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Research paper

Plasmid-DNA loaded chitosan microspheres for in vitro IL-2 expression

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Abstract

Interleukin-2 (IL-2) expression plasmid (pCXWN-hIL-2) loaded chitosan microspheres were evaluated for using in gene-based immunotherapy. Chitosan microspheres containing pCXWN-hIL-2 were prepared by using a precipitation technique. In addition, the effects of different factors such as the concentration (0.35–0.70%) and the molecular weight of chitosan (low and medium molecular weights), the plasmid amount (5–10 μg/ml) and the presence of glutaraldehyde during the encapsulation process, on microsphere characteristics were investigated. The size of microspheres changed between 1.45 and 2.00 μm. All the formulation factors affected the size of microspheres. The structure of plasmid remained unchanged during the encapsulation process and the release studies. Plasmid encapsulation efficiency of chitosan microspheres was high (82–92%). The zeta potential values of microspheres was approximately +5.2 to +12.4 mV. In vitro release properties of microspheres changed with formulation variables. In vitro release of DNA changed with the concentration and molecular weight of chitosan and initial plasmid amount. Addition of glutaraldehyde is not necessary for a formulation. MAT-LyLu, the rat prostate adenocarcinoma cell line, was used for the determination of the in vitro transfectional activity of IL-2 encoding plasmid DNA loaded chitosan microspheres and the level of IL-2 expressed into the cells was assayed using a ELISA kit. High level of IL-2 expression was obtained with plasmid-loaded chitosan microspheres. Microspheres showed similar IL-2 production as lipofectin. The molecular weight of chitosan used and the amount of plasmid influenced the in vitro IL-2 production in the cells. Encapsulation of IL-2 encoding gene into chitosan microspheres might be a useful strategy to increase the expression and to control the delivery of cytokine gene to cells.

Keywords: Interleukin-2; Chitosan; Microspheres; Gene delivery; Plasmid DNA; In vitro transfection; Gene therapy

1. Introduction

Cytokines are important modulators of the host antitumor response. The cytokine interleukin-2 (IL-2) is known to have potent antitumor activity, but its dosage tends to be extremely high, because of its short half-life [1]. The efficacy of IL-2 protein-based treatments has been limited by a significant toxicity of the systemically administered drug. In addition systemically administered IL-2 may have no effect in the intratumoral cytokine enviroment [2]. The delivery of cytokine genes could offer an attractive alternative to cytokine injection if a sustained expression of the delivered gene could be achieved [3,4].

However, there are numerous safety concerns associated with the use of viruses as vectors in humans, as well as in animals. Viral vectors expressing IL-2 at high doses may also induce IL-2 dependent toxicity, sometimes resulting in death [5]. Recently, cytokine genes have been administered via non-viral gene delivery systems to increase the stability and the efficacy of cytokine gene expression. Lipid-complexed forms of hIL-2 gene are currently in a phase I clinical trial for prostate cancer [2].

Tumour regression was obtained using IL-2 plasmid DNA/lipid complex injection in human renal carcinoma [6]. Oh et al. [7] studied mIL-2 plasmid/PEI complex in a macrophage cell line and reported highly enhanced IL-2 expression by complexation with PEI.

Chitosan is a natural polysaccharide. It has several advantageous qualities in comparison with other non-viral vectors in that it is relatively non-toxic and has high transfection efficiency [8–10]. The preparation and in vivo transfection properties of chitosan microspheres containing

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plasmids were reported by Özbaş-Turan et al. [11] and Akbuğa et al. [12].

In this study, encapsulation of hIL-2 encoding gene into chitosan microspheres was investigated. The effect of formulation variables on microsphere properties and in vitro transfection efficiency were also studied.

2. Materials and methods

2.1. Materials

Chitosans (Medium; mol. wt: ca. 400,000 Da, viscosity: ca. 200 mPa and low; mol. wt: ca. 150,000 Da, viscosity: ca. 100 mPa in 1% acetic acid at 20 °C) with a deacetylation grade of about 87% were purchased from Fluka (Germany). Sodium sulphate came from Carlo Erba, Italy. All of the cell culture media, reagents and Lipofectin were purchased from Gibco-BRL (Life Technologies, USA). Other chemicals were of pharmaceutical or molecular grade.

2.2. Plasmid construction and isolation

pCXWN-hIL-2 plasmid DNA encodes human IL-2 [13]. The plasmid DNA is of 6.3 kb and contains chicken β-actin promoter. pCXWN-hIL-2 was kindly provided by Prof. Dr Jun-ichi Miyazaki (Kumamoto University, Medical School, Japan).

