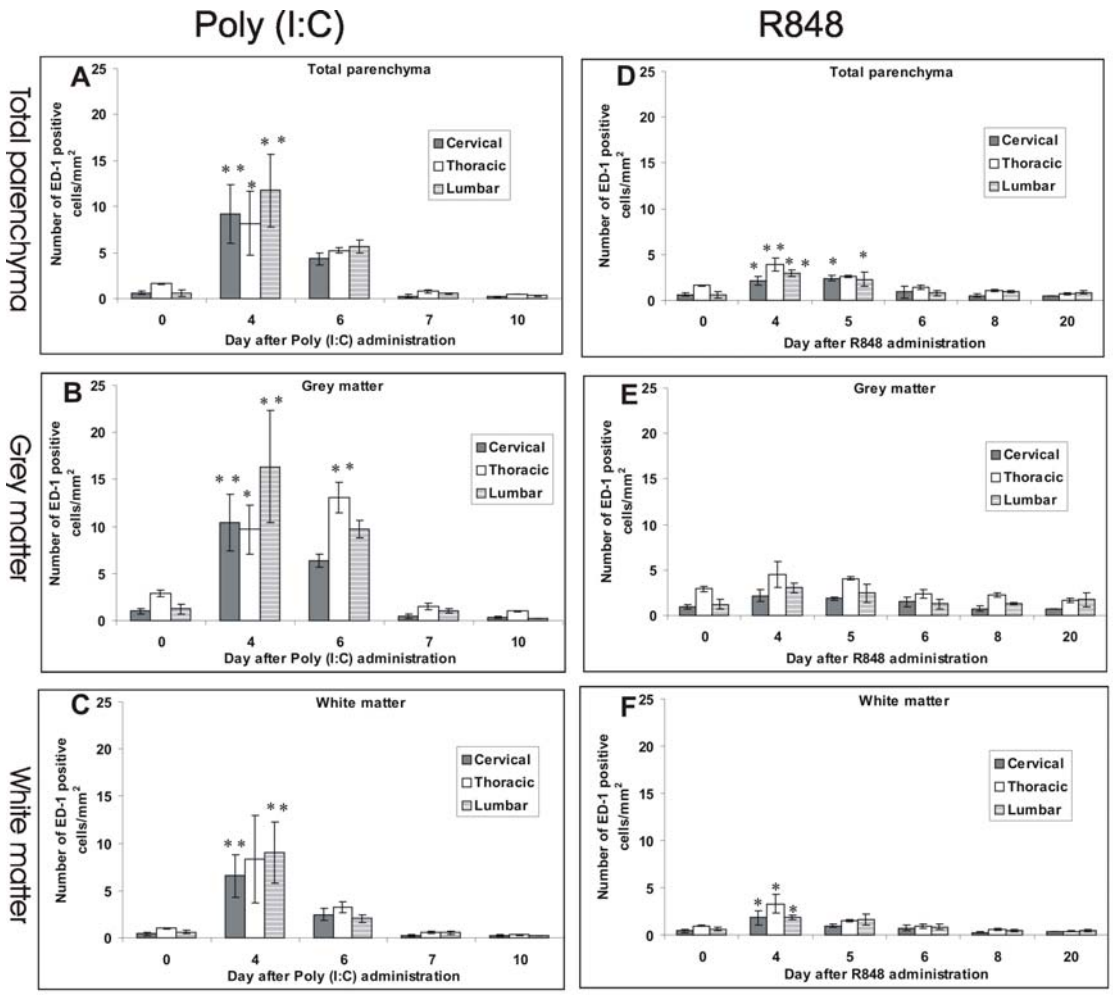


Fig. 3. **The distribution of ED-1$^+$ microglial cell in cervical, thoracic and lumbar regions of the spinal cord after poly (I:C) or R848 injection.** Error bars represent means ±SEM, n=3. *p< 0.05, ** p < 0.01 vs. respective controls (Dunnett's test after one-way ANOVA).

