Fig. 3. Effect of P/L Ratio of the Lipoplex on Gene Expression in Lungs of Normal and B16BL6 Tumor-Bearing Mice

Lipoplexes, prepared as described in Materials and Methods, were intravenously injected in normal mice or tumor bearing mice at 14 d post-inoculation of B16BL6 cells. Each mouse received 30 μg of pDNA. The lungs of the animals were harvested 24 h after injection. The luciferase activity in the lungs was determined, as described in Materials and Methods, 24 h after lipoplex injection. Open column, normal mouse; filled column, tumor-bearing mouse. Data represent the mean±S.D. ($n=4$).

in tumor-bearing mice, a relatively higher gene expression in the lungs was obtained at several P/L ratios, whereas gene expression in various another organs was very low or absent (data not shown). The rank order of increased gene expression level was for P/L ratios of 80, 8, 40, 100 and 20. The tendency observed in tumor-bearing mice reflects the *in vitro* lipofection results obtained in the absence of serum rather than those in the presence of serum.

To further examine the *in vivo* transfection efficiency, the lipoplex was intravenously injected into tumor-bearing mice at the indicated days after tumor cell inoculation. Gene expression in the lung was assayed 24 h after lipoplex injection. Two different lipoplexes with P/L ratios of 8 and 80, which showed relatively higher transgene expression (Fig. 3), were used in this study. The progression of tumor in lung was estimated by counting the number of tumor nodules on the surface of the lung. As shown in Fig. 4A, the extensive progression of tumor was not observed until at 14—15 d after tumor cell inoculation. For both lipoplexes, while tumor bearing mice showed no significant gene expression at earlier periods (until 7 d after tumor inoculation), they showed significant gene expression at a later period (over 10 d after tumor inoculation) (Fig. 4B). With the lipoplex (8 P/L ratio), significant gene expression occurred when the lipoplex was administered at 10 d after tumor inoculation, then reached a maximum at 14 d and remained constant even at 17 and 21 d. In the case of the lipoplex (80 P/L ratio), significant gene expression occurred when the lipoplex was given at 10 d after tumor inoculation, reached a maximum level at 14 d and then decreased almost to the control level up to 21 d. The level of gene expression by the lipoplex with a 80 P/L ratio was 2-times higher than that for the lipoplex with a 8 P/L ratio at earlier after tumor inoculation, but this value became 8-times lower later. There was no obvious relation between the gene expression induced by TFL-3 and the number of tumor nodules in the lung.

Fluorescence Microscopic Study of Lung The lipoplex (gWIZ-GFP/ TFL-3) with a P/L ratio of 8 or 80 was intravenously injected into tumor-bearing mice at 14 d post-tumor inoculation. In the normal lung, GFP expression was observed in endothelial cells of the microvasculature and there was no significant difference in the area of GFP expression with both lipoplexes tested (data not shown). In the tumor-
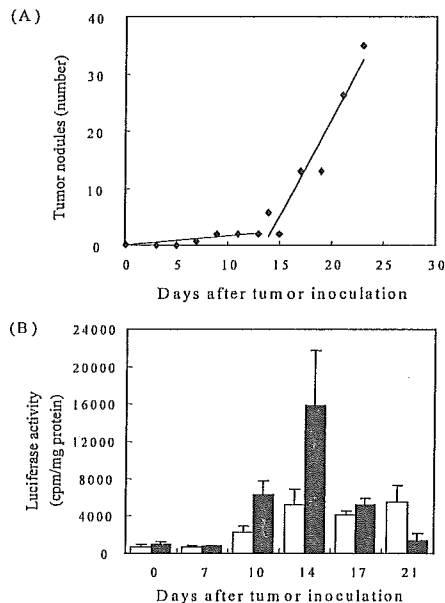

Fig. 4. Relation between Tumor Progression and Gene Expression in Lungs

(A) B16BL6 cells (5×10^4 cells in 0.2 ml PBS) were intravenously injected into a C57BL/6 mouse. At the indicated day, the mice were sacrificed. The number of tumor nodules on the surface of the lung was counted. (B) At the indicated day post-tumor inoculation, the lipoplex with a P/L ratio of 8 or 80 (30 μg of pDNA) was injected intravenously. The luciferase activity in the lungs was determined as described in Materials and Methods 24 h after lipoplex injection. Open column, lipoplex of 8 P/L ratio; filled column, lipoplex of 80 P/L ratio. Data represent the mean±S.D. ($n=4$).
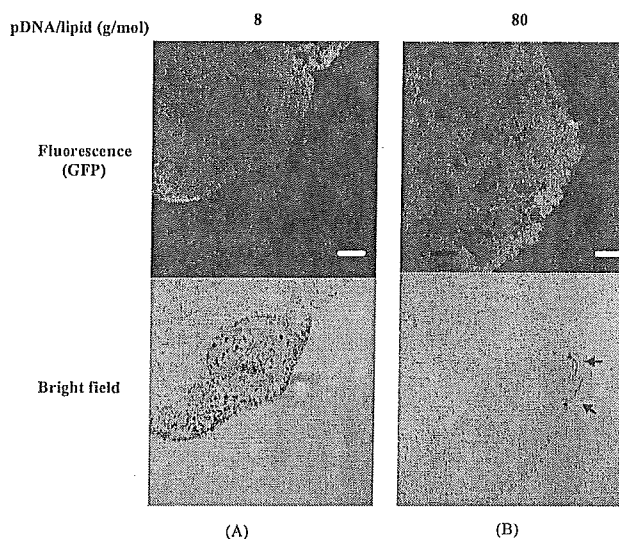

Fig. 5. Fluorescent Microscopic Observation of Expressed GFP in the Tumor-Bearing Lung

At 14 d post-tumor inoculation, the lipoplex with a P/L ratio of 8 or 80 (30 μg of pDNA) was injected intravenously. At 24 h after injection, GFP expression in the lungs was observed under a confocal laser scan microscope as described in Materials and Methods. In the left picture (A), the dark area in the bright field represents tumors in the prepared section. In the right picture (B), the black arrow heads represents very tiny tumors in the prepared section. The scale bar in the pictures represents 100 μm. The pictures shown are typical examples of multiple evaluations.

bearing lung, with the lipoplex with a P/L ratio of 8, the expressed GFP was barely detected in the tumor area (Fig. 5A). Interestingly, the strongest GFP expression was observed in the tissue around the tumor. In contrast to this observation, with the lipoplex with a P/L ratio of 80, the expressed GFP

was detected in the entire lung tissue unrelated to the area of tumor growth (Fig. 5B).

DISCUSSION

Many parameters are known to affect the efficiency of transfection by lipoplex (lipofection) *in vitro* and *in vivo*. Among these parameters, one of factors that affect lung lipofection is the interaction of lipoplex with serum. It has been reported that incubation in pure serum or serum-containing media could decrease the extent of lipoplex delivery and alter its intracellular fate[19,20] and even cause the lipoplex to disintegrate upon prolonged exposure.[18,21] However, an injected lipoplex is rapidly cleared from the circulation[18,22] and internalized in the lungs,[23] thus ruling out long-term exposure to serum. In addition, Sakurai *et al.* showed the pre-incubation of a DOTAP/Chol lipoplex with serum for as long as 30 min did not significantly impair lipofection in the lungs.[24] Accordingly, the importance of the above mentioned serum effects for lung tansgene expression following intravenous injection of lipoplex is still an open question.

