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Development of safe and effective nonviral gene therapy by eliminating CpG motifs from plasmid DNA vector

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1. ABSTRACT

Nonviral gene therapy is expected to become a regular treatment for a variety of difficult-to-treat diseases, such as cancer and virus infection. Plasmid DNA, which is used in most nonviral gene delivery systems, usually contains, unmethylated cytosine-guanine dinucleotides, so called CpG motifs. CpG motifs are recognized by immune cells as a danger signal, leading to an inflammatory response. Such inflammatory responses could affect the safety and effectiveness of nonviral gene therapy. Therefore, reducing the number of CpG motifs in plasmid DNA has been used to increase the potency of plasmid DNA-based gene therapy. Previous studies have demonstrated that CpG reduction can extend the time period of transgene expression from plasmid DNA after *in vivo* gene transfer. In this review, the biological functions of the CpG motif are briefly summarized. Then, safety issues of nonviral gene therapy are discussed from the viewpoint of the inflammatory response to the CpG motif in plasmid DNA, and the effects of the CpG motif in plasmid DNA on the transgene expression profile of nonviral gene transfer are reviewed.

2. INTRODUCTION

The success of gene therapy is much lower than initially expected. Successful application of gene therapy has been hampered by many factors including the acute inflammatory response to gene vectors, carcinogenesis and a limited therapeutic effect (1, 2). In performing gene therapy, the vector that carries the transgene is one of the most important components that determine the therapeutic outcome. Some serious side effects in clinical studies of gene therapy using viral vectors as well as improvements in the efficacy of nonviral gene transfer methods have greatly increased the importance of nonviral gene transfer methods that use no potentially harmful viral vectors in their clinical applications (1-4).

Plasmid DNA is the most frequently used DNA in nonviral gene therapy. As the properties of plasmid DNA, such as its promoter, enhancer and poly adenylation site greatly affect the level and time-course of transgene expression, optimization of the properties of plasmid DNA has been performed by modifying these functional regions (5). In particular, the promoter region has been the most

attractive region for optimization. By selecting an appropriate promoter, the period of transgene expression from the plasmid DNA is extended (6-8). Moreover, drug-inducible promoters and tissue-specific promoters have been developed to control the temporal and special profile of the transgene expression (9-11). Not only these functional sequences in plasmid DNA, but a small sequence of unmethylated CpG dinucleotides has been identified as an important factor that requires great attention. In this review, the biological role of the CpG sequence in the mammalian body is discussed with regard to inflammation and the regulation of endogenous gene expression. Then, the effect of CpG sequences in plasmid DNA on the inflammatory response to administered plasmid DNA is discussed. Finally, the relationship between the CpG sequences in plasmid DNA and transgene expression from plasmid DNA is summarized based on the recent results obtained in animal experiments.

3. BIOLOGICAL ROLE OF CpG MOTIFS IN THE BODY

In vertebrate animals, the frequency of CpG dinucleotides in genome DNA is 1 out of about 50 bases, which is much lower than the mathematical frequency of 1 out of 16 bases. Moreover, most of CpGs are methylated at the carbon 5-position of the cytosine residue, which further reduces the frequency of the unmethylated CpG dinucleotides (12). In contrast, bacterial DNA contains CpG dinucleotides almost as frequently as expected from the mathematical frequency, and most of bacterial CpGs are unmethylated (13). Mammals use this difference in CpGs between bacterial DNA and mammalian DNA, i.e., unmethylated CpG dinucleotides are recognized as a danger signal in mammals. Despite the low frequency of CpG motifs in mammalian DNA, there are some regions in mammalian genome DNA with many CpGs, called CpG islands, which have been recently discovered to play important roles in regulating the expression of a variety of genes. In this section, the latest view of the DNA recognition in mammals and the role of CpGs in gene regulation are briefly summarized.

3.1. Response to CpG dinucleotides in mammals

Unmethylated CpG dinucleotides, or CpG motifs, are recognized as a danger signal by Toll-like receptor 9 (TLR9), one of the pattern recognition receptors that recognize a pathogen-associated molecular pattern (14). It is known that a limited number of cells express TLR9. In humans, a constitutively high level of TLR9 is expressed in plasmacytoid dendritic cells and B cells and, in mice, macrophages and dendritic cells also express high level of TLR9 (15-17). Therefore, the TLR9-mediated response is mainly induced by a limited number of cells. We showed that depletion of phagocytic cells using clodronate liposomes markedly reduced the inflammatory response induced by the intravenous injection of CpG DNA complexed with cationic liposomes (18). This study also demonstrated that splenectomy hardly reduced the response, which suggests that cells outside the spleen are at least involved in the CpG DNA-induced response.