4. Discussion

Immune system and CNS have close interactions. There are several routes by which peripheral cytokines can either cross the BBB or signal to the CNS through other signalling molecules (Dantzer, 2001; Licinio and Wong, 1997). Further, the expression of a cluster of TLRs in microglia and astrocytes indicates an intact innate immune system in the CNS (Bsibsi et al., 2002). Local inflammation can regulate the regeneration in the injury spinal cord and activated microglia might play a role in such processes. It is important to find reagents that might influence the local inflammatory state in the spinal cord. Poly (I:C) and R848 are TLRs ligands and potential therapeutics. Peripheral

administration of poly (I:C) or R848 not only induces certain kinds of cytokines, which involve in defensive innate immune response, but also causes “sickness behaviour”, which is owed to the effects of its induced cytokines on CNS (Fortier et al., 2004; Katafuchi et al., 2003; Kent et al., 1992; Toien and Mercer, 1995). In this study, we analyzed microglia response in the spinal cord of rats after i.p. injection of poly (I:C) or R848.

Microglia are highly abundant throughout the CNS and are considered as sensors of many kinds of pathological alterations (Kreutzberg, 1996). The main function of microglia are believed to be defensive but contribute to the onset of or to exacerbation of in many diseases (Nakajima and Kohsaka, 2004). Under physiological conditions, they are quient cells with no recognised functions and characterized by a ramified morphology along with downregulated immunophenotype (Kreutzberg, 1996). Under pathological conditions, microglial cells become reactive (Ayoub and Salm, 2003). Microglia activation process is a graded phenomenon, characterized by morphology change (retracted processes and hytrophy; amoeboid morphology under strongly pathological envieonment), proliferation, increased expression of certain cell markers (such as MHCII), production/secreation of cytokines and/or changes in functional activities (such as phagocytosis) (Mertsch et al., 2001; Watkins and Maier, 2003). And the changes in cell markers, cytokine production can occur without the morphological change or proliferation (Watkins and Maier, 2003).

In our study, a significant but transient increase of ED-1$^+$ spinal cord microglia was observed 4 days after a single i.p. injection of poly (I:C) or R848. ED-1 stains CD68, a lysosomal membrane protein, which is mainly found on phagocyting macrophages and reactive microglia (Damoiseaux et al., 1994; Walker, 1998). ED-1 staining has been widely applied to show microglia activation (Beiter et al., 2005; Beschorner et al., 2002). Up-regulation of CD68 indicates that cells are phagocytically active because they accumulate lysosomal vacuoles (Walker, 1998). Though phagocytical activity of spinal cord microglia significant increased 4 day after poly (I:C) or R848 peripheral administration activated microglial cells showed a ramified morphology (Figure 1D). Further, few activated microglia expressed MHCII (detected by OX6

antibody) or EMAPII, a cytokine that is associated with the late activation cascade of microglia (Schluesener et al., 1997). These data suggeste that the microglia are not fully activated. Similar phenomenon was reported by Beiter et al (Beiter et al., 2005) and Liu et al (Liu et al., 1998). They observed incomplete rat spinal cord microglia activation in experimental autoimmune neuritis and speculated these cells might prepare for evens that finally do not develop. But the exact role of these incompletely activated microglia remain elusive.

$P2X_4R$ is an ATP-gated ion channel and has been demonstrated to increase expression in spinal cord injury (Inoue et al., 2004). Tsuda and colleagues found that $P2X_4R$ in microglia was involved in pain signaling (Tsuda et al., 2003). However, in our experiments, $P2X_4R$ expression was restricted to few PVCs and did not occur in parenchymal microglia.

Briefly, a significant but transient increase of incompletely activated microglia was induced in rat spinal cord after poly (I:C) or R848 peripheral administration. Though the exact role of these cells remains elusive this should be considered in the design of clinical trials, as both TLR ligands have been used in patients.