Our earlier study showed that the size of lipoplex is a major determinant in the lipofection efficiency of TFL-3 in the presence of serum with A431, a human epidermoid carcinoma cell line, and SBC-3, a human lung cancer cell line.[25] Therefore, in this study, we focused on the effect of lipoplex size as the result of from interaction with serum proteins on *in vitro* lipofection and on their relation with *in vivo* lipofection in tumor-bearing mice. As shown in Figs. 1 and 2, the importance of lipoplex size was also confirmed in *in vitro* lipofection with B16BL6 cells in the absence or presence of serum. Interestingly, in the presence of serum, the size of lipoplex that has over P/L ratio of 40 was decreased relative to absence of serum (Figs. 1A, 2A). This may result from that the larger lipoplex as a result of aggregation of smaller lipoplexes was dispersed by the interposition of serum proteins.[10] The *in vitro* lipofection of the lipoplex in the presence of serum (Fig. 2) showed no obvious relation with *in vivo* lipofection in tumor-bearing mice (Fig. 3). Probably, the alteration in the size of lipoplex, resulted from aggregate formulation with anionic serum proteins after intravenous injection, is not a major factor in determining *in vivo* lipofection efficiency in our system. Otherwise, an interaction with serum proteins would not lead to a predominant change in the size of the lipoplex after an intravenous injection. During the intravenous injection of lipoplex, the lipoplex mixes with about 50—100 μl of serum, presuming cardiac flow through the tail vein and abdominal veins to be *ca.* 300 μl of serum per 3 s.[26] In this situation, *i.e.* 50—100 μl serum/200 μl of lipoplex dispersion, the serum concentration is much considerably diluted compared with our *in vitro* experimental conditions (50% (v/v)). In the presumed *in vivo* condition, at the lipoplex/serum ratio, the cationic membrane surfaces are only partially associated with anionic serum proteins, which therefore may not act as bridges between lipoplexes, thereby not inducing lipoplex aggregation well.

Previous studies using TFL-3 showed a high transfection efficiency *in vitro*, even in the presence of serum, and *in vivo* a low toxicity was reported by intraperitoneal injection.[9] Accordingly, TFL-3 would be expected to be applicable for *in vivo* use with systemic injection. Here, we report that tumor-related gene expression in the lung is achieved by using TFL-3 (Fig. 3). In our artificial metastasis model of murine melanoma, some micrometastases were established in the lung due to the direct intravenous injection of the cells. Once established, the pulmonary metastases secrete factors that promote angiogenesis and then begin to proliferate progressive, resulting in an enlarged vascular bed and an increase in vascular permeability. Simberg *et al.*[27] recently reported that hypervascularization in the liver induced by VEGF did not increase level of lipofection with a DOTAP/CHOL lipoplex, despite the increased entrapment of lipoplex in the liver. Based on the fact that enlarging the vascular bed and increasing vascular permeability in the liver were not sufficient to promote efficient lipofection, they concluded that the lungs might be an organ that is simply susceptible than liver. Accordingly, the predominant tumor-related gene expression in this study may have been obtained due to the following reasons: (a) the lung is a more lipofectable organ, (b) hypervascularization occurred due to tumor progression, and (c) extensive proliferation of tumor cells occurred. But, we can not exclude influence of alteration of biological conditions related to tumor progression on the resulting gene expression in the lung.

The P/L ratio of a lipoplex of TFL-3 affected the degree of gene expression in tumor-bearing lungs (Fig. 3). In addition, pulmonary gene expression was dependent on the time after tumor cell inoculation (Fig. 4). These observations lead to the possibility that the lipoplex causes gene expression in a different part of the lung. This area-specific gene expression may be related, in part, to the pattern of interaction of lipoplexes with tumor cells, pulmonary endothelial cells and normal cells. To assess this, a fluorescent microscopic study was carried out. The findings showed that the lipoplex with a P/L ratio of 8 expressed GFP over the surrounding tumor cells, while the lipoplex, with a P/L ratio of 80, expressed it in the entire lung tissue without any specificity (Fig. 5). The first pass entrapment (60—80% of the liposomes are entrapped within minutes after intravenous injection)[18,22,28] can be attributed to the highly extended lung capillary bed and was proposed to explain the predominant gene expression in the lungs.[28,29] Ito *et al.*[30] recently reported that vascular endothelial cells, probably under angiogenesis, in the lungs of tumor bearing mice, which demonstrates an increased uptake of cationic liposome–DNA complexes and transgene expression compared to normal mice. In addition, they showed that the increased uptake of liposome–DNA complexes by tumor cells over normal cells is also responsible for the increased transgene expression. The alveolar macrophages present in a tumor microenvironment could be less responsible for the transgene expression, since it has been reported that macrophages are less phagocytic or are inactivated by factors produced by the tumor cells.[31,32] Accordingly, the P/L ratios of lipoplexes showing the largest difference in physicochemical properties, as shown in Figs. 1 and 2, affect the microdistribution of lipoplexes, their interaction with various cells and uptake by those cells after intravenous injection, may result in the area-specific gene expression in tumor-bearing lungs.

Although the present study demonstrates that the increased gene expression in tumor-bearing lungs is probably due to an increased uptake of lipoplex by tumor cells and pulmonary

endothelial cells of normal or angiogenic vessels over the surrounding normal cells, it is possible that other factors such as endosomal release, nuclear uptake, and increased transcription and translation, may play a role in the different degree and distribution pattern observed for gene expression in the lung. A further examination of these individual phenomena may aid in the development of an effective gene delivery vector. However, a note of caution is that the experimental metastases model used in this study, although performed in such a way that the lung metastases are established, has some limitations. It does not mimic the full sequence of events that primary tumors undergo. These cancer cells may undergo additional changes. Thus, it may not be predictive of the responses of these tumors to a systemically delivered gene therapy agent. Therefore, the results of the present study, although relevant for lung cancer therapy, represent only an intermediate step on the path toward the development of a systemic gene transfer with a broad utility.

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Gene Expression in Primary Cultured Mouse Hepatocytes with a Cationic Liposomal Vector, TFL-3: Comparison with Rat Hepatocytes

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We recently reported that a cationic liposomal vector, TFL-3, could be used to achieve significant gene expression in primary cultured rat hepatocytes (Nguyen *et al., Biol. Pharm. Bull.*, 26, 880–885 (2003)). A combination of hepatocyte transplantation and hepatocyte-targeted gene transfer represents a potentially important strategy for expanding treatment options for liver disease. A widely applied approach to support cross-species is necessary before human applications can be realized. Therefore, in this study, we examined the utility of TFL-3 in another species of rodent hepatocytes, namely mouse hepatocytes. Gene expression in mouse hepatocytes by TFL-3 was successful and the level was higher than those in rat hepatocytes that we recently reported on. Interestingly, it appears that both the degree and rate of gene expression were dependent on the incubation time prior to lipofection as well as on the density of cells per dish, but these parameters were independent of the amount of pDNA associated with the cells. These significantly suggest that the culture time prior to and following lipofection, which are related to the biological condition of the cells, may be one of major factors that affect gene expression in hepatocytes and non- or less dividing cells.

Key words gene delivery; cationic liposome; hepatocyte; non-dividing cell; plasmid quantification

The liver is an important target organ for gene therapy, because hepatocytes synthesize a wide variety of proteins, perform a variety of post-translational modifications, and are involved in numerous diseases. Therefore, hepatocyte-targeted gene transfer would represent an important strategy for expanding treatment options for liver diseases. The successful gene expression in mouse hepatocytes in living animals was recently achieved by a rapid injection of a large amount of naked pDNA into a mouse tail vein (the hydrodynamics-based procedure).[1,2] Despite the many desirable features of the procedure such as simplicity, convenience, and high efficiency, this procedure is not suitable for the treatment of liver disease because it seriously damage the hepatocytes.[2,3]

We recently reported that a cationic liposomal vector, TFL-3[4] composed of a cationic lipid, DC-6-14, with helper lipids dioleoylphosphatidylethanolamine and cholesterol (1 : 0.75 : 0.75 molar ratio), achieved sufficient gene expression in primary cultured rat hepatocytes.[5] Hence, TFL-3 may be a suitable vector system for successful gene expression in hepatocytes and it may be an attractive candidate for use in *in vivo* or *ex vivo* gene therapy.