Plasmid was amplified in *Escherichia coli* DH5α, extracted by the alkaline lysis method, and purified by phenol/chloroform extraction followed by PEG:NaCl extraction and ethanol precipitation [14]. The quantity and quality of the purified plasmid DNA were assessed spectrophotometrically at 260 and 280 nm (Shimadzu UV-Biospec 1601, Japan) and also by electrophoresis in agarose gels.

2.3. Cell line

The rat prostate adenocarcinoma cell line (MAT-LyLu) was obtained from the European Type Culture Collection (ECAC 94101454). Cells were cultured in RPMI 1640 (Sigma, USA) supplemented with 2 mM L-glutamine (Sigma, USA), 250 nM dexamethasone (Applichem, Germany), 10% foetal bovine serum (FBS; Gibco, USA) and 0.1% antibiotic solution (penicillin (10,000 units/ml), streptomycin (10 mg/ml) and amphotericin (10 mg/ml)) in humidified atmosphere (5% CO_2, 95% air, 37 °C) (Heto-Holten, Denmark). Cells were grown until confluent then trypsinized with trypsin solution (0.05% trypsin and 0.05% EDTA) for 5 min. The detached cells were collected by centrifugation at $100 \times g$, 3 min (Hettich, Germany) and resuspended in 5 ml medium and counted using a hemocytometer.

2.4. Preparation of plasmid-loaded chitosan microspheres

Plasmid-loaded chitosan microspheres were prepared according to earlier reports of Berthold et al. [15] and Aral et al. [16] Briefly, pCXWN-hIL-2 plasmid DNA (500 or 1000 μg) was added to 50 ml of sodium sulphate solution (20%, w/v) and this mixture was dropped into 50 ml of acidic solution of chitosan (0.35, 0.50 and 0.70%, w/v) and stirred (Ika-Werk, Germany) for an hour at 500 rpm. Formed microspheres were washed with bidistilled water three times and separated by centrifugation at 12,000 × g then freeze-dried (Leybold-Lyovac, Germany). Microspheres were stored at 4 °C in a desiccator.

In order to study the effect of formulation variables (concentration and molecular weight of chitosan, plasmid amount, use of glutaraldehyde as a cross-linker) on microsphere properties, different microsphere formulations were prepared (Table 1). Each formulation batch was prepared at least three times. Encapsulation efficiency was calculated by measuring the difference between the total amount of DNA added in the preparation medium and the amount of non-entrapped DNA remaining in the aqueous supernatant suspension after the coacervation process. For this purpose, the supernatant was spectrophotometrically analyzed at 260 and 280 nm for DNA concentration [11,17].

Table 1
Codes, formulation and properties of pCXWN-hIL-2 loaded chitosan microspheres ($n = 5$)

Code	Chitosan conc. (%)	Amount of pDNA (μg)	Glutaraldehyde[a] (ml)	Encapsulation efficiency (%) ± SD	Particle size (μm ± SD)
I_1	0.50	500	–	88.1 ± 0.4	1.8 ± 0.4
I_2	0.35	500	–	89.5 ± 1.5	1.6 ± .04
I_3	0.70	500	–	82.9 ± 2.7	2.0 ± 0.4
I_4	0.35	1000	–	82.1 ± 1.9	1.5 ± 0.4
I_5	0.35	500	1	92.1 ± 1.7	1.5 ± 0.4
I_6	0.70	500	1	87.3 ± 1.8	2.0 ± 0.4
I_7	0.50[b]	500	–	85.2 ± 2.8	1.5 ± 0.4

[a] Glutaraldehyde solution (12.5%).
[b] Low molecular weight chitosan.

2.5. Characterization of pCXWN-hIL-2 loaded chitosan microspheres

The morphological examination of the microspheres was performed by scanning electron microscopy (SEM) (Joel, JSM 5200, Japan). Size of microspheres was determined by using an ocular micrometer in a light microscope (Olympus BH, Japan), ($n = 1000$ particles). The zeta potential was determined in pH 7.4 phosphate buffered saline (PBS, BP) with Malvern 3000 HS_A Instruments (UK).

