Effects of GM-CSF gene transfer using silica-nanoparticles as a vehicle on white blood cell production in dogs

Eun Wha Choi, Il Seob Shin, Young Jin Chae, Hye Cheong Koo, Jong Hwa Lee, Tae Ho Chung, Yong Ho Park, Dae Yong Kim, Cheol Yong Hwang, Chang Woo Lee, and Hwa Young Youn

Objective. We sought to test two concepts: that nanoparticles can be used for in vivo gene delivery and that canine granulocyte-macrophage colony-stimulating factor (GM-CSF)/nanoparticles can have possibility to be used to treat transient (acute) canine leukopenia.

Materials and Methods. We have generated a novel fluorescent-silica nanoparticle binding of canine GM-CSF gene; canine GM-CSF gene was inserted between the cytomegalovirus promoter and poly-adenylation sequences of simian virus 40, and the gene construct was ligated to fluorescent silica nanoparticles functionalized with tertiary amine.

Results. When the GM-CSF/nanoparticles were injected into normal dogs, the GM-CSF was expressed in peripheral blood mononuclear cells for at least 9 days and there were significant increases in white blood cell counts, as confirmed by complete blood count, differential count, and flow cytometry. Significant increases in expression of major histocompatibility complex class II on granulocytes and in serum GM-CSF were also observed. Readministration of the nanoparticles was also effective and expression in various tissues was confirmed by reverse transcriptase polymerase chain reaction.

Conclusions. These GM-CSF/nanoparticles may be useful for correction of acute leukopenia, such as chemotherapy-induced myelosuppression without developing neutralizing antibodies.

Neutropenia is a major factor contributing to infection, morbidity, mortality, and inadequate dosing in animals and people undergoing chemotherapy against cancer [1]. A nontoxic treatment capable of either accelerating recovery from or preventing chemotherapy-induced myelosuppression would clearly be beneficial. Granulocyte-macrophage colony-stimulating factor (GM-CSF) is a growth factor that stimulates granulopoiesis and acts in antimicrobial defense, peripheralization of hematopoietic progenitors, and as a vaccine adjuvant [2]. Canine GM-CSF shares 53% to 75% homology with human, feline, ovine, porcine, bovine, and murine GM-CSF [3]. Moreover, murine and human GM-CSF do not cross-react in biologic activity or receptor binding, and their amino acid sequences are only 56% identical, making GM-CSF species-specific and one of the less well-conserved among the myeloid growth factors [2]. Although recombinant human GM-CSF has been used to control acute canine leukopenia in veterinary clinics, its use has been associated with the development of neutralizing antibodies to the heterologous recombinant human GM-CSF [1]. Thus development of a species-specific recombinant GM-CSF protein or GM-CSF therapeutic gene would be advantageous.

Although recombinant DNA technology and large-scale culture techniques have made it possible to produce various recombinant proteins in amounts suitable for research, clinical, and industrial purposes [4], production of recombinant proteins has faced a number of practical problems [5]. Viral vectors are commonly used for therapeutic gene delivery, but their applications are limited due to their requirements for specific cell membrane receptor-mediated infection and
the risks of excessive immune response and insertional mutagenesis [6,7]. The technology of nano substances is currently being investigated, because these particles are of suitable size and are stable after insertion into cells. Although silica-nanoparticles are effective in cell transfection, their in vivo ability to express genes of interest over long periods of time and their clinical applicability have yet to be clarified.

Because nanoparticles do not use the packaging mechanisms of viruses, they would not be expected to have size limitations. Moreover, nanoparticles cannot integrate into the host genome because they lack integrase. Thus, nanoparticles would not show the toxicity or immune response, such as virus vector, but they would be expected to act during the short term. However, nanoparticles would be advantageous and useful in condition requiring transient gene expression, such as acute leukopenia induced by chemotherapy. Thus, we intend to apply this concept to normal animals before applying to leukopenic animals.