Among the currently available therapeutic options, hepatocyte transplantation is a promising procedure that could replace liver transplantation because it would overcome the shortage of available donors as well as a variety of technical difficulties.[6—9] Therefore, a combination of hepatocyte transplantation and *in vitro* hepatocyte-targeted gene transfer using TFL-3, leading to the efficient expression of functional proteins, such as enzymes or growth factors, represent an important strategy for expanding the treatment options for liver disease. However, a widely applied approach to support cross-species is needed before human applications: the utility of TFL-3 on gene expression in non-dividing mammalian cells should be tested with different species. Therefore, in this study, we further examined the utility of TFL-3 on transgene expression in another rodent hepatocyte, namely primary cultured mouse hepatocytes.

MATERIALS AND METHODS

Materials TFL-3 was a generous gift from Daiichi Pharmaceutical Co. Ltd. (Tokyo, Japan). The pDNA, pGL3- Control, encoding luciferase was purchased from Promega (WI, U.S.A.). Collagenase TYPE I was purchased from Funakoshi (Tokyo, Japan). Cell culture reagents were obtained from Nissui Pharmaceutical Co. Ltd., (Tokyo, Japan). Other reagents were of analytical grade. LipofectAMINE was purchased from Life Technologies (MD, U.S.A.).

Cell Preparation and Culture Parenchymal hepatocytes were isolated from an adult male 4 ddY mouse weighing 17—22 g (Japan SLC, Shizuoka, Japan) or adult male Wistar rats weighing 170—200 g (Japan SLC, Shizuoka, Japan) using the in situ perfusion method, as described earlier.[5,10] Animal experiments were evaluated and approved by the Animal and Ethics Review Committee of Faculty of Pharmaceutical Sciences, The University of Tokushima. The isolated hepatocytes were plated at a density of 0.75×10^6 or 1.5×10^6 cells in 60 mm diameter collagen type I coated dish (IWAKI, Tokyo, Japan), and cultured at 37 °C under an atmosphere of humidified 5% CO_2 for 4 h to allow the cells to adhere to the dish. In each preparation, the viability of isolated cells was checked by trypan blue dye exclusion.

Lipofection and Luciferase Assay Lipofection was carried out using procedures as described in our earlier paper.[5] Briefly, pDNA/TFL-3 complexes were prepared at a ratio of 3 μg of pDNA to 30 nmol of cationic lipid. If necessary, rhodamine-labeled phosphatidylethanolamine (0.2% (mol/mol)) (Molecular probe, OR, U.S.A.) was incorporated in the liposome as a marker. At 18 or 42 h following the 4 h of preculture, the complexes were added to the dish at a final concentration of 1 μg pDNA/ml of media, which contained 5% heat-inactivated FBS (lipofection). After a 4 h lipofection period, the cells were further incubated in complete media for various times until used in the assays.

Luciferase activity was determined by a previously re-

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ported.[5] Briefly, the cells were lysed by the addition of 900 μl of cell culture lysis reagent (CCLR, Promega). An aliquot of the cell lysate was removed and the luciferase activity determined according to the manufacture's recommended protocol (Promega). The protein content of the lysate was measured with a DC protein assay kit (Bio-Rad Laboratories, CA, U.S.A.) and the data are represented as light counts/min/μg of protein.

Measurement of pDNA Delivered in the Hepatocytes
The pDNA delivered to hepatocytes was quantitatively determined by using a method recently developed in our laboratory[11] with minor modifications. Briefly, at various times post-lipofection, the cells were suspended in 0.5 ml of lysis buffer (0.5% Nonidet P-40, 10 mM Tris, 10 mM NaCl, 3 mM MgCl$_2$, pH 7.4) to destabilize the plasma membrane. Aliquots of the suspension were subjected to PCR study. To extract intracellular DNA (containing pDNA), proteinase K (Merck, Frankfurter, Germany) was added to the fraction to a final concentration of 0.1 mg/ml. After an overnight incubation at 37 °C, proteins were eliminated by phenol/chloroform treatment; and the DNA was precipitated by the addition of ethanol. The precipitate was dissolved in TE buffer (10 mM Tris–HCl, 1 mM EDTA (pH 8.0)) and was used as the DNA sample. The concentrations of DNA were determined by measurement of the absorbance at 260 nm with a Shimadzu UV-1200 spectrophotometer (Shimadzu, Kyoto, Japan).

Part of the luciferase region in the pDNA pGL3 was amplified by PCR (TaKaRa PCR Thermal Cycler (MP TP3000, TAKARA Bio, Shiga, Japan)) with the primers RHY008 (5'-GTACACGTTCGTCACATCTC-3') and RHY010(5'-CCT-GATACCTGGCAGATGGA-3') (Proligo Japan, Kyoto, Japan). The reaction mixture (100 μl) consisted of an adequate amount of template, 100 pmols of primers, 1.6 mM dNTP, 2.5 units of Taq DNA polymerase (Ex Taq), 1.5 mM MgCl$_2$, 50 mM KCl and Tris buffer (pH 8.3) (TaKaRa Bio, Shiga, Japan). The PCR was performed by denaturation at 94 °C for 3 min, followed by 30 cycles at 94 °C for 1 min, annealing at 55 °C for 1 min, and extension at 72 °C for 2 min. After the PCR procedure, a 10 μl aliquot of the reaction mixture was subjected to agarose gel (1%) electrophoresis, and the amplified pDNA was quantified by measuring the fluorescence intensity of ethidium bromide bound to the DNA with an ATTO imaging analyzer (AE-6911) (Tokyo, Japan).

Measurement of TFL-3 Associated with Hepatocytes
At various time points post-lipofection, cells were washed twice with 3 ml of cold phosphate-buffered saline (PBS, pH 7.4) and harvested by the addition of 1 ml of 0.25% trypsin solution, followed by incubation at 37 °C for 5 min. The cells were then collected by centrifugation (1000 rpm, 5 min, and 4 °C). The collected cells were lysed by incubation with 2 ml CCLR at room temperature for 2 h. An aliquot was used for the measurement of rhodamine intensity using a fluorescent spectrophotometer, F-4500 (Hitachi, Tokyo, Japan). The protein content of the sample was determined with the DC protein assay kit. The data are expressed as the amount of liposome (pmol) per μg of protein.

Statistics All values are expressed as the mean±S.D. Statistical analyses were performed using the GraphPad In-Stat software program (GraphPad Software, CA, U.S.A.). The level of significance was set at $p < 0.05$.

RESULTS

Effect of Culture Time Prior to Lipofection on Gene Expression in Primary Cultured Mouse Hepatocytes
Mouse hepatocytes were lipofected for 4 h at either 18 or 42 h after a 4 h-preculture. Luciferase activity was determined after 24, 48 or 72 h (Fig. 1). Gene expression was gradually increased in a post-lipofection culture time-dependent manner. In addition, the gene expression in cells that had been cultured for a longer period (42 h) before lipofection was much higher, in total, than in cells cultured for shorter periods (18 h) before lipofection. Interestingly, gene expression mediated by TFL-3 was much higher (approximately 15 fold) than those mediated by LipofectAMINE. This indicates that TFL-3 is superior to LipofectAMINE in terms of lipofection in primary mouse hepatocytes.

Measurement of pDNA Delivered in and TFL-3 Associated with the Hepatocytes Following a 4 h lipofection by TFL-3, the amount of pDNA transported into the hepatocytes was quantitatively determined at either 6 h or 48 h post-lipofection (Fig. 2A). The successful delivery of pDNA to mouse as well as rat hepatocytes occurred at an earlier time point (6 h after lipofection). No significant difference in amounts was found between mouse and rat hepatocytes. The amount dramatically decreased for a longer time point (48 h after lipofection). It appears that the culture time prior to lipofection has an effect on the retention of transported pDNA (Fig. 2A, insertion), although this difference would not reflect gene expression, since the restored pDNA was less than 1% of pDNA delivered at earlier time points.

Under the same experimental conditions, the amount of TFL-3 associated with hepatocytes was also examined (Fig. 2B). There was no significant difference at any of the time points after lipofection. It thus appears that the culture time prior to lipofection has no effect on the amount of TFL-3 bound to the hepatocytes. The amount of bound liposome decreased slightly with increasing time of post-lipofection.