TLR9 is composed of an extracellular domain, a transmembrane domain and a cytoplasmic domain. TLR9 is localized in the endoplasmic reticulum and is delivered to the endolysosomal compartments, where TLR9 meets CpGs (19). As TLR9 recognizes CpG DNA in the endosome, the intracellular localization of CpG-containing DNA is also an important factor that determines the inflammatory response (20). Binding of CpGs to TLR9 recruits signaling adaptor molecules, such as MyD88, which leads to the activation of nuclear factor-κB and activation of gene expression of cytokines and co-stimulatory molecules (21, 22). In addition, recognition of CpG motifs by TLR9 has been reported to be related to the development of autoimmunity (23, 24). An example is the activation of B cells by the immune complexes of DNA and autoantibody in an autoimmune mouse model (25), which led to the secretion of rheumatoid factors through the TLR9-dependent signaling pathway.

3.2. CpG motifs in epigenetic regulation of gene expression

In the nucleus of eukaryotes including mammalian cells, genetic information is preserved in a DNA-protein structure called chromatin. Chromatin consists of the repeat of nucleosome, which contains DNA wrapped around histone proteins. Gene expression from the genome DNA is initiated by the binding of the transcription complex to DNA. Therefore, the interaction of DNA with histone, which sterically affects the transcription complex-DNA interaction, is an important regulatory factor for gene expression. Here, DNA methylation plays a central role in the regulation of the histone-DNA interaction. DNA methylation occurs on cytocine at the CpG dinucleotides and most of the CpGs in the mammalian genome are methylated. CpG islands are the short CpG-rich regions and generally located around promoter. It is reported that methylation status of CpG motifs in CpG islands are controlled in order to regulate the expression of endogenous genes (26). DNA methylation is recognized and bound by methyl-CpG-binding domain proteins, which forms repressor complexes with histone deacetylase (HDAC). HDACs remove the acetyl group from histones. Hyperacetylated histones are generally associated with chromatine decondensation, which increases accessibility of DNA to binding proteins and the transcription activity (27, 28). However, hypoacetylated histones are generally associated with chromatin condensation, which reduces transcription activity (27, 28).

4. CpG MOTIFS IN PLASMID DNA VECTOR

As plasmid DNA is a bacteria-derived DNA, it consists of bacteria-derived regions which contains many CpG motifs other than the cassette for transgene expression such as promoter, cDNA, enhancer and polyA (Table 1). As it has been considered that bacteria derived sequences such as replication ori are not essential for transgene expression, these regions initially attracted less attention than the transgene expression cassette. Some recent studies have shown that bacteria derived sequences in plasmid DNA can also affect the profile of transgene expression after gene transfer. Li *et al.* demonstrated that the PCR-

Table 1. Number of CpG motifs in the typical functional regions of plasmid DNA

Replication ori		Drug resistance gene		Promoter		poly A	
pUC ori	45	Ampicillin	49	CMV	31	BGH poly A	3
pMB1 ori	48	Kanamycin/Neomycin	73	SV40	10	SV40 poly A	0
R6K ori	0	zeocin	51	EF1	94		
		hygromycin	103	ROSA26	213		

The number of CpG motifs in one strand of each region was summarized, so those in double stranded plasmid DNA should be doubled.

amplified fragment of the transgene expression cassette reduced the level of transgene expression but resulted in more sustained gene expression (29). Chen *et al.* reported that simply eliminating bacteria-derived regions by using restriction endonuclease was effective in obtaining more persistent transgene expression than parental plasmid DNA (30). In their following study, they further optimized their expression cassette by obtaining a minicircle DNA vector containing only the expression cassette, which showed more sustained transgene expression than the conventional plasmid and plasmid DNA fragment obtained by restriction endonuclease (31). These pieces of experimental evidence suggest that bacteria-derived sequences have negative effects on the transgene expression from plasmid DNA.