References

Akiyama, Y., Stevenson, G.W., Schlick, E., Matsushima, K., Miller, P.J., Stevenson, H.C., 1985. Differential ability of human blood monocyte subsets to release various cytokines. J. Leukocyte Biol. 37, 519-523.

Alexopoulou, L., Holt, A.C., Medzhitov, R., Flavell, R.A., 2001. Recognition of double-stranded RNA and activation of NF-kappaB by Toll-like receptor 3. Nature 413, 732-738.

Ayoub, A.E., Salm, A.K., 2003. Increased morphological diversity of microglia in the activated hypothalamic supraoptic nucleus. J. Neurosci. 23, 7759-7766.

Beiter, T., Artel, M.R., Traumann, K., Schlusener, H.J., 2005. Experimental autoimmune neuritis induces differential microglia activation in the rat spinal cord. J. Neuroimmunol. 160, 25-31.

Beschorner, R., Nguyen, T.D., Gözalan F., Pedal, I., Mattern, R., Schluesener, H.J., Meyermann, R., Schwab, J.M., 2002. CD14 expression by activated parenchymal microglia/macrophages

and infiltrating monocytes following human traumatic brain injury. Acta. Neuropathol. 103; 541–549.

Bsibsi, M., Ravid, R., Gveric, D., van Noort, J.M., 2002. Broad expression of toll-like receptors in the central nervous system. J. Neuropathol. Exp. Neurol. 61, 1013-1021.

Buates, S., Matlashewski, G., 2001. Identification of genes induced by a macrophage activator, S-28463, using gene expression array analysis. Antimicrob. Agents Chemother. 45, 1137-1142.

Carter, W.A., Strayer, D.R., Brodsky, I., Lewin, M., Pellegrino, M.G., Einck, L., Henriques, H.F., Simon, G.L., Parenti, D.M., Scheib, R.G., 1987. Clinical, immunological, and virological effects of ampligen, a mismatched double-stranded RNA, in patients with AIDS or AIDS-related complex. Lancet. I, 1286-1292.

Damoiseaux, J.G., Dopp, E.A., Calame, W., Chao, D., MacPherson, G.G., Dijkstra, C.D., 1994. Rat macrophage lysosomal membrane antigen recognized by monoclonal antibody ED1. Immunology 83, 140-147.

Dantzer, R., 2001. Cytokine-induced sickness behavior: where do we stand? Brain. Behav. Immun. 15, 7-24.

Diebold, S.S., Kaisho, T., Hemmi, H., Akira, S., Reis, e Sousa, C., 2004. Innate antiviral responses by means of TLR7-mediated recognition of single-stranded RNA. Science 303, 1529-1531.

Dockrell, D.H., Kinghorn, G.R., 2001. Imiquimod and resiquimod as novel immunomodulators. J. Antimicrob. Chemother. 48, 751-755.

Fortier, M.E., Kent, S., Ashdown H., Poole S., Boksa., P., Luheshi G.N., 2004. The viral mimic, polyinosinis:polycytidylic acid (Poly I:C), induces fever in rats via an interleukin-1 dependent mechanism. Am. J. Physiol. Regul. Integr. Comp. Physiol. 287, R759-766.

Gillespie, D., Hubbell, H.R., Carter, W.A., Midgette, P., Elsasser, W., Mullaney, R., Strayer, D.R., 1994. Synergistic inhibition of AZT-resistant HIV by AZT combined with poly(I):poly(C12U) without synergistic toxicity to bone marrow progenitor cell elements. In Vivo 8, 375-380.

Heil, F., Hemmi, H., Hochrein, H., Ampenberger, F., Kirschning, C., Akira, S., Lipford, G., Wagner, H., Bauer, S., 2004. Species-specific recognition of single-stranded RNA via toll-like receptor 7 and 8. Science 303, 1526-1529.