To determine whether canine GM-CSF linked to nanoparticles can be effective in induction of leukocytosis, we have constructed novel fluorescent silica-nanoparticles binding of canine GM-CSF and have evaluated the use of nanoparticles as vehicle for gene delivery. These GM-CSF/nanoparticles were injected into normal dogs, and their duration of expression and sites of action were determined. We also evaluated the effects of GM-CSF/nanoparticles on leukocyte expression of major histocompatibility complex (MHC) class II and cytokine mRNA.

Materials and methods

Construction of the therapeutic gene
Canine GM-CSF gene was linked to the human oncostatin M signal sequence for efficient secretion into body fluids and the construct was inserted between the cytomegalovirus promoter for high level expression and the simian virus 40 adenylation sequence for efficient termination of transcription (Fig. 1A).

Construction of nanoparticles and determination of the therapeutic gene to nanoparticle ratio
Rhodamine B isothiocyanate was modified with the silane compound, 3-aminopropyltriethoxysilane, and mixed with tetraethoxysilane, and polymerization was initiated by the addition of
ammonia solution. The resulting water soluble silica-nanoparticles were surface modified with cationic N-trimethoxysilylpropyl-N,N,N-trimethylammonium chloride, (MeO)3Si-PTMA, to prepare them for use as gene delivery carriers. Positively charged fluorescent magnetic nanoparticles-PTMA was hybridized with negatively charged plasmid to find the most suitable ratio. Size homogeneity of the particles was confirmed by transmission electron microscopy (JEOL Ltd., Tokyo, Japan). To determine a suitable binding ratio, various amounts of the therapeutic gene were ligated with a fixed amount of fluorescent magnetic nanoparticles-PTMA, at ratios of 0:100, 1:20, 1:50, 1:100, 1:200, and 1:0 (therapeutic gene: silica nanoparticles, w/w), at pH 7.4 for complete binding for 2 hours at 4°C, and the degree of binding was then determined by electrophoresis.

Experimental animals
A total of 12 mongrel dogs determined to be healthy after routine physical examination, complete blood counts (CBC), serum biochemistry analysis and urinalysis were used under protocols approved by the Institutional Animal Care and Use Committee of Seoul National University (approval number: SNU-060217-1). All efforts were made to minimize animal suffering. Dogs were housed separately in metal cages and maintained on commercial dry dog food and water ad libitum. Cages were kept at a constant temperature (22–25°C) and humidity (50–55%). Animals were acclimated for at least 5 weeks prior to use. Five micrograms GM-CSF/nanoparticles (1:100, w/w), suspended in phosphate-buffered saline (PBS) and filter sterilized, were administered intravenously to each of six dogs in the experimental group, and an equivalent amount of suspended and sterilized nanoparticles was administered to each of six dogs in the control group.

Detection of therapeutic gene transcription from PBMC
Total RNA was isolated from peripheral blood mononuclear cells (PBMC) using QIAamp RNA Blood Mini kit (52304, Qiagen, GmbH, Hilden, Germany) and reverse transcription polymerase chain reaction (RT-PCR) was performed according to the manufacturer’s instructions (Maxime RT-PCR Pre-Mix, 25131; iNtRON Biotechnology, Sungnam, Korea). To detect transcription of the therapeutic gene in the experimental group, we used a forward primer homologous to the fusion portion of the human oncostatin M signal sequence (GM-SIZE-F: 5'-CTCTCTGCTCAACAGAG GACCCT-3') and a reverse primer homologous to canine GM-CSF (GM-SIZE-R: 5'-CTCCTTGATCACGTGTAGGCGG -3').

CBC and differential count
Blood was collected between 9 AM and 10 AM from the jugular vein of each dog. CBC was determined by automatic analyzer (Celltac alpha MEK-6318; Nihon Kohden, Tokyo, Japan) and differential count was determined manually in blood smears.