As already described, the CpG motif is a typical sequence that is frequently observed in bacteria DNA as well as plasmid DNA, a bacteria-derived DNA. Therefore, plasmid DNA usually contains many CpG motifs. Unmethylated CpG motifs could be methylated and the methylation status of CpG motifs is associated with histone modification. As these changes influence the transgene expression from plasmid DNA, the transgene expression from plasmid DNA can be regulated via CpG motifs by the same mechanism as the epigenetic regulation of endogenous DNA. In addition, CpG motifs are TLR9 ligands so that CpG motifs in plasmid DNA have been shown to induce an inflammatory response. In the following section, the effect of CpGs in plasmid DNA on the response to the administered DNA and the transgene expression is discussed.

4.1. Inflammatory response to plasmid DNA

When plasmid DNA containing CpG motifs is administered *in vivo*, it can induce an inflammatory response if CpG motifs are recognized by TLR9 (Figure 1). As TLR9 is expressed in limited types of cells and TLR9 generally interacts with CpG motifs in endosomes, the intracellular distribution of the DNA as well as the distribution of the DNA in the body determines the response against DNA. It has been shown that gene transfer of naked plasmid DNA sometimes induces an inflammatory response. Moreover, carrier-based gene delivery, in which liposomes and polymers are used to efficiently deliver plasmid DNA, is frequently associated with a more severe inflammatory response than naked plasmid DNA, which is probably due to the differences in their distribution in the body and in cells (32, 33). Compared with carrier-based gene therapy, naked DNA-based gene delivery generally results in a weaker inflammatory response to the plasmid DNA. In this section, the inflammatory responses to naked plasmid DNA or plasmid DNA/carrier complex are separately discussed, because the intracellular distribution of plasmid DNA has been reported to be quite different between the naked DNA delivery and the carrier-based DNA delivery. In addition, TLR9-independet inflammatory response against plasmid DNA is also discussed.

4.1.1. Inflammatory response to plasmid DNA

Tissue injection of naked plasmid DNA is the simplest form of *in vivo* gene delivery. Therefore, it has often been used in nonviral gene therapy including some clinical trials (4). In particular, intramuscular injection is frequently used to deliver naked DNA (34). When a plasmid DNA containing CpG motifs is administered intramuscularly, an inflammatory response is induced in a CpG-dependent manner. It has been reported that CpG motifs in plasmid DNA vector induce the expression of chemokines and MHC class II in muscle (35). In the case of DNA vaccine, in which plasmid DNA encoding antigen is administrated to induce an antigen-specific response, the inflammatory response induced by CpG motifs is advantageous because it can boost the immune response (36). However, the inflammatory response to plasmid DNA is not favorable for other forms of gene therapy because the inflammatory response is a harmful side effect and in some cases, transgene-expressing cells are eliminated by the response. To avoid the inflammatory response following intramuscular injection of plasmid DNA, Reyes-Sandoval *et al.* administered plasmid DNA that had been methylated *in vitro* (37). As a result, they succeeded in reducing the immune response induced by plasmid DNA. In order to increase the transgene expression level, various physical stimulations, such as electroporation and sonoporation, ca be applied after local DNA administration. However, these physical stimulations may induce an inflammatory response, probably in a CpG-independent manner (38-40), although the degree of inflammation is much less compared with that induced by carrier-based delivery.

Simple intravascular injection of naked plasmid DNA generally results in little or no transgene expression. However, it can still induce an inflammatory response. For example, in mice, systemic injection of naked plasmid DNA with many CpG motifs at regular doses of about 1 mg/kg produced no detectable level of TNF-α in serum while a large naked plasmid DNA injection induced TNF-α secretion in the blood circulation, and less TNF-α was produced when large amount of plasmid DNA with few CpGs was injected (33). As a mode of naked DNA delivery for systemic administration, so called hydrodynamic