Effect of the Density of Hepatocytes on Lipofection Efficiency A greater gene expression was detected with larger numbers of hepatocytes (Fig. 3). The gene expression for the
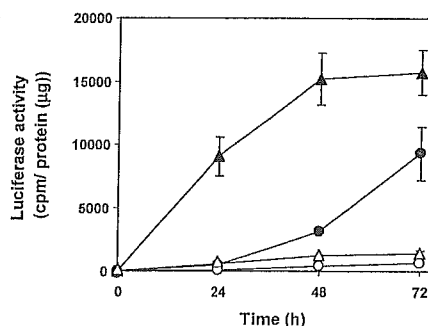

Fig. 1. Effect of Culture Time Prior to Lipofection on Gene Expression in Primary Cultured Mouse Hepatocytes

Isolated mouse hepatocytes (1.5×10^6 cells) were plated in a collagen type-I coated dish (60 mm) and incubated for 4 h (preculture). At 18 h (●, ○) or 42 h (▲, △) after preculture, the cells were lipofected with either pDNA/TFL-3 complexes (closed symbols) or pDNA/LIPOFECTAMINE complexes (open symbols) for 4 h. After removal of the lipoplexes, the cells were further incubated for periods up to 72 h. During incubation at the indicated time points, the luciferase activity (cpm/μg of total protein) in hepatocytes was determined as described in the Materials and Methods. The data represent the mean±S.D.
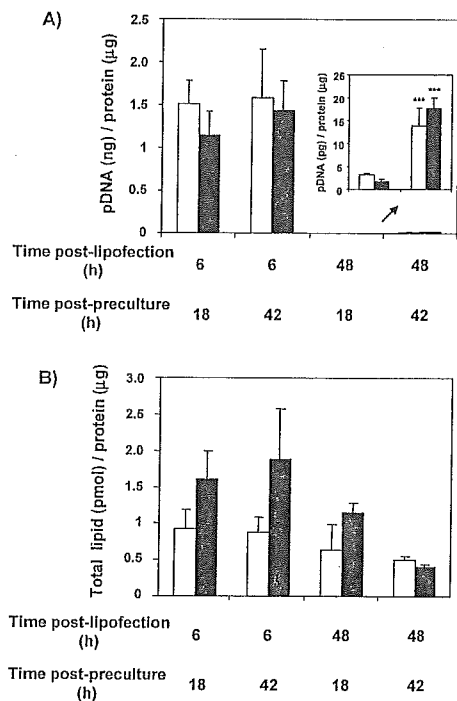
Fig. 2. Amount of pDNA Delivered in and TFL-3 Associated with the Hepatocytes

Isolated mouse (□) and rat (■) hepatocytes (1.5×10^6 cells) were plated and incubated for 4 h (preculture). At 18 h or 42 h after preculture, the cells were lipofected for 4 h. At 6 h or 48 h post-lipofection, the amounts of pDNA in the cells (A) and TFL-3 bound to the cells (B) were determined as described in the Materials and Methods. The data represent the mean±S.D.
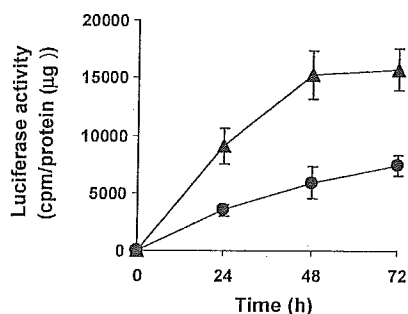

Fig. 3. Effect of Density of Hepatocytes on Lipofection Efficiency

Isolated mouse hepatocytes (0.75×10^6 (●) or 1.5×10^6 (▲) cells) were plated in a collagen type-I coated dish (60 mm) and incubated for 4 h (preculture). At 42 h after preculture, the cells were lipofected for 4 h. At 6 h or 48 h post-lipofection, the amounts of pDNA in the cells and TFL-3 bound to the cells were determined as described in the Materials and Methods. The data represent the mean±S.D. After removal of the lipoplexes, the cells were further incubated up to 72 h. During incubation at the indicated time points, luciferase activity (cpm/μg of total protein) in hepatocytes was determined, as described in the Materials and Methods. The data represent the mean±S.D.

case of higher cell density was relatively 2—3 folds larger that those for the case of a lower cell density. At a higher cell density, gene expression increased, reaching a maximum level up to 48 h. To the contrary, at a lower cell density, gene expression still gradually increased in a culture time postlipofection-dependent manner.

In addition, the viability of hepatocytes was monitored throughout this study. Viability gradually decreased in a culture time-dependent manner with or without lipofection (data not shown), although the efficiency of gene expression relatively increased.

DISCUSSION

Among the currently available therapeutic options, hepatocyte transplantation is a promising procedure. Therefore, a combination of hepatocyte transplantation and in vitro hepatocyte-targeted gene transfer represents an important strategy for expanding the treatment options for liver disease. TFL-3 may be a candidate for in vitro gene transfer targeted to hepatocytes, since we recently reported that TFL-3[4] achieved sufficient gene expression in primary cultured rat hepatocytes.[5] However, a widely applied approach to support cross-species is needed with TFL-3 before human applications. Therefore, in this study, we further examined the utility of TFL-3 on transgene expression in another rodent hepatocyte. If some differences on the gene expression between mouse hepatocytes and rat ones were obtained although both animals are rodent. This would suggest that there is a species difference on the gene expression by hepatocytes.

As a first matter, the issue of whether TFL-3 can be successfully used to achieve gene expression in primary cultured mouse hepatocytes was examined. As shown in Fig. 1, efficient gene expressions could be achieved using TFL-3 and the level of gene expression was dependent on the culture time prior to lipofection. In addition, the transgene activity was superior to that of the commercially available LipofectAMINE under our experimental conditions, which is consistent with the result showing the LipofectAMINE does not work very well in the presence of serum.[12] These observations are consistent with the result obtained with rat hepatocytes.[4] These findings suggest that TFL-3 could be useful vector for achieving a sufficient level of gene expression in rodent hepatocytes.

Interestingly, gene expression in mouse hepatocytes was relatively much higher (2—2.5 fold) than those in the rat hepatocytes. In addition, the peak for maximum gene expression level in mouse hepatocytes appeared to move to a later time point post-lipofection, compared to the results reported for rat hepatocytes.[5] This is supported by the report of Weglarz et al.[13,14] demonstrating that the peak for DNA replication after a partial hepatectomy in rats, presumably in hepatocytes, occurs at 24 h after the operation while it takes place at 44 h in mice. It appears that the difference in DNA replication is due to the difference in the length of G1 between rat and mouse hepatocytes.[15] This suggests that there may be a species difference in exogenous gene expression by the hepatocytes as a result of transfection with TFL-3.

The time of culture prior to lipofection had no effect on the amount of pDNA delivered to rodent hepatocytes (Fig. 2A), while gene expression was obviously dependent on the time of culture prior to lipofection (Fig. 2).[5] Similar results have also been reported for other mammalian line cells.[16] These findings suggest that gene expression is not necessarily related to the amount of pDNA delivered by TFL-3, but rather to the biological condition of the hepatocytes that influences, for example, the process of transcription of the delivered pDNA. Of course, the delivery of a sufficient amount of pDNA to the cell and nucleus to permit gene expression is an absolute requisite.

As described in Fig. 3, gene expression increased with increasing cell density. This observation is inconsistent with our earlier results for rat hepatocytes[5] and other's.[17] This

discrepancy may be due to differences in experimental conditions; 0.75×10^6 and 1.5×10^6 cells for the mouse study and 1.5×10^6 or 3×10^6 cells for the rat study.[5] Gene expression in rat hepatocytes might have already reached the maximum level at a lower cell density (1.5×10^6). Hence, the resulting gene expression may decrease at higher cell density (3.0×10^6). Although the reason for why efficient gene expression was achieved for a lower cell density of hepatocytes is currently unclear, the increased cellular interaction at higher cell density might induce some signal transduction, which would inhibit protein production in the cells. Further study will be required to confirm this assumption.