Flow cytometry
Antibodies to canine MHC class I (H58A), MHC class II (CAT81A), pan-lymphocyte (DH52A), B lymphocyte (F46A), CD172a (DH59B), CD4 (DH29A), CD8 (CADO46A), and CD14 (CAM36A) were obtained from VMRD (Pullman, WA). About 50 µL (15 µg/mL) of each monoclonal antibody was incubated for 30 minutes on ice with 100 µL cells (1 × 10⁷ cells/mL) in each well of a V-bottomed 96-well microplate. Plates were washed three times with the first washing buffer (PBS 450 mL, ACD 50 mL, 20% NaN₃ 5 mL, γ-globulin–free horse serum [Sigma-Aldrich, St Louis, MO, USA] 10 mL, 250 mM ethylenediamine tetraacetate acid 20 mL, and 0.5% phenol red 1 mL), with centrifugation at 670g for 5 minutes. Each pellet was disrupted by vortexing, mixed with 50 µL of a 1:100 dilution of fluorescein isothiocyanate–conjugated goat anti-mouse IgG₂a antibody and a 1:200 dilution of phycoerythrin–conjugated goat anti-mouse IgG₃ antibody (Caltag Lab, Burlingame, CA, USA), and incubated on ice for 30 minutes in the dark. The cells were washed three times with a second washing buffer, identical to the first, but excluding horse serum), with 200 µL 2% PBS-formaldehyde (38% formalin 20 mL, PBS 980 mL) and kept in the refrigerator. Canine leukocyte subpopulations were analyzed by flow cytometry (a FACSort flow cytometer) using the CellQuest program (Becton Dickinson Immunocytometry Systems, San Jose, CA, USA) and FCS Express software (De Novo software, Thornton, Ontario, CA).

ELISA for detection of serum level of canine GM-CSF
Serum samples were frozen at –70°C, and canine GM-CSF concentration was assessed by indirect enzyme-linked immunosorbent assay (ELISA), using commercial recombinant canine GM-CSF (1546-GM/CF, R&D Systems, Minneapolis, MN, USA) as standard. One hundred microliters of sera diluted 1:1, 1:5, 1:10, and 1:50 were used to coat the plates, followed by sequential incubation with goat anti-canine GM-CSF antibody (0.5 µg/mL, AF1546, R&D Systems) and horseradish peroxidase–conjugated rabbit anti-goat IgG (H+L) (1:5000, 81-1620; Zymed, San Francisco, CA, USA) according to the manufacturer’s instructions.

ELISA for detection anti-canine GM-CSF antibodies
IgG antibodies to canine GM-CSF was measured in the sera of control and treated dogs collected on 0, 7, 14, and 28 days after injection with nanoparticles or GM-CSF/nanoparticles. Recombinant canine GM-CSF (2 ng/well; R&D Systems) was coated overnight at 4°C in PBS. The excess binding sites were blocked with 1% bovine serum albumin in PBS. Dog sera were tested at 1: 200 dilution, and the amount of specific antibody bound to canine GM-CSF was determined using peroxidase–conjugated rabbit anti-dog IgG (1:10000, Sigma A6792) as secondary antibody and 3',3',5,3'-tetramethylbenzidine as substrate. Stop solution (2 M H₂SO₄) was added and color development was read at a wavelength of 450 nm and a reference range wavelength of 540 nm using an automatic plate reader.

Semi-quantitative RT-PCR analysis of cytokines
Semi-quantitative RT-PCR to measure cytokine gene expression was performed as described previously, using the same primer sequences, optimal PCR annealing temperatures and cycle numbers [8]. All reactions were performed using the PCR Thermal Cycler Dice TP600 (TaKaRa, Otu, Japan).

Histopathology
One dog euthanized on day 3 after GM-CSF/nanoparticles (7.5 µg/750 µg) administration. Tissue samples, including those of liver, kidney, lung, heart, muscle, pancreas, spleen, uterus, ovary, and brain were fixed in 10% neutral buffered formalin, embedded in paraffin, and sectioned at 5 µm. For light microscopy, samples were stained with hematoxylin and eosin using routine histological techniques.

Confirmation of therapeutic gene transcription from tissue
To confirm that the vector delivered the gene to various tissues, total RNAs were prepared from dog tissue samples obtained 3 days...
after injection of the GM-CSF/nanoparticles or nanoparticles alone, using RNeasy Mini Kits (74104; Qiagen, GmbH, Hilden, Germany), followed by RT-PCR.