2.6. Agarose gel electrophoresis of pDNA

The integrity of the free and the encapsulated plasmid DNA was analyzed by electrophoresis. Plasmid DNA (500 ng equivalent of DNA in each lane) was applied into a 0.8% agarose gel containing tris–boric acid–EDTA buffer (TBE, pH 8.3) and ethidium bromide (0.5 μg/ml) at constant voltage (80 V) (Horizontal gel apparatus system, ATTO, Japan). Plasmid was visualized under UV-light (Vilber Lourmat, USA) and the conformation of DNA was checked using the gel documentation system (Kodak Digital Science, DC40 Camera and 1D Image Analysis Software, USA). The percentage of DNA in the supercoiled conformation was determined using the following equation [18]:

$$\text{SC}\ (\%) = \frac{V_{SC} - V_O}{[(V_{SC} + V_L + V_{OC}) - (3 \times V_O)]}$$

V_{SC}, volume of super coiled (SC) DNA; V_L, volume of linear DNA; V_{OC}, volume of open circular DNA; V_O, volume of background.

2.7. In vitro release studies

Plasmid DNA release from chitosan microspheres was determined by incubating the microspheres in PBS at 37 ± 0.5 °C. At appropriate time intervals samples were centrifugated and the supernatant was replaced by fresh medium, the amount of DNA released in the supernatant was measured spectrophotometrically at 260 nm using the supernatant of non-loaded chitosan microspheres for basic correction. Released samples were checked with agarose gel electrophoresis as described above after precipitation with ethanol. Each formulation batch was tested in duplicate ($n = 6$).

2.8. In vitro transfection

In vitro transfection studies were performed in MAT-LyLu, a rat prostate adenocarcinoma cell line (ECAC 94101454). The cells (5×10^4 cells/well) were seeded in 24-well plates (Greiner, Germany) and allowed to grow for 18–24 h for obtaining 75–80% confluence. After dilution with medium, 100 μg of plasmid loaded microspheres having an amount of DNA equivalent to 150–300 ng per well were added to the cells and incubated for 48 h. Then the cells were washed with PBS to remove all non-penetrated microspheres. Fresh medium was added to cells and cells were incubated for an additional 6 h. Medium was removed and the cells were washed with ice cold PBS and collected by centrifugation. The cells were then lysed by cold-heat treatment (Incubation at −20 °C for 30 min in a deep-freezer and then 37 °C for 30 min). Cell debris was removed by centrifugation at $12{,}000 \times g$. The supernatant was removed and expression of IL-2 was determined by ELISA (h-Interleukin-2 ELISA kit, Roche, Germany).

Lipofectin was used according to the manufacturer's procedures. Each well received 1 μg lipofectin that was complexed with 1–2 μg of DNA.

In some experiments, ascorbate (Roche, Germany) was added to the medium in order to investigate the effect of ascorbate on transfection property of the cells.

2.9. Statistical analysis

The significance of data was determined by the Student's *t*-test. A value of $P < 0.05$ was considered to indicate significant difference.

3. Results and discussion

A scanning electron micrograph (SEM) of a pCXWN-hIL-2 loaded chitosan microsphere is given in Fig. 1. Microspheres prepared according to different formulations are about 1.45–2.00 μm in size and spherical in shape (Table 1). All the formulation factors studied affected the size of microspheres ($P < 0.05$). Particle size increased as the chitosan concentration increased from 0.35 to 0.70% ($P < 0.05$) (Table 1). Smaller microspheres were obtained

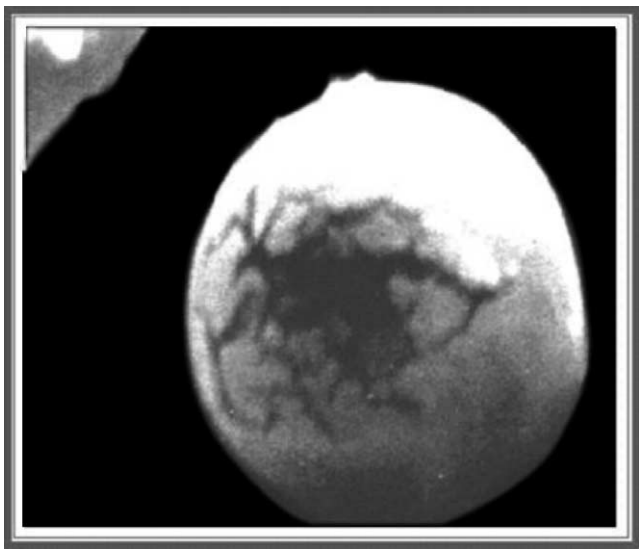

Fig. 1. SEM photograph of pCXWN-hIL-2 loaded chitosan microsphere.

with low molecular weight of chitosan (150,000 Da) ($P < 0.05$). These results showed similarity with the data of Mao et al. [17].