The results presented here indicate that the conditions used for lipofection are critical for achieving an efficient gene expression in primary cultured hepatocytes. This strongly indicates that an increased gene expression in rodent hepatocytes could be achieved by optimizing the lipofection conditions, such as the time of culture prior to lipofection, the time used for lipofection and the density of the cells. In addition, the results described here and earlier[5] demonstrate that the TFL-3 may be a suitable non-viral vector system for successful gene expression in non- or less-dividing cells. Therefore, for developing further efficient delivery system with TFL-3 for clinical use, it will be important to collect more information on the biological conditions that affect the transcription process of target cells.

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RESEARCH ARTICLE

Stimulatory effect of polyethylene glycol (PEG) on gene expression in mouse liver following hydrodynamics-based transfection

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Abstract

Background Rapid intravenous injection of a large volume of plasmid DNA (pDNA), i.e. a transfection procedure based on hydrodynamics, is known to be an efficient and liver-specific method of *in vivo* gene delivery. However, the gene expression is transient.

Methods We investigated the effect of addition of polyethylene glycol (PEG) to a solution of naked pDNA (luciferase) on the expression of the gene in mouse liver following transfection by the hydrodynamics-based technique. In addition, the mechanism leading to the enhancement of the gene expression was studied.

Results The addition of 1% (w/v) PEG2000 to the pDNA solution enhanced the resulting gene expression in the liver. Increasing the PEG2000 concentration to more than 1 and up to 10% (w/v) rather diminished the gene expression level. By contrast, increasing the molecular weight of PEG to over 2000 up to 10000 did not affect the level of gene expression. Histopathological and serum-chemistry examinations indicated that hydrostatic or osmotic pressure increased tissue and hepatocellular damage in a PEG-concentration-dependent manner, and resulted in a decrease in gene expression. Quantitative evaluation showed that the enhanced gene expression resulted from stabilization of the pDNA introduced into the hepatocytes and an enhancement of the transport of *intact* pDNA to the nucleus.

Conclusions For most gene therapy applications and gene function studies, sustained expression of the introduced gene(s) is necessary. This simple method to achieve enhanced gene expression in liver may have a great potential for a wide variety of laboratory studies in molecular and cellular biology as well as possibly for future clinical applications in humans. Copyright © 2005 John Wiley & Sons, Ltd.

Keywords polyethylene glycol (PEG); hydrodynamics-based transfection; gene delivery; liver; hepatocytes; naked DNA

Introduction

The transfection of plasmid DNA (pDNA) into living animals is an indispensable tool in molecular and cellular biology. The avalanche of new genes discovered as part of the human genome project has further increased the importance of gene transfer in understanding gene function. However,

the analysis of gene expression in living animals has been limited since it has required complicated laboratory procedures such as the implantation of transfected cells or generation of transgenic animals. There is an obvious need for simple methods that allow for efficient gene transfer into cells to achieve high transgene expression *in vivo* for gene function studies.

One of the alternatives is the direct use of naked pDNA. Since the initial report in the early 1990s of the successful expression of a reporter gene in muscle by simple intramuscular injection of pDNA [1], there have been many *in vivo* studies demonstrating successful transgene expression in liver [2–5], lung [6], heart [7], and skin [8]. The single use of naked pDNA has the advantage that it does not require the laborious procedures for preparation and purification connected with the use of viral vectors; it lacks the immunogenecity and oncogenic effects of viral vector systems, and the hazard of endogenous virus recombination [9,10]. On the other hand, the use of naked pDNA has a severe limitation in that it requires local or regional administration or surgical procedures. In addition, the level of transgene expression resulting from such local injection is relatively low and restricted to the injection site.

Another adapted alternative, the hydrodynamics-based procedure, has been reported by Liu et al. [11] and Zhang et al. [5]. By rapid injection of pDNA in a large volume into a mouse tail vein they achieved high levels of foreign gene expression in mouse liver, particularly in hepatocytes. Under these conditions, a high hydrostatic pressure is generated in the inferior vena cava and in the vein linked to that vessel because the volume and speed of injection exceed the cardiac output. The mechanism of gene transfer by this procedure is not clearly understood but must be related to an enhanced uptake and intracellular processing of the injected pDNA in the hepatocytes. To date, the hydrodynamics-based transfection procedure has been utilized by the gene therapy community for the evaluation of therapeutic activities of various genes (see, for reviews, Liu and Knapp [12] and Horweijer and Wolff [13]). Other reported applications of this technique include studies to define the regulatory functions of DNA sequences [14–16], investigations to evaluate gene suppression activity of siRNA [17–20], and experiments to establish animal models for viral infection [21]. Despite many desirable features of the hydrodynamics-based procedure such as simplicity, convenience, and high efficiency, further improvements to this procedure are required because the transgene expression is transient.

Polyethylene glycol (PEG) is a hygroscopic, water-soluble, and relatively chemically inert synthetic polymer [22] having a variety of effects on biological systems [23–26]. In earlier days, PEG was used as a cell-cell fusogen for the production of somatic hybrids [27]. Dehydration of lipid membranes by PEG has been suggested to be critical for the ability of this polymer to induce membrane fusion [28,29]. Osmotic effects are also considered to be of importance. An osmotic imbalance due to the osmolyte gradient between the exclusion layer and the bulk PEG solution is assumed to result in stress that is considered to lead to an increase in membrane permeability [30].

Most recently, Zhang et al. [31] proposed that the hydrodynamic injection induces a transient irregularity of the heart function, a sharp increase in venous pressure, an enlargement of liver fenestrae, and enhancement of membrane permeability of the hepatocytes. This report led us to hypothesize that PEG, if added to a large volume of pDNA solution, might increase the membrane permeability of the hepatocytes and consequently allow large amounts of pDNA into the hepatocytes, resulting in enhanced transgene expression in the liver compared to the regular hydrodynamics-based procedure. In this study we examined this hypothesis and we show that it is correct.

Materials and methods

Materials and animals

Plasmid pGL3-Control (pDNA) containing the cDNA of firefly luciferase driven by the SV40 promoter, the luciferase assay kit and cell culture lysis reagent (CCLR) were purchased from Promega (WI, USA). PEGs with average molecular weights of 2000, 6000 and 10 000 were from Boehringer Mannheim (Mannheim, Germany). Evans Blue was purchased from Nacalai Tesque (Kyoto, Japan). Collagenase type I and bromo-2′-deoxyuridine (BrdUrd) were purchased from Sigma (MO, USA). RPMI1640 medium was purchased from Nissui Pharmaceutical (Tokyo, Japan). Antibiotics (penicillin and streptmicin) were purchased from ICN Biomedical (OH, USA). All other reagents were of analytical grade.

Mice and hydrodynamic injection

ddY male mice, 4 weeks old, 18–20 g, were purchased from Japan SLC (Shizuoka, Japan). All animal experiments were evaluated and approved by the Animal and Ethics Review Committee of the Faculty of Pharmaceutical Sciences, The University of Tokushima. Animals were injected via the tail vein with a large volume of saline (2 ml) containing 40 µg of pDNA in 5 s according to a previously published procedure [11]. Saline was used as a carrier solution for injection. The indicated concentrations of PEG with the estimated average molecular weights were added to the carrier solution.

Luciferase assay

Luciferase activity in the tissue was determined according to an established procedure [11,32] with minor modifications. Briefly, approximately 200 mg of liver tissue were homogenized in 1 ml of the CCLR and then frozen and thawed three times, followed by centrifugation for

10 min at 4 °C and 20 000 *g*. The supernatant was diluted, if needed, using the CCLR. A 10-μl aliquot of the diluted sample was used to determine the luciferase activity using luciferase assay reagent according to the manufacturer's recommended protocol (Promega, WI, USA). The protein content of the sample was measured with the DC protein assay kit (Bio-Rad Laboratories, CA, USA), and the data are expressed as light counts/s/mg of protein.