Inoue, K., Tsuda, M., Koizumi, S., 2004. ATP- and adenosine-mediated signaling in the central nervous system: chronic pain and microglia: involvement of the ATP receptor P2X4. J. Pharmacol. Sci. 94, 112-114.

Katafuchi, T., Kondo, T., Yasaka, T., Kubo, K., Take, S., Yoshimura, M., 2003. Prolonged effects of polyriboinosinic:polyribocytidylic acid on spontaneous running wheel activity and brain interferon-alpha mRNA in rats: a model for immunologically induced fatigue. Neuroscience 120, 837-845.

Kent, S., Bluthe, R.M., Kelley, K.W., Dantzer, R., 1992. Sickness behavior as a new target for drug development. Trends Pharmacol. Sci. 13, 24-28.

Kreutzberg, G.W., 1996. Microglia: a sensor for pathological events in the CNS. Trends Neurosci. 19, 312-318.

Licinio, J., Wong, M.L., 1997. Pathways and mechanisms for cytokine signaling of the central nervous system. J. Clin. Invest. 100, 2941-2947.

Liu, L., Persson, J.K., Svensson, M., Aldskogius, H., 1998. Glial cell responses, complement, and clusterin in the central nervous system following dorsal root transection. Glia 23, 221-238.

Manetti, R., Annunziato, F., Tomasevic, L., Gianno, V., Parronchi, P., Romagnani, S., Maggi, E., 1995. Polyinosinic acid:polycytidylic acid promotes T helper type 1-specific immune responses by stimulating macrophage production of interferon-a and interleukin-12. Eur. J. Immunol. 25, 2656-2661.

Mertsch, K., Hanisch, U.K., Kettenmann, H., Schnitzer, J., 2001. Characterization of microglial cells and their response to stimulation in an organotypic retinal culture system. J. Comp. Neurol. 431, 217-227.

Nakajima, K., Kohsaka, S., 2004. Microglia: neuroprotective and neurotrophic cells in the central nervous system. Curr. Drug Targets Cardiovasc. Haematol. Disord. 4, 65-84.

Nakamura, Y., 2002. Regulating factors for microglial activation. Biol. Pharm. Bull. 25, 945-953.

Nguyen, M.D., Julien, J.P., Rivest, S., 2002. Innate immunity: the missing link in neuroprotection and neurodegeneration? Nat. Rev. Neurosci. 3, 216-227.

Piehl, F., Lidman, O., 2001. Neuroinflammation in the rat--CNS cells and their role in the regulation of immune reactions. Immunol. Rev. 184, 212-225.

Popovich, P.G., 2000. Immunological regulation of neuronal degeneration and regeneration in the injured spinal cord. Prog. Brain Res. 128, 43-58.

Prewitt, C.M., Niesman, I.R., Kane, C.J., Houle, J.D., 1997. Activated macrophage/microglial cells can promote the regeneration of sensory axons into the injured spinal cord. Exp. Neurol. 148, 433-443.

Robinson, R.A., DeVita, V.T., Levy, H.B., Baron, S., Hubbard, S.P., Levine, A.S., 1976. A phase I±II trial of multiple-dose polyriboinosic±polyribocytidylic acid in patients with leukemia or solid tumors. J. Natl Cancer Inst. 57, 599-603.

Salazar, A.M., Levy, H.B., Ondra, S., Kende, M., Scherokman, B., Brown, D., Mena, H., Martin, N., Schwab, K., Donovan, D., Dougherty, D., Pulliam, M., Ippolito, M., Graves, M., Brown, H., Ommaya, A., 1996. Long-term treatment of malignant gliomas with intramuscularly administered polyinosinic±polycytidylic acid stabilized with polylysine and carboxymethyl-cellulose: an open pilot study. Neurosurgery 38, 1096-1101.

Sauder, D.N., Smith, M.H., Senta-McMillian, T., Soria, I., Meng, T.C., 2003. Randomized, single-blind, placebo-controlled study of topical application of the immune response modulator resiquimod in healthy adults. Antimicrob. Agents Chemother. 47, 3846-3852.