The zeta potential of microspheres was measured at pH 7.4 in PBS. The zeta potentials of I_2,I_3,I_4 and I_7 formulations are 6.9 ± 1.0, 10.1 ± 0.8, 5.2 ± 1.8 and 12.4 ± 1.5 mV, respectively. Microspheres had a slightly positive surface charge. Zeta potential was slightly increased when the concentration of chitosan increased. The amount of plasmid did not affect the zeta potential value.

As seen in Table 1, plasmid encapsulation efficiency of microspheres was about 85%. Plasmid encapsulation efficiency was affected by the initial plasmid amount ($P < 0.05$) (I_2 and I_4). The higher encapsulation efficiency was obtained with the lower amount of plasmid. However, the other factors (chitosan concentration and using low molecular weight of chitosan and the addition of glutaraldehyde) studied did not affect the encapsulation efficiency significantly ($P > 0.05$).

To determine whether the encapsulation procedure affected the integrity of plasmid DNA, agarose gel electrophoresis was applied after microsphere preparation. Agarose gel electrophoresis of released plasmid showed that plasmid DNA was present in supercoiled form (Fig. 2). As seen in this gel photograph, there was no additional band.

In order to select the release medium, bidistilled water, tris-acetate buffer (pH 5.5, BP), tris-chloride buffer (pH 7.4, BP) and phosphate buffered saline (pH 7.4, BP) were tested as a medium. Release profiles of plasmid-loaded chitosan microspheres in different release media are given in the inset of Fig. 5. The slowest release was obtained in bidistilled water and the faster DNA release was in pH 5.5. However, a significant difference was not found between the release profiles of microspheres obtained in the PBS and tris-chloride buffer at pH 7.4 ($P > 0.05$). Therefore PBS was used as a medium because of its suitability for cell culture studies.

In vitro release profiles of pCXWN-hIL-2 plasmid from chitosan microspheres are shown in Figs. 3–6.

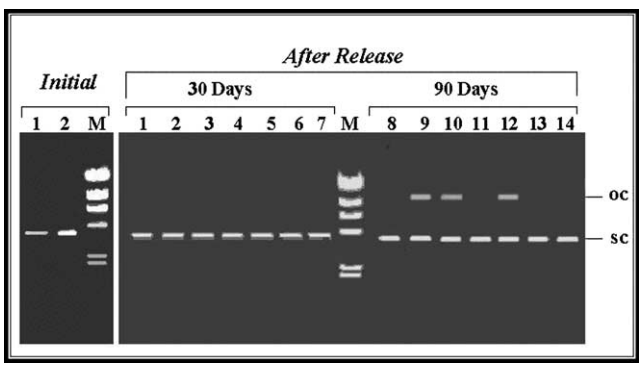

Fig. 2. Gel photographs of pDNA; after isolation (lanes 1 and 2) and release studies (lanes 1–7 and 8–14). M is *Hind* III digested λ DNA marker. Lanes 1–7 are pDNA released from microspheres (I_1–I_7) after 30 days. Lanes 8–14 are pDNA released from microspheres (I_7–I_1) after 90 days.

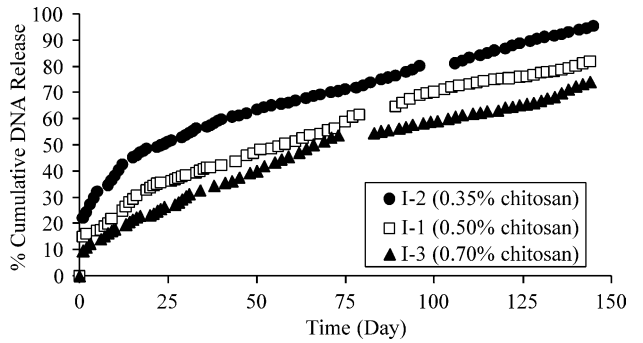

Fig. 3. Effect of chitosan concentration on release behaviour of pCXWN-hIL-2 loaded chitosan microspheres ($n = 6$).