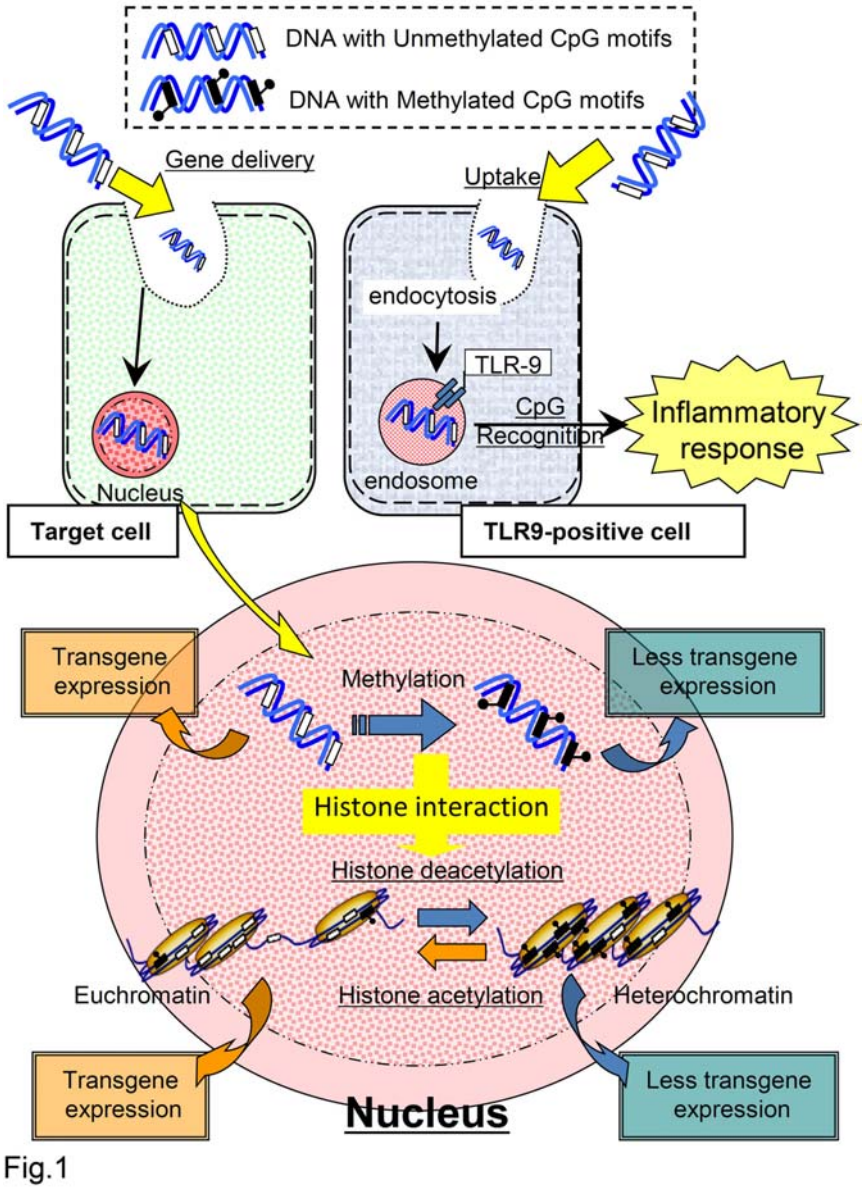

Figure 1. Schematic image of the role of CpG motifs in nonviral gene therapy. For nonviral gene therapy, plasmid DNA should be delivered to the nucleus of target cells. After intranuclear delivery of plasmid DNA, plasmid DNA may be methylated at the CpG motif, which decreases the level of transgene expression from the DNA. Plasmid DNA in the nucleus is likely to interact with histone. The level of transgene expression from plasmid DNA closely associated with histone, a heterochromatine-like structure, is lower than the DNA loosely associated with histone, a euchromatin-like structure. Plasmid DNA administered to mammals is often taken up by TLR9-positive cells. Plasmid DNA with unmethylated CpG motifs is recognized by TLR9 in endosomes, which induces an inflammatory response.

injection, in which a large volume of naked plasmid DNA solution is rapidly injected, is one of the most efficient methods to obtain a high level of transgene expression. Although the level is not very high, hydrodynamic injection of plasmid DNA at regular dose increases TNF-α in a CpG-dependent manner (41). However, hydrodynamic injection of saline without DNA increases the serum IL-6 concentration, which suggests that the gene delivery method itself may produce an inflammatory response in some situations.

4.1.2. Inflammatory response to plasmid DNA delivered by carrier

Carrier-based nonviral gene delivery is also an accepted delivery method because it is associated with multiple functions such as the controlled delivery of plasmid DNA. Cationic liposomes and polymers are frequently complexed with plasmid DNA as carriers to increase delivery efficiency. However, complexing plasmid DNA with cationic lipids not only increases the delivery efficiency, but also increases inflammatory responses.