Schluesener, H.J., Seid, K., Kretzschmar, J., Meyermann, R., 1998. Allograft-inflammatory factor-1 in rat experimental autoimmune encephalomyelitis, neuritis, and uveitis: expression by activated macrophages and microglial cells. Glia 24, 244-251.

Schluesener, H.J., Seid, K., Zhao, Y., Meyermann, R., 1997. Localization of endothelial-monocyte-activating polypeptide II (EMAP II), a novel proinflammatory cytokine, to lesions of experimental autoimmune encephalomyelitis, neuritis and uveitis: expression by monocytes and activated microglial cells. Glia 20, 365-372.

Schwab, J.M., Conrad, S., Elbert, T., Trautmann, K., Meyermann, R., Schluesener, H.J., 2004. Lesional $RhoA^{+}$ cell numbers are suppressed by anti-inflammatory, cyclooxygenase-inhibiting treatment following subacute spinal cord injury. Glia 47, 377-386.

Schwab, J.M., Frei, E., Klusman, I., Schnell, L., Schwab, M.E., Schluesener, H.J., 2001. AIF-1 expression defines a proliferating and alert microglial/macrophage phenotype following spinal cord injury in rats. J. Neuroimmunol. 119, 214-222.

Schwab, J.M., Brechtel, K., Nguyen, T.D., Schluesener, H.J., 2000. Persistent accumulation of cyclooxygenase-1 (COX-1) expressing microglia/macrophages and upregulation by endothelium following spinal cord injury. J. Neuroimmunol. 111, 122-130.

Steinman, L., 2004. Elaborate interactions between the immune and nervous systems. Nat. Immunol. 5, 575-581.

Strayer, D.R., Carter, W.A., Brodsky, I., Cheney, P., Peterson, D., Salvato, P., Thompson, C., Loveless, M., Shapiro, D.E., Elsasser, W., 1994. A controlled clinical trial with a specifically configured RNA drug, poly(I).poly(C12U), in chronic fatigue syndrome. Clin. Infect. Dis. 18 (Suppl. 1), S88-S93.

Toien, O., Mercer, J.B., 1995. Poly I:C-induced fever elevates threshold for shivering but reduces thermosensitivity in rabbits. Am. J. Physiol. 268, R1266-R1272.

Tsuda, M., Shigemoto-Mogami, Y., Koizumi, S., Mizokoshi, A., Kohsaka, S., Salter, M.W., Inoue, K., 2003. P2X4 receptors induced in spinal microglia gate tactile allodynia after nerve injury. Nature 424, 778-783.

Walker, D.G., 1998. Wood, P.L. (Ed.). Inflammatory markers in chronic neurodegenerative disorders with emphasis on Alzheimer' disease. Neuroinflammation, mechanism and management. Totowa, Humana Press, Inc., pp 61-90.

Watkins, L.R., Maier, S.F., 2003. Glia: a novel drug discovery target for clinical pain. Nat. Rev. Drug Discov. 2, 973-985.

Watkins, L.R., Maier, S.F., 2002. Beyond neurons: evidence that immune and glial cells contribute to pathological pain states. Physiol. Rev. 82, 981-1011.

List of publications

Full articles:

1. **Zhang Z.**, Trautman K., Artelt M. and Schluesene H.J. 2006. Lesional accumulation of $P2X_4$ receptor$^+$ monocytes after experimental brain injury. *Experimental Neurology.* 197, 252-257.

2. **Zhang Z.**, Trautman K., Artelt M. and Schluesene H.J. 2006. Bone morphogenetic protein-6 up-expression in early-activated astrocytes following traumatic brain injury. *Neuroscience*. 138, 47-53.

3. **Zhang Z.**, Weinschenk T. and Schluesener H.J. 2006. siRNA binding proteins of microglial cells: PKR is an unanticipated ligand. *Journal of Cellular Biochemistry*. 97, 1217-1229.