All release profiles of the microspheres are similar and exhibit a small burst release of about 10–20% in the first 24 h and then slow release at constant rate. Effect of chitosan concentration ($I_1 - I_3$) on pDNA release from microsphere is shown in Fig. 3. DNA release significantly changed with the chitosan concentration of microspheres ($P < 0.05$). Microspheres prepared with the highest concentration of chitosan (I_3) provided the lowest DNA release. These data are consistent with previous reports of Jong et al. [19] and Aral et al. [16] concerning the reporter gene-loading chitosan microspheres. Microspheres prepared with high molecular weight of chitosan indicated slow DNA release ($P < 0.05$). As shown in Fig. 4, as the molecular weight of chitosan increased, the release of plasmid DNA from microspheres decreased (I_1 and I_7). This indicates that molecular weight of chitosan is important in DNA release properties as earlier reported by Xu and Du [20]. Moreover, DNA release was affected by the amount of plasmid entrapped. An increase in DNA content of microspheres (compare I_2 and I_4) resulted in a decrease in plasmid release ($P < 0.05$) (Fig. 5). These data are consistent with our previous findings [11].

Chitosan microspheres were prepared using sodium sulphate as a precipitant according to method of Berthold et al. [15]. The microspheres are charged positively although sulphate ions are used as precipitant. The zeta potential of free microspheres (having no plasmid) was

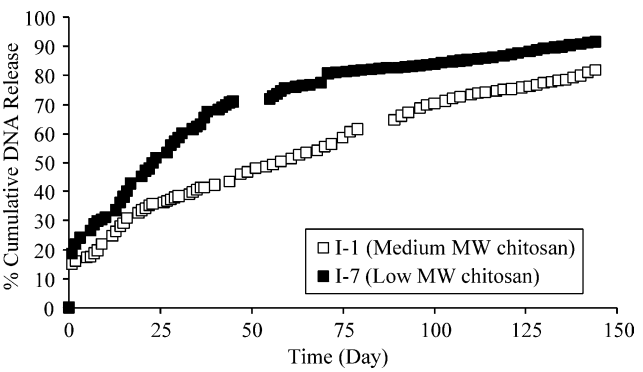

Fig. 4. Effect of the molecular weight of chitosan on release behaviour of pCXWN-hIL-2 loaded chitosan microspheres ($n = 6$).

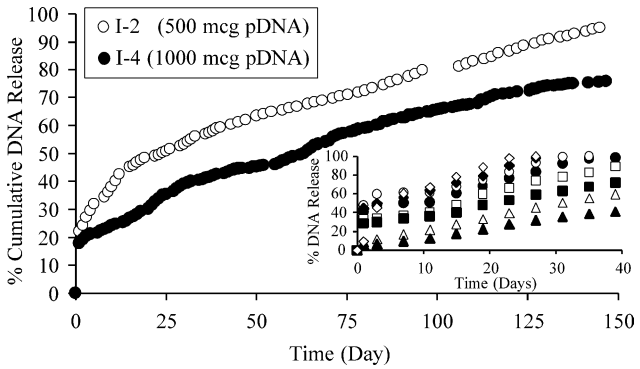

Fig. 5. Effect of initial plasmid amount on release behaviour of pCXWN-hIL-2 loaded chitosan microspheres. Inset: effect of release medium on the DNA release from microspheres (Bidistilled water (△I_2, ▲I_4), tris-chloride buffer pH 7.4 (●I_2, ○I_4), PBS; (□I_2, ■I_4), tris-acetate buffer pH 5.5 (◆I_2, ◇I_4)) ($n = 6$).

approximately 7.0 mV (data not shown). Addition of plasmid had no great effect on the zeta potential values of microspheres (Table 1). As seen in agarose gel photograph of microspheres, full complexation is not present between DNA and chitosan. Encapsulation of pDNA is dominant in this system. This indicates that only a part of amino groups are neutralized during microsphere formation. The residual amino groups would be responsible for the positive zeta potential.

Truong-Le et al. [21] have shown that glutaraldehyde could effectively stabilize nanoparticles without damaging the plasmid DNA, therefore glutaraldehyde was used in this study. No damage was observed in DNA structure after using glutaraldehyde as a co-crosslinker (Fig. 2). The experiment using glutaraldehyde as a cross-linker reduced the pDNA release from microspheres (compare I_2 and I_5) ($P < 0.05$) and (compare I_3 and I_6) ($P < 0.05$) (Fig. 6). This may be due to stronger microsphere structure. However, as reported by Mao et al. [17] particles formed of low pH can remain physically stable at physiological pH without crosslinking.