Serum-chemistry assay

Mice were injected using the hydrodynamics-based procedure (2 ml/mouse/5 s) in the presence of either 0, 1 or 10% (w/v) PEG2000. Blood was collected by heart puncture by using a heparinized syringe under ether anesthesia at 3 h, 12 h, 24 h and 7 days after the injection. Blood samples were collected in a tube containing an SST gel and a clot activator (Eiken, Tokyo, Japan), allowed to stand for 30 min at room temperature, and then centrifuged at 3000 rpm for 15 min to collect serum. Sera were stored at −80 °C until use. A Hitachi 7170 automatic analyzer (Tokyo, Japan) was used to measure aspartate aminotransferase (AST) and alanine aminotransferase (ALT).

Vascular permeability assay

In order to quantify blood vessel permeability in the liver after hydrodynamic injection, Evans Blue dye solution (0.2 mg in 2 ml of saline) containing 0, 1 or 10% (w/v) PEG2000 was injected into the tail vein with the hydrodynamics-based procedure. For the same purpose, the dye (0.2 mg in 0.2 ml of saline) was injected either 5 or 30 min after the hydrodynamic injection of saline containing 0, 1 or 10% (w/v) PEG2000 had been administered. At 6 h after the first injection, the liver was removed, and the dye was extracted. The ground liver tissue (200 mg) was dissolved in 1 ml of tissue solubilizer (2 N KOH in isopropanol) at 60 °C overnight. The solution was allowed to cool before the addition of 2 ml of ethyl acetate; the mixture was then vortexed, and 2 ml of 1 N HCl were added, vortexed again, and centrifuged at 2000 *g* for 15 min. Absorption of the upper phase at 626 nm was recorded. Concentrations were determined from a standard curve of Evans Blue dye.

Histological evaluation

To evaluate tissue and cellular damage in the liver following hydrodynamic injection, mice were injected with saline or saline containing the indicated concentrations of PEG2000. At 10 min after injection, the mice were sacrificed and livers were collected. The livers were separated and the separate lobes were fixed in 20% neutral-buffered formalin. After embedding in paraffin, the lobes were sectioned in slices of 5 μm thickness. The paraffin sections were used for histopathological analysis (hematoxylin and eosin staining). The histopathological scores were recorded under microscope observation.

Isolation of hepatocytes

Hepatocytes were isolated from the livers by the *in situ* perfusion method established previously [33]. Briefly, mice were anesthetized with sodium pentobarbital and the portal vein was cannulated. Hanks' balanced salt solution containing 0.5 mM EGTA and 10 mM HEPES was perfused at approximately 40 ml/min for 4 min. Then, 0.0125% collagenase type I in RPMI1640 medium containing antibiotics (100 units/ml penicillin and 100 μg/ml streptmicin), prepared just prior to use, was perfused at 20 ml/min for 6 min. Following these perfusions, the liver was carefully removed and dissected, and the cells were dispersed in cold Hanks' solution and the suspension was filtered over a cell strainer (Becton Dickinson Labware, NJ, USA) with a 100-μm mesh to obtain a single-cell suspension. Parenchymal cells (hepatocytes) were separated from non-parenchymal cells by differential centrifugation at 50 *g* for 90 s. The precipitate (hepatocytes) was resuspended in RPMI1640 medium containing antibiotics supplemented with 5% heat-inactivated fetal bovine serum (Sigma, MO, USA). In each preparation, the viability of the isolated cells was determined by trypan blue exclusion.

Isolation of nuclei of hepatocytes

The isolated hepatocytes (1×10^6 cells) were suspended in 0.5 ml of lysis buffer (0.5% Nonidet P-40, 10 mM Tris, 10 mM NaCl, 3 mM $MgCl_2$, pH 7.4) to destabilize the plasma membrane. The nuclear fraction in the suspension was collected as a precipitate by centrifugation (1400 *g*, 5 min, 4 °C). To prevent the detection of extranuclear pDNAs, the nuclear fraction was treated with *Hind*III (Nippon Gene, Tokyo, Japan), for which a recognition site exists in the amplified region of pGL3 (37 °C, 1 h). The treated solution was centrifuged again. The precipitate was used as the nuclear fraction.

Quantitative determination of pDNA delivered in the hepatocytes

The pDNA delivered to hepatocytes was quantitatively determined using a method recently developed in our laboratory [34] with minor modifications. Briefly, the isolated hepatocytes (1×10^6 cells) were suspended in 0.5 ml of lysis buffer (0.5% Nonidet P-40, 10 mM Tris, 10 mM NaCl, 3 mM $MgCl_2$, pH 7.4) to destabilize the plasma membrane. Aliquots of the suspension were subjected to polymerase chain reaction (PCR) study. Part of the luciferase region in the pGL3 pDNA was amplified by PCR using a thermal cycler (MP TP3000; TaKaRa Bio, Shiga, Japan) with primers RHY008

(5′-GTACACGTTCGTCACATCTC-3′) and RHY010 (5′-CCTGATACCTGGCAGATGGA-3′) (Proligo Japan, Kyoto, Japan). The reaction mixture (100 μl) consisted of an adequate amount of template, 100 pmol of the primers, 1.6 mM dNTP, 2.5 units of Taq DNA polymerase (Ex Taq), 1.5 mM MgCl$_2$, 50 mM KCl and Tris buffer (pH 8.3) (TaKaRa Bio). The PCR was performed by denaturation at 94 °C for 3 min, followed by 30 cycles at 94 °C for 1 min, annealing at 55 °C for 1 min, and extension at 72 °C for 2 min. After the PCR procedure, 10 μl of the reaction mixture were subjected to agarose gel (1%) electrophoresis, and the amplified pDNA was quantified by measuring the fluorescence intensity of ethidium bromide bound to the DNA with an ATTO imaging analyzer (AE-6911, Tokyo, Japan).

Quantitative determination of pDNA delivered in the nuclei of hepatocytes

To extract nuclear DNA (containing pDNA), the nuclear fraction prepared as described above was resuspended in 0.5 ml of Tris-HCl buffer (10 mM Tris, 10 mM NaCl, 3 mM MgCl$_2$, pH 7.4) containing 10 mM EDTA and 0.1% sodium dodecyl sulfate (SDS), and proteinase K (Merck, Frankfurt, Germany) was added at a final concentration of 0.1 mg/ml. After incubation at 37 °C overnight, proteins were eliminated by phenol/chloroform treatment and the DNA was precipitated by the addition of ethanol. The precipitate was dissolved in TE buffer (10 mM Tris-HCl, 1 mM EDTA; pH 8.0) and used as a pDNA sample. Concentrations of DNA were determined by measurement of the absorbance at 260 nm with a Shimadzu UV-1200 spectrophotometer (Kyoto, Japan).

For quantitative analysis, the prepared DNA sample was divided into two portions to determine the amount of pDNA delivered in nuclei and the amount of intact pDNA in the nuclei that kept the luciferase-coding region in the sequence intact.

The amount of pDNA delivered in the nuclei
Part of the luciferase region in the pGL3 pDNA was amplified by PCR (TaKaRa PCR thermal cycler, MP TP3000; TaKaRa Bio) with primers RHY008 (5′-GTACACGTTCGTCACATCTC-3′) and RHY010 (5′-CCTGATACCTGGCAGATGGA-3′) (Proligo Japan). The reaction mixture (100 μl) consisted of an adequate amount of template, 100 pmol of the primers, 1.6 mM dNTP, 2.5 units of Taq DNA polymerase (Ex Taq), 1.5 mM MgCl$_2$, 50 mM KCl and Tris buffer (pH 8.3) (TaKaRa Bio). The PCR was performed by denaturation at 94 °C for 3 min, followed by 30 cycles at 94 °C for 1 min, annealing at 55 °C for 1 min, and extension at 72 °C for 2 min. After the PCR procedure, 10 μl of the reaction mixture were subjected to agarose gel (1%) electrophoresis, and the amplified pDNA was quantified by measuring the fluorescence intensity of ethidium bromide bound to the DNA with an ATTO imaging analyzer (AE-6911).