4. **Zhang Z.**, Trautman K., Artelt M. and Schluesene H.J. 2006. Early infiltration of CD8+ microglia/macrophages to lesion locates following traumatic brain injury. *Neuroscience*. (In press).

5. Li XB, **Zhang Z**, Schluesener HJ, Xu SQ. 2006. Exosome and its applications in tumor immunotherapy. *Journal of Cellular and Molecular Medicine*. 10, 364-375. (Equal first author, review).

6. Su Y.H., **Zhang Z.,** Trautman K. and Schluesener H.J. 2005. Stimulators of the innate immune system, TLR and NOD2 ligands, induce cell proliferation in the rat intact spinal cord. *Journal of Neuropathology and Experimental Neurology* 64, 991-997*.* (Equal first author).

7. **Zhang Z.**, Trautman K. and Schluesener H.J. 2005. Microglia activation in rat spinal cord by systemic injection of TLR 3 and TLR7/8 agonists. *Journal of Neuroimmunology* 164, 154-160.

8. **Zhang Z.**, Trautman K. and Schluesener H.J. 2005. Repeated ip injection of poly (I:C) activates spinal cord microglia and astrocyte in rat. *Neuroreport* 16, 1495-1499.

9. **Zhang Z.**, Guo K.T. and Schluesener H.J. 2005. The immunostimulatory activity of CpG-oligonucleotides on microglia cells is affected by polyguanosine motif. *Journal of Neuroimmunology* 161, 68-77.

10. **Zhang Z.**, Weinschenk T. and Schluesener H.J. 2005. Uptake, Intracellular Distribution and Novel Ligands of Immunostimulatory CpG Oligodeoxynucleotides in Microglial Cells. *Journal of Neuroimmunology* 160, 32-40.

11. **Zhang Z.** and Schluesener H.J. 2005. Protein pigpen is one of the cellular binding proteins of therapeutic oligonucleotides. *Cytotherapy* 7, 186-194.

12. **Zhang Z.**, Weinschenk T. and Schluesener H.J. 2005. Uptake, Intracellular Distribution and Novel Ligands of Immunostimulatory CpG Oligodeoxynucleotides in Glioblastom Cells. *Molecular and Cellular Biochemistry* 272, 35-46.

13. Li X., **Zhang Z.**, Beiter T., Schluesener H.J. 2005. Nanovesicular vaccines: exosomes. *Arch Immunol Ther Exp (Warsz).* 53, 329-335. (Review)

14. **Zhang Z.**, Blank M. and Schluesener H.J. 2004. Nucleic Acid Aptamers in Human Viral Disease. *Arch. Immunol. Ther. Exp. (Warsz)* 52, 307-315. (Review)

15. Schittenhelm J., **Zhang Z.**, Guo K.T., Trautmann K., Iglesias-Rozas J.R., Meyermann R., Schluesener H.J.. Frizzled9 Expression in Primitive Neuroectodermal Tumours (Submitted, equal first author).

16. **Zhang Z.,** Schittenhelm J., Guo K., Buhring H.J., Trautmann K., Meyermann R., and Schluesener H.J. Frizzled 9 (FZD9) is upregulated in human astrocytomas. *Neuropathology and Applied Neurobiology*. (In press).

Posters and abstract:

1. Zhang Z., Weinschenk T. and Schluesener H.J. Uptake, Intracellular Distribution and Novel Ligands of Immunostimulatory CpG Oligodeoxynucleotides in Microglial Cells. Poster Seminar of the Graduate College of "Cellular mechanisms of immune-associated processes", GK 794, July 2003, Tuebingen, Germany.

2. Zhang Z., Weinschenk T. and Schluesener H.J. Uptake, Intracellular Distribution and Novel Ligands of Immunostimulatory CpG Oligodeoxynucleotides in Microglial Cells. Molecular Neuropathology and 48th Annual Meeting of Germany Society of Neuropathology and Neuroanatomy, October 2003, Berlin, Germany.