The integrity of the incorporated DNA is retained after the encapsulation process and throughout the 90 days release (Fig. 2). However, in some formulations (I_3,I_5 and I_6) after 90 days the conformation of pDNA slightly

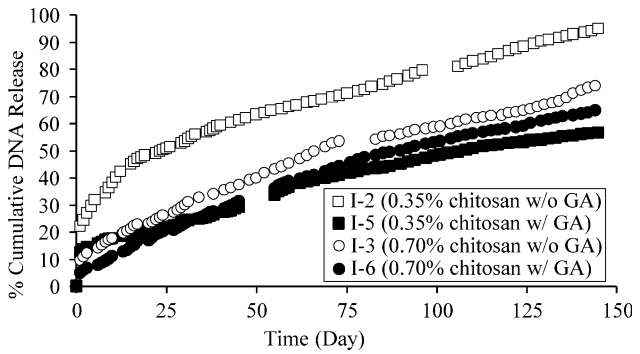

Fig. 6. Release profiles of pCXWN-hIL-2 loaded chitosan microspheres prepared with (w/) or without (w/o) glutaraldehyde ($n = 6$).

changed and the open circular form of DNA appeared in the gel (Fig. 2). As seen in the figure the change in pDNA topology was not observed in the gel photograph of chitosan microspheres prepared with low molecular weight chitosan (I_7).

3.1. In vitro transfection results

To determine the in vitro transfectional activity of IL-2 encoding plasmid DNA-loaded chitosan microspheres, the MAT-LyLu cell line was used and protein production was measured using ELISA. As seen in Fig. 7, high level cytokine expression was measured in cells incubated with plasmid loaded chitosan microspheres. A significant difference was not found between the control group and empty chitosan microspheres ($P > 0.05$). Lipofection reagent was used in comparison. Transfection efficiencies of chitosan and lipofection in the cell line are depicted in Fig. 7. Chitosan microspheres showed similar IL-2 production as lipofectin. When comparing the protein values at 48 and 72 h, higher IL-2 production was obtained at 48 h after transfection with microspheres (Fig. 7). This may be due the release of surface DNA from microspheres within the first 48 h. As mentioned above, a burst effect was seen in the release profiles of microspheres within the first day (Figs. 3–5).

When a comparison was made, higher transfection activity was obtained with microspheres containing higher amount of DNA (I_2 and I_4) ($P < 0.05$). On the other hand, microspheres prepared with low molecular weight chitosan (I_7) expressed slightly higher level of IL-2 than the high molecular weight chitosan (I_1) ($P < 0.05$) (Fig. 8). Using glutaraldehyde during the microsphere preparation (compare I_3 and I_6) reduced the in vitro protein production ($P < 0.05$) (Fig. 8). These results are in agreement with the previous report of Mao et al. [17]. Huang et al. [22] studied the uptake of chitosan nanoparticles by A549 cells and noted that cellular uptake of chitosan nanoparticles was concentration dependent, our results showed similarity to this report of Huang et al. [22]. Transfection increased

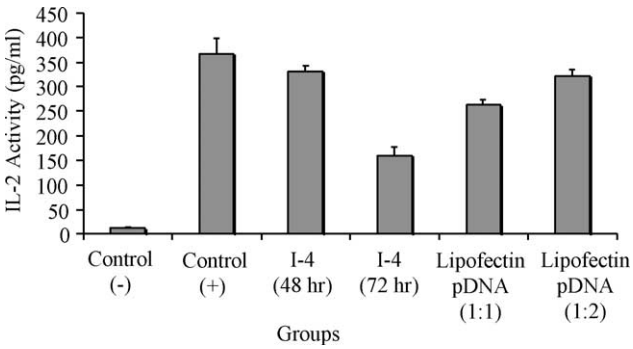

Fig. 7. Comparison of IL-2 expression in MAT-LyLu cells transfected with chitosan-pDNA microspheres and lipofectin-pDNA complexes. hIL-2 was used as a control (+). Cell culture medium incubated with empty chitosan microspheres were used as a control (−) ($n = 4$).