Incubation of cationic lipids/plasmid DNA complex, or lipoplex, with macrophages or dendritic cells stimulates the cells to release more inflammatory cytokines than that with naked plasmid DNA (42, 43). Like the situation *in vitro*, intravenous administration of lipoplex to mice results in more inflammatory cytokine production than that produced by naked plasmid DNA (18, 33). Moreover, systemic delivery of plasmid DNA complexed with cationic lipid induces an acute inflammation with adverse hematologic changes and liver damage (44, 45). Therefore, the inflammatory response to plasmid DNA is a more serious problem for carrier-based gene therapy than that for naked gene therapy. On the other hand, little inflammatory response was observed when plasmid DNA was complexed with polyethyleneimine, a cationic polymer frequently used as a nonviral vector (46). Such differences in the inflammatory response were discussed in relation to the altered intracellular distribution of plasmid DNA. Saito *et al.* reported that after endocytocis, polyplexes tend to escape from the endosome more effectively than lipoplexes (47). As the endosome is the place where TLR9 recognizes CpG DNA, an efficient escape of polyplex from the endosome may be a reason for the less inflammatory response against polyplexes.

Results from *in vitro* experiments using cultured macrophages and dendritic cells show that the secretion of inflammatory cytokine from these cells induced by lipoplex is CpG-dependent. Therefore, to avoid the inflammatory response against lipoplex, Yew *et al.* developed a CpG-depleted plasmid DNA vector. As a result, the systemic delivery of a CpG depleted plasmid DNA/cationic lipid complex induced a weaker inflammatory response, less liver damage and fewer hematologic changes (48). The effect of CpGs in plasmid DNA on the inflammatory response was more dramatically demonstrated by Hyde *et al* (49). They constructed plasmid DNAs with different numbers of CpG dinucleotides (from 0 to 317 CpGs), and complexed the plasmid DNAs with a cationic lipid formulation. The complex was administered to the airways of mice as an aerosol. Administration of plasmid DNA with 317 and 193 CpGs induced almost the same degree of lung inflammation. Plasmid DNA with 1 CpG still induced lung inflammation, although the level of inflammation was lower than that produced by plasmid DNA with 193 and 317 CpGs. Administration of plasmid DNA without CpG hardly induced lung inflammation compared with the mock group. These results suggest that even one CpG in plasmid DNA can induce an inflammatory response. Therefore, elimination of CpG sequences is an effective approach to reducing the degree of inflammatory response to plasmid DNA delivered as a complex with carriers.

4.1.3. TLR9-independent recognition and inflammatory response to plasmid DNA

Although TLR9 is the receptor for CpG DNA, other proteins are also involved in the recognition of both endogenous and exogenous DNA in a CpG motif-independent manner. Recently, several candidates for the receptors recognizing DNA in the cytosol have been reported. Ishii *et al.* reported the importance of TANK-binding kinase 1 in the immunostimulation by DNA vaccines (50). Separately, Takaoka *et al.* found that DAI (DNA-dependent activator of IFN-regulatory factors) recognizes cytosolic DNA irrespective of the presence of CpGs (51). They demonstrated that recognition of non-CpG DNA by DAI in fibroblasts resulted in type I interferon production. More recently, Unterholzner *et al.* identified IFI16, a member of the PYHIN protein family, as a cytosolic censor for DNA (52). They found that microbial DNA is recognized by IFI16 in monocytes, which results in type I interferon production. Therefore, inflammatory responses induced by TLR9-independent pathway should be considered in the future development of gene therapy.

4.2. Effect of CpG motifs in plasmid DNA on transgene expression from the DNA

Recently, CpG depletion from plasmid DNA is regarded to be effective not only in reducing the inflammatory response but also in increasing the duration of transgene expression from the DNA. Prolongation of transgene expression by eliminating bacteria-derived regions to produce a minicircle vector may also be because of the reduction in CpG motifs. In the following section, the effect of the number of CpG motifs in plasmid DNA on the transgene expression from the plasmid DNA is discussed first followed by an examination of how CpG-elimination is related to the transgene expression from plasmid DNA.

4.2.1. Relationship between transgene expression profile from plasmid DNA and the number of CpG motifs

The profile of transgene expression after the administration of plasmid DNA with different numbers of CpG by the hydrodynamics-based procedure has been investigated frequently regarding the transgene expression from plasmid DNA in naked form (41, 48, 53-55). In those studies, administration of CpG-depleted plasmid DNA by the hydrodynamics-based procedure resulted in prolongation of the gene expression in most cases. Our group has also reported that the number of CpG motifs in plasmid DNA is inversely correlated with the duration of transgene expression from the plasmid DNA. By using a mouse lung metastasis model, we demonstrated that administration of interferon-gamma (IFNγ)-expressing plasmid DNA with less CpGs by the hydrodynamic injection method was more effective in inhibiting tumor growth in the lung than the conventional IFN-expressing plasmid DNA with many CpGs (41, 55). Moreover, a single administration of IFNγ-expressing plasmid DNA with much fewer CpGs suppressed the onset of atopic dermatitis in Nc/Nga mice while multiple administration of conventional IFNγ-expressing plasmid DNA could not (53). Therefore, our studies suggest the possibility that prolonging the transgene expression period is an effective approach to improve the therapeutic effect after gene therapy.