The amount of intact pDNA in the nuclei
Aliquots of pDNA sample were subjected to agarose gel (1%) electrophoresis. After electrophoresis, the gel was stained for 15 min at room temperature in ethidium bromide solution (0.5 mg/ml). The bands of pDNA were visualized by ultraviolet irradiation and the region of the gel containing intact pDNA (non-degradated) was excised. The intact pDNA was purified from the agarose with a GENECLEAN kit (BIO101, CA, USA). The recovered intact pDNA was subjected to PCR, and the amplified pDNA was quantified, as described above.

Statistics

All values were expressed as the mean ± standard deviation (S.D.). Statistical analyses were performed using GraphPad InStat software (GraphPad Software, CA, USA). The level of significance was set at $p < 0.05$.

Results

Effect of PEG concentration on gene expression in the liver

Luciferase activity in tissues was determined at 12 and 24 h after the hydrodynamic injection of 40 μg pDNA containing 1, 2, 5 or 10% (w/v) PEG2000. Injection without PEG (regular hydrodynamic injection) served as a control. In all our experiments low luciferase activity was detected in lung, spleen and kidney (data not shown); extensive gene expression was detected in only liver. At 12 h after injection, the expressed luciferase activities in the liver were lower in the PEG-containing groups than in the control livers (Figure 1). In addition, the expression levels decreased with increasing PEG concentration (Figure 1). Surprisingly, with prolonged time after injection, luciferase activity was enhanced, irrespective of the PEG concentration. By contrast, the luciferase level induced by the regular hydrodynamic injection rather declined as time proceeded. It is worthwhile to note that the highest concentration of PEG (10% (w/v)) diminished the expressed luciferase level induced by regular hydrodynamic injection. It seems that there is an optimal concentration of PEG to cause enhancement of gene expression. On the basis of the result described in Figure 1, 1% (w/v) PEG2000 was adopted for all subsequent experiments.

We also examined the effect of PEG on gene expression induced by conventional injection (40 μg pDNA in 200 μl of saline). At 12 and 24 h after the injection, the gene expression levels were much lower than those induced by the hydrodynamic injection, and no enhancing effect of PEG on the gene expression was observed (data not shown).
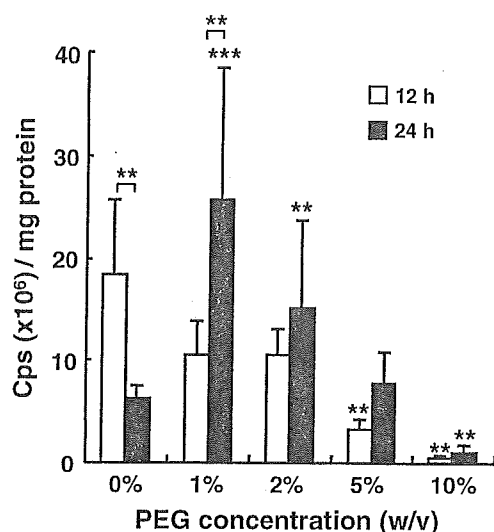
Figure 1. Effect of PEG concentration on gene expression in the liver. Mice were injected with a large volume of pDNA solution (40 μg pDNA) containing 0, 1, 2, 5 or 10% (w/v) PEG2000 using the hydrodynamics-based procedure (2 ml/mouse/5 s). Luciferase activity in the liver was determined at 12 and 24 h after injection. Data are presented as mean ± S.D. (n = 3). Statistical significance against 0% PEG2000 (regular hydrodynamic injection) was analyzed by Student's t-test. Statistical significance between gene expressions levels at different time points (12 and 24 h) after hydrodynamics-based transfection was also analyzed by Student's t-test. $^{**}p < 0.01$; $^{***}p < 0.005$
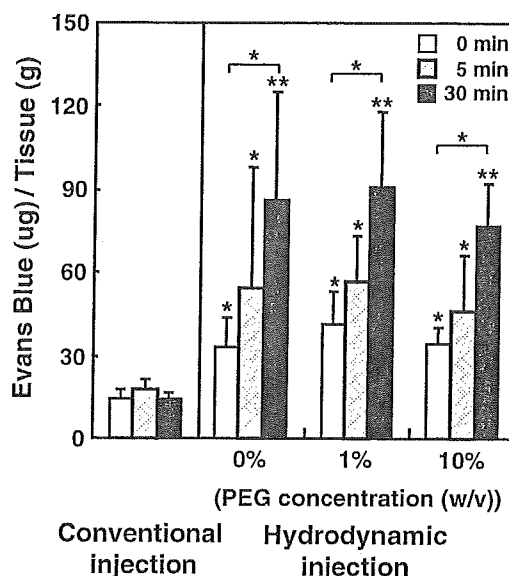

Figure 2. Effect of PEG on vascular permeability and/or hepatocyte-membrane permeability. Evans Blue dye (0.2 mg in 2 ml of saline) containing 0, 1 or 10% (w/v) PEG2000 was injected into the tail vein with the hydrodynamics-based procedure. The dye (0.2 mg in 0.2 ml of saline) was injected at either 5 or 30 min after the hydrodynamic injection of saline containing 0, 1 or 10% (w/v) PEG2000. At 6 h after the first injection, the liver was removed, the dye was extracted and quantified. Data are the mean ± S.D. (n = 3). Statistical significance against conventional injection was analyzed by Student's t-test. Statistical significance between gene expression levels at different time points after hydrodynamics-based transfection was analyzed by one-way ANOVA. $^{*}p < 0.05$; $^{**}p < 0.01$

Effect of PEG chain length on gene expression in the liver

PEG chain length may affect the permeability-enhancing effect of PEG on hepatocyte membranes, possibly resulting in a further enhancement of gene expression in the liver. To test this, we added 1% (w/v) of PEG6000 or PEG10000 instead of PEG2000 to the pDNA solution and injected it hydrodynamically into mice. The livers showed no significant increase in luciferase levels at 12 or 24 h after injection, compared to the values obtained with 1% (w/v) PEG2000 (data not shown). On the basis of this result and those described in Figure 1, we adopted 1% (w/v) PEG2000 to add to the pDNA solution for all subsequent experiments.

Effect of PEG on vascular permeability and/or hepatocyte membrane permeability

To examine the effect of PEG on the vascular permeability and/or hepatocyte membrane permeability, the mice were treated with the hydrodynamic injection or a conventional injection. Subsequently, they were treated with a conventional injection of Evans Blue solution (200 μl), which is one of the most commonly used markers for membrane permeability [35]. The hydrodynamic injection resulted in a significant increase in the level

of Evans Blue in the liver, compared to the conventional injection (Figure 2). This effect was observed regardless of the PEG concentration and time after hydrodynamic injection. Interestingly, the concentration of Evans Blue in the liver increased with increasing the time interval between the two injections.

Liver damage following rapid injection with a large volume of PEG-containing pDNA solution

To examine whether PEG might cause more severe liver damage relative to the regular hydrodynamics-based procedure, liver histology was examined by histopathological evaluation. Figure 3 shows liver sections of mice receiving a small volume of pDNA solution (a conventional injection) or a large volume of pDNA solution with or without PEG2000 (1 or 10% (w/v)) within 5 s (the hydrodynamic injection).