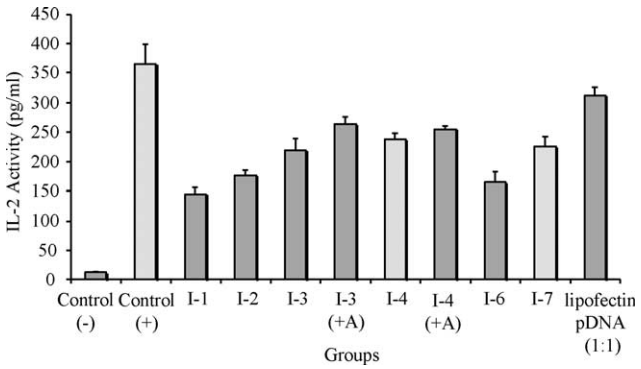

Fig. 8. In vitro expression of IL-2 in the cells transfected with pCXWN-hIL-2 plasmid loaded microspheres. Microspheres were prepared in different formulations (I_1–I_7). hIL-2 was used as a (+) control. The effect of ascorbate (+A) on transfection was investigated. Cell culture medium incubated with empty chitosan microspheres were used as a control ($n = 4$).

when the chitosan concentration was increased (compare I_1 and I_3).

Briefly, the molecular weight of chitosan and the amount of plasmid are important in the in vitro IL-2 production of chitosan microspheres containing pCXWN-hIL-2. In this study, ascorbate was added into the medium during the in vitro cell transfection because of outstanding effect. Ascorbate is required for normal defence and functions in cellular redox systems [23,24]. On the other hand, addition of ascorbate into the medium, increased the level of protein production slightly as seen in Fig. 8 ($P < 0.05$).

Fast, brownian motion of small complexes and nanoparticles may prevent them from reaching the cells. This may be some low transfection results [8]. In this respect, microspheres may offer advantages over nanoparticles.

On the other hand, there have been reports that non-viral carriers bind to cells via their net positive charge. Surprisingly a decrease of surface charge of nanoparticles did not affect the transfection efficiency of chitosan nanoparticles [17]. In this study, the effect of zeta potential on endocytosis is not clear. Increases in the plasmid amount (compare I_2 and I_4) and the molecular weight (compare I_3 and I_7) of chitosan did not significantly affect the zeta potential of microspheres ($P > 0.05$), but these factors had effects on the transfection ($P < 0.05$). The relationship between transfection activity and cell uptake of plasmid/chitosan complexes has not to be clearly illustrated [10,17].

Cell transfection mechanism of chitosan–DNA microspheres and nanoparticles is not clear but cell-dependent [9]. There are several hypotheses to explain the transfection mechanism of chitosan [8,10]. Ishii et al. [10] reported that cell uptake of plasmid/chitosan complexes might be thermal and energy dependent and that complexes might be endocytosed and possibly released from endosomes due to swelling of complex, causing the endosome rupture.

To date, no attempts have been made to encapsulate a cytokine gene in a microsphere system. Our results suggest that encapsulation of IL-2 encoding plasmid into chitosan microspheres might be a useful strategy for increasing the expression and the delivery of cytokine genes to immune cells. Chitosan is preferred as a delivery system because of non-toxicity. With these microsphere systems, manipulation of the protein amount is also possible by changing the formulation factors.