3. Zhang Z., Weinschenk T. and Schluesener H.J. siRNA Binding Proteins of Microglial Cells. Poster Seminar of the Graduate College of "Cellular mechanisms of immune-associated processes", GK 794, July 2004, Tuebingen, Germany.

4. Zhang Z., Weinschenk T. and Schluesener H.J. siRNA Binding Proteins of Microglial Cells. Molecular Neuropathology and 49th Annual Meeting of Germany Society of Neuropathology and Neuroanatomy, September 2004, Cologne, Germany.

5. Zhang Z., Schittenhelm J., Guo K., Buhring H.J., Trautmann K., Meyermann R., and Schluesener J.H. Frizzled 9 (FZD9) is selectively expressed in neoangiogenic regions of human glioblastomas. Molecular Neuropathology and

50th Annual Meeting of Germany Society of Neuropathology and Neuroanatomy, October 2005, Graz, Austria.

Oral Presentations:

1. Zhang Z. Cyclooxygenase (COX) and nonsteroidal anti-inflammatory drugs (NSAIDs). Seminar of Institute of Brain Research, November 2002, Tuebingen, Germany.

2. Zhang Z. Analysis of pathological vascular endothelial cell by DAN-aptamer. Winter seminar of the Graduate College of “Cellular mechanisms of immune-associated processes”, GK 794, January 2003, Tuebingen, Germany.

3. Zhang Z. Acupuncture and pain management. Weekend seminar of the Graduate College of “Cellular mechanisms of immune-associated processes”, GK 794, July 2003, Oberjoch, Germany.

4. Zhang Z. Specificity of siRNA-mediated RNAi in mammalian cells. Weekend seminar of the Graduate College of “Cellular mechanisms of immune-associated processes”, GK 794, April 2004, Blaubeuren, Germany.

Acknowledgements

I thank Professor Hermann J. Schluesener for excellent advice and guidance of my scientific study.

Thanks to all present and former colleagues at the Institute of Brain Research, University Hospital Tuebingen, for their kindly help and fulfilling my life with great experience.

I thank my lovely family for love, support, encouragement and everything during my abroad.

The experiments were partly supported by the DFG.

Zhiren Zhang received a scholarship from the DFG from Sep. 2002 to Feb. 2004.

Zhiren Zhang was a scholar in the doctoral programme of “Cellular mechanisms of immune-associated processes”, Graduate College 794, Tuebingen, Germany from Sep. 2003 to March 2006.

.

Curriculum Vitae

Name:	Zhiren Zhang
Place of birth:	Yongfeng, Jiangxi Province, P.R. China
Date of Birth:	Sep. 27th, 1974
Nationality:	Chinese
Marital status:	Married

Education

1982 - 1987	Primary School, Yongfeng, Jiangxi Province, P.R. China
1987 - 1993	Middle School, Yongfeng, Jiangxi Province, P.R. China
1993 - 1998	Bachelor of Medicine, Institute of Environmental Medicine, Department of Public Health, Tongji Medical University, P.R. China
1998 - 2001	Master of Medicine, Department of Public Health, Tongji Medical School, Huazhong University of Science and Technology, P.R. China
2002 - Present	Ph. D. candidate of Prof. Dr. H. J. Schlüsener, Institute of Brain Research, Eberhard-Karls-Universität Tübingen, Germany
2002 – 02/2006	Scholar of the Graduate College "Cellular mechanisms of immune-associated processes", GK 794, Tübingen, Germany
09/2002 - 02/2004	Scholarship for a Ph.D. student within the Graduate College "Cellular mechanisms of immune-associated processes", Tübingen, Germany
03/2004 - present	„Wissenschaftlicher Mitarbeiter" at the Institute of Brain Research, University of Tübingen