Comparison on leukocyte counts of dogs injected with GM-CSF plasmid, GM-CSF/nanoparticles and recombinant GM-CSF protein, respectively
Leukocyte counts of dogs injected with GM-CSF plasmid at a dose of 1 μg/kg body weight (n = 3), GM-CSF/nanoparticles at a dose of 1 μg/kg body weight intravenously (n = 2) and recombinant GM-CSF protein at a dose of 5 μg/kg subcutaneously (n = 3), respectively, were determined by automatic analyzer at 0, 6, 12, 18, 24, 30, 48, and 72 hours after injection.

Statistical analysis
All results are expressed as means with standard error of the mean. Between group comparisons were assessed by Student’s t-test, whereas within-group comparisons were analyzed by paired t-test. Differences with a confidence level of ≥95% were considered statistically significant (p < 0.05). All statistical analyses were performed using SPSS version 12.0.

Results

Construction of nanoparticles and determination of the therapeutic gene to nanoparticle ratio
Size homogeneity of nanoparticles (45–55 nm) was determined by transmission electron microscopy (Fig. 1B). The ratio of nanoparticles to the GM-CSF construct was set at 1:100 (w/w, Fig. 1C).

Detection of therapeutic gene transcription from PBMC
After injection with GM-CSF/nanoparticles, a single band of 262 bp was seen in agarose gel electrophoresis from day 1 to day 9 (Fig. 2A), demonstrating that the transcription of the therapeutic gene lasted for at least 9 days in GM-CSF/nanoparticle group. But no band was detected in dogs administered nanoparticles alone (data not shown).

CBC and differential counts
CBC analysis showed significantly increased peripheral white blood cell counts on days 1 and 6 in all dogs administered GM-CSF/nanoparticles (p < 0.05, Fig. 3A). Neutrophils most markedly increased and the numbers of eosinophil and monocyte also increased (Fig. 3B–F). But those increases were not observed in dogs administered nanoparticles alone.

Flow cytometry
On day 6, the G+M (granulocyte plus monocyte) fraction was significantly greater (p = 0.04) and the lymphocyte fraction was significantly lesser (p = 0.04) in dogs administered GM-CSF/nanoparticles than in dogs administered nanoparticles alone. Moreover, the proportion of granulocyte expressing MHC class II in dogs administered GM-CSF/nanoparticles was significantly greater on day 6 than at baseline (p = 0.018), but those increase was not observed in dogs administered nanoparticles alone (p = 0.59) (Table 1, Fig. 4). There were no other within or between group differences in any other surface molecules tested (MHC class I [H58A], pan-lymphocyte [DH52A], B lymphocyte [F46A], CD4 [DH29A], and CD8 [CADO46A]).

Expression of serum GM-CSF
Dogs administered with canine 5 μg/500 μg GM-CSF/nanoparticles showed an average 3.2-fold increase in serum GM-CSF at day 6 (2.44 ± 0.22 ng/mL) compared to baseline (0.76 ± 0.38 ng/mL), whereas dogs administered nanoparticles alone showed no significant change in serum GM-CSF concentrations from baseline. Time-course (24 hours) serial samples were assayed from one dog after 7.5 μg/750 μg GM-CSF/nanoparticles injection. The highest value of GM-CSF was 13.89 ng/mL and this is a 5.2-fold increase over baseline (2.67 ng/mL).

ELISA for detection anti-canine GM-CSF antibodies
Absorbance examined on day 7, 14, and 28 postinjection with nanoparticles alone or GM-CSF/nanoparticles showed no significant change compared to baseline (day 0) and there was no difference in absorbance between the two groups (Fig. 5).
Reinjection of GM-CSF/nanoparticles

Thirty days after first injection led to effective leukocytosis (data not shown) and the patterns of leukogram were similar with those examined at first injection of Figure 3.