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References

[1] M.T. Lotze, L.W. Frana, S.O. Sharrow, R.J. Robb, S.A. Rosenberg, In vivo administration of purified human interleukin-2. Half life and immunologic effects of Jurkat cell-line derived interleukin-2, J. Immunol. 134 (1985) 157–166.
[2] A. Belldegrun, C-L. Tso, A. Zisman, J. Naitoh, J. Said, A.J. Pantuck, A. Hinkel, J. DeKernion, R. Figlin, Interleukin-2 gene therapy for prostate cancer: phase I clinical trial and basic biology, Hum. Gene. Ther. 12 (2001) 883–892.
[3] G.J. Prud'homme, B.K. Lawson, Y. Chang, A.V. Theofitopoulos, Immuno-therapeutic gene transfer into muscle, Trends. Immunol. 22 (2001) 149–155.
[4] J. Jiang, E. Yamato, J. Miyazaki, Intravenous delivery of naked plasmid DNA for in vivo cytokine expression, Biochem. Biophys. Res. Commun. 289 (2002) 1088–1092.
[5] C.L. Addison, T. Braciak, R. Ralston, W.J. Müller, J. Gauldie, F.L. Graham, Intratumoral injection of an adenovirus expressing interleukin-2 induced regression and immunity in a murine breast cancer model, Proc. Natl. Acad. Sci. USA 92 (1995) 8522–8526.
[6] D.M. Hoffman, R.A. Figlin, Intratumoral interleukin-2 for renal-cell carcinoma by direct gene transfer of a plasmid DNA/DMRIE/DOPE lipid complex, World J. Urol. 18 (2000) 152–156.
[7] Y.K. Oh, D. Suh, J.M. Kim, H-G. Choi, K. Skin, J.J. Ko, Polyethyleneimine-mediated cellular uptake, nucleus trafficking and expression of cytokine plasmid DNA, Gene Ther. 9 (2002) 1627–1632.
[8] P. Erbacher, S. Zou, T. Bethinger, A.M. Steffan, J.S. Remy, Chitosan based vector/DNA complexes for gene delivery: biophysical characteristics and transfection ability, Pharm. Res. 15 (1998) 1332–1339.
[9] K.W. Leong, H.Q. Mao, V.L. Truong-Le, K. Roy, S.M. Walsh, J.T. August, DNA-polycation nanospheres as non-viral gene delivery vehicles, J. Cont. Rel. 53 (1998) 183–193.
[10] T. Ishii, Y. Okayata, T. Sato, Mechanism of cell transfection with plasmid/chitosan complex, Biochim. Biophys. Acta 1514 (2001) 51–64.
[11] S. Özbaş-Turan, C. Aral, L. Kabasakal, M. Keyer-Uysal, J. Akbuğa, Co-encapsulation of two plasmids in chitosan microspheres as a non-viral gene delivery vehicle, J. Pharm. Pharmaceu. Sci. 6 (2003) 27–32.
[12] J. Akbuğa, C. Aral, S. Özbaş-Turan, L. Kabasakal, M. Keyer-Uysal, Transfection efficiency of chitosan microspheres: effect of DNA topology, S.T.P. Pharm. Sci. 13 (2003) 99–103.
[13] H. Niwa, K-I. Yamamura, J-I. Miyazaki, Efficient selection for high-expression transfectans with a novel eucaryotic vector, Gene 108 (1991) 193–199.
[14] J. Sambrook, E.F. Fritsch, T. Maniatis, Molecular Cloning: a Laboratory Manual, Cold Spring Harbor Laboratory Press, New York, 1989.

[15] A. Berthold, K. Cremer, J. Kreuter, Preparation and characterization of chitosan microspheres as drug carriers for prednisolone sodium phosphate as model for anti inflammatory drugs, J. Cont. Rel. 39 (1996) 17–25.
[16] C. Aral, S. Özbaş-Turan, L. Kabasakal, M. Keyer-Uysal, J. Akbuğa, Studies of effective factors of plasmid DNA-loaded chitosan microspheres: I. Plasmid size, chitosan concentration and plasmid addition techniques, S.T.P. Pharm. Sci. 10 (2000) 83–88.
[17] H-Q. Mao, K. Roy, V.L. Troung-Le, K.A. Janes, K.Y. Lin, Y. Wang, J.T. August, K.W. Leong, Chitosan–DNA nanoparticles as gene carriers: synthesis characterization and transfection efficiency, J. Cont. Rel. 70 (2001) 399–421.
[18] S. Ando, D. Putnam, D.W. Pack, R. Langer, PLGA microspheres containing plasmid DNA: preservation of supercoiled DNA via cryopreparation and carbohydrate stabilization, J. Pharm. Sci. 88 (1) (1999) 126–130.
[19] Y.S. Jong, J.S. Jacob, K-P. Yip, G. Gardner, E. Seitelman, M. Whitney, S. Montgomery, E. Mathiowitz, Controlled release of plasmid DNA, J. Cont. Rel. 47 (1997) 123–134.
[20] Y. Xu, Y. Du, Effect of molecular structure of chitosan on protein delivery properties of chitosan nanoparticles, Int. J. Pharm. 250 (2003) 215–226.
[21] V.L. Truong-Le, J.T. August, K.W. Leong, Controlled gene delivery DNA–gelatin nanospheres, Hum. Gene Ther. 9 (1998) 1709–1717.
[22] M. Huang, Z. Ma, E. Khor, L-Y. Lim, Uptake of FITC-chitosan nanoparticles by A549 cells, Pharm. Res. 19 (2002) 1488–1494.
[23] I.L. Chapple, Reactive oxygen species and antioxidants in inflammatory diseases, J. Clin. Periodontol. 24 (1997) 287–296.
[24] J. Chandiere, R. Ferrari-Illion, Intracellular antioxidants: from chemical to biochemical mechanisms, Food Chem. Toxicol. 37 (1999) 949–962.