The effect of CpG elimination from plasmid DNA on transgene expression was observed not only with naked DNA delivery but also with carrier-based DNA delivery. Yew *et al.* intravenously administered a lipoplex of plasmid DNA with few or many CpG motifs (48). They found that administration of lipoplex with less CpG motifs

resulted in higher and more sustained transgene expression in the lung than that of lipoplex with many CpG motifs. The same trend was observed when plasmid DNA complex was administrated into the airways by aerolization (49). Administration of CpG-free plasmid DNA produced higher and more sustained gene expression in the lung. Not only in the case of lipoplex, but also in the case of plasmid DNA/cationic polymer (polyplex), CpGs in plasmid DNA had an effect on the transgene expression. DeWolf *et al.* administered CpG-rich or CpG-free plasmid DNA complexed with liposome or polyethyleneimine and found that the depletion of CpG motifs within the plasmid DNA of lipoplex and polyplex enhances the degree and duration of transgene expression (56).

4.2.2. Factors that link CpG motifs with transgene expression

Compared with the established role of CpG motifs in the induction of an inflammatory response, the mechanism whereby CpG-depleted plasmid DNA generally produces more sustained transgene expression is still unclear. To date, several putative mechanisms have been postulated. Persistence of plasmid DNA within cells is a prerequisite for the long-term transgene expression and, therefore, CpG-reduced plasmid DNA might remain longer than CpG-rich plasmid DNA after administration. However, there has been little evidence to support this hypothesis. Another possible mechanism involves the inflammatory response. As it has been demonstrated that inflammatory cytokines such as TNF-α and IFNs suppress transgene expression, the inflammatory response induced by CpG motifs may reduce and suppress transgene expression (57, 58). Therefore, avoiding the inflammatory response by eliminating CpGs from plasmid DNA can positively affect transgene expression. However, reduced inflammatory response alone cannot be the mechanism whereby CpG depletion has a positive effect on transgene expression because CpG depletion can also improve transgene expression after naked DNA delivery, in which the inflammatory response is induced to a much lower extent than carrier-based DNA delivery. Moreover, the time courses of transgene expression from plasmid DNA administered by hydrodynamic delivery were almost identical between the control mice and those injected with lipoplexes to induce inflammatory responses (55). Another putative mechanism is CpG methylation (Figure 1). As enzymatically methylated plasmid DNA shows lower transgene expression *in vivo* than the unmethylated plasmid DNA (55), CpG methylation has a negative effect on the transgene expression from plasmid DNA. Therefore, eliminating the CpG motifs reduces the number of methylation target sites, which can prevent silencing by methylation and extend the period of transgene expression. The other hypothetical mechanism is the interaction of histone with plasmid DNA, which is related to the methylation of DNA (Figure 1). Endogenous DNA in the nucleus usually interacts with histone. It has been reported that administered plasmid DNA also interacts with histone after it reaches the nucleus (59). The interaction of histone with plasmid DNA, a heterochromatin-like structure, reduces the level of transcription. However, the weak interaction of plasmid DNA with histone, a euchromatin-like structure, transcribes more mRNA. As interaction of DNA with histone is suggested to be regulated by CpG motifs, changing the number of CpG motifs changes the interaction mode of plasmid DNA with histone, which might affect the transgene expression profile.

5. CONCLUSION

Although the number of CpG motifs in plasmid DNA contributes to the safety and effectiveness of nonviral gene therapy remains a matter of debate, CpG-free plasmid DNA is much less likely to induce an unexpected response such as inflammation than CpG-replete plasmid DNA. Especially in performing carrier-based nonviral gene therapy, the response to CpG motifs is boosted compared with that of naked DNA-based, which could interfere with the evaluation of the effect of transgene products not only in animal experiments but in clinical trials. These considerations clearly indicate that the use of CpG-free plasmid DNA is desirable even in animal experiments. As CpG elimination has great advantages and seems to have few disadvantages, a CpG-depleted or CpG-free DNA vector will be the standard DNA for use as nonviral gene therapy in the future.

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