Semi-quantitative RT-PCR analysis of cytokines

Interleukin (IL)-10 mRNA expression in PBMC was significantly higher on day 6 than at baseline in dogs injected with GM-CSF/nanoparticles \((p = 0.015, \text{Fig. 6D})\). In contrast, expression of IL-18, IL-1\(\beta\), and transforming growth factor-\(\beta\) mRNA was not significantly changed (Fig. 6A–C), and expression of IL-6 and interferon-\(\gamma\) mRNA could not be detected.

Histopathological examination

No abnormal histopathological findings in organs, such as the liver, kidney, heart, muscle, spleen, lymph nodes, pancreas, uterus, ovary and brain, were observed (data not shown). However, it was not possible to detect fluorescent nanoparticles on prepared slides by confocal microscope.

Confirmation of therapeutic gene transcription from tissue

We also assessed expression of the therapeutic gene in various tissues from one dog administered GM-CSF/nanoparticles by RT-PCR. The specific GM-CSF bands of 262 bp were detected in RNA samples from various tissues such as liver, heart, kidney, lung, muscle, spleen, lymph node, brain, and pancreas (Fig. 2B); however, no band was detected in RT-PCR from the other dog administered nanoparticles alone (data not shown).

Comparison on time profiles of leukocyte counts of dogs injected with GM-CSF plasmid, GM-CSF/nanoparticles and recombinant GM-CSF protein, respectively

As shown at Figure 7, single administration of GM-CSF plasmid combined with nanoparticles at a dose of 1 \(\mu\)g/kg body weight induced increase in significantly leukocyte counts, but the same amount of GM-CSF plasmid, which was not conjugated with nanoparticles, could not. Single
administration of recombinant GM-CSF protein at a rate of 5 μg/kg induced increase in leukocyte counts until 24 hours. After 24 hours, leukocyte counts decreased gradually and returned to the level of baseline at 72 hours after administration; whereas the dogs administered GM-CSF plasmid combined with nanoparticles at a dose of 1 μg/kg body weight showed increased leukocyte counts at 72 hours after administration compared to baseline.

### Discussion

In this study, we sought to test two concepts: that nanoparticles can be used for in vivo gene delivery and that canine GM-CSF/nanoparticles can have possibility to be used to treat transient (acute) canine leukopenia.

Plasmid DNA is a relatively safe alternative to viral vectors for gene therapy, because their toxicity is generally very low, and large-scale production is relatively easy. The relative inefficiency of plasmids in gene transduction, however, has prevented their widespread application [9]. In this study, single administration of GM-CSF plasmid combined with nanoparticles at a dose of 1 μg/kg body weight induced increase in significantly leukocyte counts, but the same amount of GM-CSF plasmid, which was not conjugated with nanoparticles, could not induce the same effect.

Recombinant protein needs continuous infusion twice a day or once a day for its activity [10,11]. Unfortunately, the high cost and low yield of recombinant proteins produced in some systems and the further difficulties with posttranslational protein processing and purification have slowed the progress of producing therapeutic and other beneficial proteins [5].

The surfaces and cores of nanoparticles are readily modified during synthesis. We added tertiary amine to the silica for efficient binding of negatively charged plasmids, leading to successful carriage into cells. Incorporation of a fluorescent dye into the silica shell had an additional advantage, the significant increase in photochemical stability, resulting in minimal photobleaching even after multiple exposures, making it possible to use these particles for optical tracking and monitoring of gene delivery. Following injection of a high dose of fluorescent nanoparticles (25–100 mg/kg) into mice, the fluorescent nanoparticles could be detected by confocal laser scanning microscope [12]. In contrast, GM-CSF–coupled silica nanoparticles injected into dogs could not be detected by confocal microscope, which may have been due to the small volume of injected silica nanoparticles relative to the large body surfaces of the dogs (0.15–0.5 mg/kg).

When the diameter of silica-nanoparticle was >100 nm, solubility in solvent was decreased and when a diameter was <30 nm, homogeneity of particles was not consistent. Thus, we decided that a silica-nanoparticle diameter of 50 nm is a relevant size for gene delivery and size homogeneity was determined by transmission electron microscopy. The silica-nanoparticles retained their monodisperse size distribution after binding of the target gene. At a silica-nanoparticle to plasmid ratio >1:100 (w/w), we did not observe a band indicative of excess plasmid DNA, indicating the complete complexing of DNA and nanoparticle. However, at a weight ratio ≤1:50 (w/w), excess plasmid was observed.

Generally well-known, one of the main drawbacks of viral vectors is the immune responses elicited against both the vector and the vector-encoded protein expressed by infected cells [13]. We did not detect any acute immune responses associated with inflammation, judging from the result that white blood cell counts did not increase in dogs injected with nanoparticles only. Results of ELISA for detection anti-canine GM-CSF antibodies and reinjection of GM-CSF/nanoparticles 30 days after first injection led to effective leukocytosis, indicating that these animals had not generated antibodies against these GM-CSF/nanoparticles. As silica-nanoparticle cannot be degraded within the endocytic processing pathway, silica-nanoparticle with 50-nm diameters is too large to be presented by MHC class II, resulting in no production of anti–silica-nanoparticle antibody. Nevertheless, before application into small animal practice or clinic, mid-/long-term effects or safety of repeated injections should be tested in more large number of experimental dogs.

In general, for peripheralization of the hematopoietic progenitor cells, GM-CSF was preinjected 4 to 5 days earlier. However, the leukogram pattern of blood collected on day 1 showed increased numbers of white blood cells, suggesting that GM-CSF treatment not only induced a marked expansion of hematopoietic stem cells from the mitotic

### Table 1. Flow cytometric analysis of change of granulocyte plus monocyte fraction (G+M), total lymphocyte fraction (L), and fraction of granulocytes expressing MHC class II (G with MHC class II) after gene delivery of GM-CSF/nanoparticles

<table>
<thead>
<tr>
<th></th>
<th>Before (%)</th>
<th>After (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>G+M</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nano-only</td>
<td>55.77 ± 2.32</td>
<td>63.57 ± 3.3</td>
</tr>
<tr>
<td>GM-CSF/nano</td>
<td>58.67 ± 3.33</td>
<td>80.11 ± 4.39*</td>
</tr>
<tr>
<td>L</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nano-only</td>
<td>44.23 ± 2.32</td>
<td>36.43 ± 3.31</td>
</tr>
<tr>
<td>GM-CSF/nano</td>
<td>41.36 ± 3.33</td>
<td>19.89 ± 4.39*</td>
</tr>
<tr>
<td>G with MHC class II</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nano-only</td>
<td>3.52 ± 0.34</td>
<td>7.81 ± 6.84</td>
</tr>
<tr>
<td>GM-CSF/nano</td>
<td>3.50 ± 1.52</td>
<td>11.4 ± 0.89*</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± standard error of the mean.

GM-CSF = granulocyte-macrophage colony-stimulating factor; MHC = major histocompatibility complex.

Significant differences between groups or time are indicated (*,†, p < 0.05):

*Significant difference between two groups.
†Significant difference in data originated from the same group, but determined at a different time analyzed by a paired t-test.
pool in bone marrow as we know, but also stimulated the release of mature granulocytes from the storage pool in bone marrow into bloodstream.

GM-CSF, interferon-γ, and IL-3 can induce MHC class II on pure cultures of human polymorphonuclear leukocyte (PMN) in vitro, suggesting that granulocytes may be able to participate directly in the immune response as antigen-presenting cells [14]. Lloyd and Oppenheim [14] suggested that, depending on whether PMN can provide all the necessary signals for T-cell activation, PMN can either stimulate or inhibit the disease process through their effects on the immune response [14]. But it has not been definitively demonstrated that PMN can synthesize and express MHC class II in vivo. In addition, there has been a very limited number
of studies addressing this issue only in humans and mice [15–17], with no canine study. To our knowledge, this study is the first to show that GM-CSF can induce expression of MHC class II molecules on canine granulocytes in vivo.

Immunostimulation effects of GM-CSF, such as antimicrobial defense or action as vaccine adjuvant, have been generally well-known. We focused on the fact that, revealed recently, GM-CSF also has an immunosuppression effect in autoimmune thyroiditis [18]. The hypothesis, which Lloyd and Oppenheim [14] addressed in their study, could explain these ambilateral aspects of GM-CSF of immunosuppression activity due to MHC class II expression on granulocytes by GM-CSF. If immunostimulation is required, MHC class II expression by antigen-presenting cells will be increased along with increased cytokine secretion. For example, secretion of IL-1 increases the expression of IL-2 and IL-2R, thus leading to activation of the immune system. If, however, there is no antigen stimulation or presentation of a self-antigen, the MHC class II of granulocytes would be increased, whereas the total MHC class II expression would not change or be decreased or slightly increased, due to insufficient T cell activation caused by the scarcity of cytokines or costimulatory signals, resulting in immune equilibrium or immune anergy. Further research on the role of MHC class II expression on granulocytes should be conducted by the careful examination of the immunoregulatory properties of GM-CSF.

Gangi et al. [18] suggested that IL-10 secreted by CD4+CD25+ regulatory T cells in response to GM-CSF played a crucial role in the prevention or healing of autoimmune thyroiditis [18]. Our result on mRNA expression was similar with the study of Gangi et al.; we observed no qualitative differences in expression of IL-18, IL-1β, and transforming growth factor-β mRNA following injection of GM-CSF/nanoparticles, whereas IL-10 mRNA expression in PBMC increased relative to baseline, indicating that IL-10 may be involved in the immunomodulatory effects of GM-CSF treatment.

Dogs administered GM-CSF/nanoparticles showed a significant increase in serum GM-CSF relative to baseline. When the amount of administered GM-CSF increased from 5 to 7.5 µg, the increases in CBC counts and serum GM-CSF concentration were also amplified. Delivered gene with nanoparticles was expressed in various tissues, which is similar with the results of the

Figure 5. Assay of anti–granulocyte-macrophage colony-stimulating factor (GM-CSF) antibodies. Absorbance of antibody (Abs) against GM-CSF was determined in dog sera on 0, 7, 14, and 28 days after injection with nanoparticles alone (control group) or GM-CSF/nanoparticles (GM-CSF/nano group). Compared to control group, absorbance of anti–GM-CSF antibodies in GM-CSF/nano group was not significantly different. Also, any titer of sera collected on day 7, 14, and 28 showed no significant change compared to baseline (day 0). ELISA = enzyme-linked immunosorbent assay.

Figure 6. Expression of (A) interleukin (IL)-10, (B) IL-18, (C) IL-1β, and (D) transforming growth factor-β mRNA (mean with SEM) in peripheral blood mononuclear cells (PBMC) of dogs administered nanoparticles alone (n = 3) and granulocyte-macrophage colony-stimulating factor (GM-CSF)/nanoparticles (n = 6) at baseline and on day 6.
The specific therapeutic gene was expressed in vivo for at least 9 days after nanoparticle injection, as well as in all the organ tissues we examined. Target gene delivery using silica-nanoparticles under the current conditions has limited long-term expression. We are planning to modify the surface of silica coat with specific peptide acting as linker to gene, which would improve the short-term gene expression needed, such as the case of acute leukopenia. Because repeated injections are possible and the effect of GM-CSF is dose-dependent, we can control the duration and degree of leukocytosis by adjusting the injection frequency and dose. We are examining the preventive and treatment effects of GM-CSF/nanoparticles by applying it into the dogs with leukopenia. This study is the first to describe use of a silica-nanoparticle delivery system, subsequent functional gene expression over long periods of time and readministration effect in a large animal model.

Acknowledgments

We would like to thank Sook Shin (Department of Veterinary Microbiology) for her outstanding technical assistance. This work was supported by the Korea Research Foundation Grant funded by the Korean Government (MOEHRD) (KRF-2006-311-E00526). Further support was also provided by other Korea Research Foundation Grants (KRF-2006-005-J02902 and KRF-2006-005-J02903) and the Research Institute of Veterinary Science, College of Veterinary Medicine, Seoul National University and the Brain Korea 21 Program for Veterinary Science.