While no histological abnormalities were observed in the liver following the normal injection (Figure 3A), injection via the hydrodynamics-based procedure caused significant liver damage regardless of the presence or absence of PEG (Figures 3B, 3C, and 3D). Following the hydrodynamic injection, hepatocytes showed degeneration with
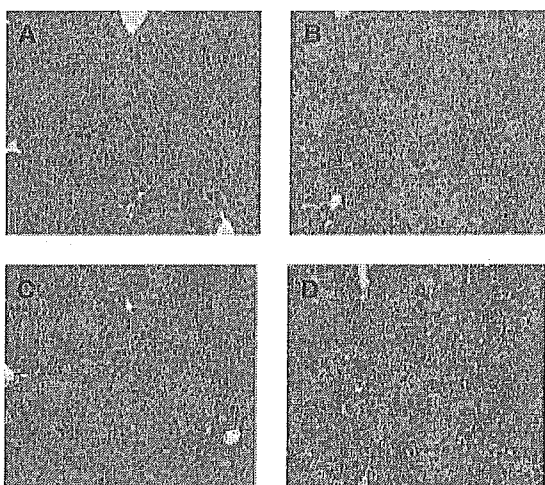
Figure 3. Microscopic images of the liver following conventional or hydrodynamic injection. Mice were treated with 0.2 ml of saline by conventional injection (A) or with 2 ml of saline containing 0 (B), 1 (C) or 10% (w/v) (D) PEG2000 using the hydrodynamic injection. At 10 min after each injection, the liver was removed, fixed in 20% neutral-buffered formalin, sectioned at 5 μm and stained with hematoxylin and eosin. Magnification 50×
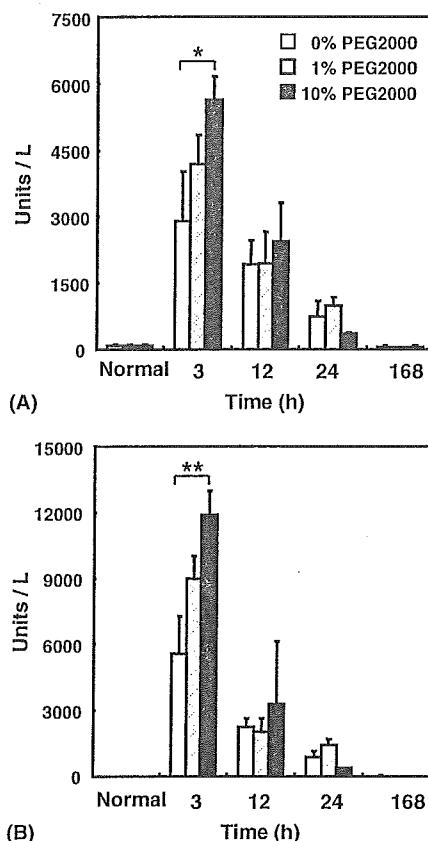

Figure 4. Profiles of serum enzymes (AST (A) and ALT (B)) following the hydrodynamic injection. Mice received a large-volume intravenous injection of saline (2 ml/mouse/5 s) and blood was collected at the indicated times. Serum AST and ALT activities were determined. Normal represents age-matched, untreated mice. The results are expressed as the mean ± S.D. (n = 3). Statistical significance between gene expression levels with different PEG concentrations (0, 1 and 10%) was analyzed by one-way ANOVA. $*p < 0.05$; $**p < 0.01$

the cytoplasm displaying decreased basophilia as well as a fine granular appearance and necrobiosis, sometimes with erythrocytes in the cytoplasm. The histopathological scores are summarized in Table 1. Hydrodynamics-based injection with 1% (w/v) PEG2000 caused significantly less liver damage than the hydrodynamics injection with none or 10% (w/v) PEG2000.

Hepatocyte damage was also determined by measurement of serum levels of the liver enzymes aspartate aminotransferase (AST) and alanine aminotransferase (ALT). Figure 4 shows the concentration profiles of AST and ALT following the hydrodynamic injection with none, 1% (w/v) or 10% (w/v) PEG2000. In this study, we excluded time points less than 3 h, after injection, because of the extensive blood dilution by the large volume of injected solution. We observed a strong but transient elevation of liver enzymes following hydrodynamic injection, dependent on PEG concentration. The highest levels were detected 3 h after injection. Enzyme levels then decreased gradually and returned to normal by 168 h (7 days). Surprisingly, the hydrodynamic injection with 1% (w/v) PEG2000 showed no significant difference in

the level of ALT and AST compared to the hydrodynamic injection without PEG.

Quantification of pDNA transported into hepatocytes and nuclei as a result of hydrodynamic injection

The amount of pDNA delivered into the hepatocytes or their nuclei was determined by PCR at 12 and 24 h

Table 1. Histopathological observations after hydrodynamic injection with various concentrations of PEG2000

Injection group	Conventional injection (200 μl)			Hydrodynamic injection (2 ml)								
PEG concentration (% (w/v))	—			0			1			10		
Grade	+	++	+++	+	++	+++	+	++	+++	+	++	+++
Number of mice (n = 5)	0	0	0	0	1	4	0	3	2	0	0	5

The histopathological scores were recorded by microscopic examination of liver sections as described in Figure 3. +: slight, ++: moderate, +++: severe.
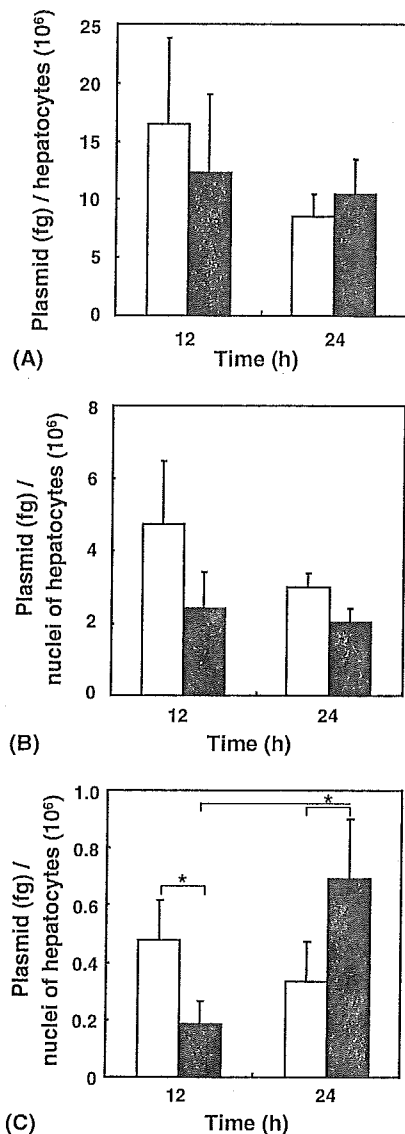
(A)

(B)

(C)

Figure 5. Total amount of pDNA delivered to hepatocytes (A) or to hepatocyte nuclei (B) or intact pDNA delivered to hepatocyte nuclei (C) following hydrodynamic injection. The amount of pDNA delivered into the hepatocytes or nuclei of the hepatocytes was determined at 12 or 24 h after hydrodynamic injection of 40 μg pDNA with or without 1% (w/v) PEG2000. The amount of intact pDNA transported into the nuclei of the hepatocytes was also determined. The results are expressed as the mean ± S.D. (n = 3). Statistical significance was analyzed by Dunnett's test. *$p < 0.05$

after the injection. With the hydrodynamic injection method substantial amounts of pDNA were transported into hepatocytes (Figure 5A). By contrast, with the conventional injection method, pDNA levels in the hepatocytes were below the detection limit (data not shown). Although at 24 h pDNA levels in hepatocytes were somewhat lower than at 12 h, this difference was not significant (Figure 5A). Approximately 20–30% of the pDNA in the hepatocytes was transported in the nuclei (Figure 5B). There was no statistically significant difference between the 12 and 24 h time points, although

pDNA transported by the hydrodynamic injection without PEG tended to decrease.

Since, obviously, only intact intranuclear pDNA reflects gene expression, it is necessary to distinguish between intact and partially or completely degraded DNA. Thus, we also determined only intact pDNA transported in the nuclei as a consequence of the hydrodynamic injection. At the earlier time point (12 h), the regular hydrodynamic injection, i.e. without PEG, transported a larger amount of intact pDNA in the nuclei than the hydrodynamic injection with PEG ($p < 0.05$) (Figure 5C). The amount of intact pDNA transported into the nuclei by the regular hydrodynamic injection did not change with time, but the amount transported by the hydrodynamic injection with PEG increased approximately 3.5-fold between 12 and 24 h. The fraction of intact pDNA as a percentage of total pDNA in the nuclei was 10.3 ± 0.7% at 12 h and 10.8 ± 3.7% at 24 h ($p = 0.85$), following the hydrodynamic injection without PEG. These values were 7.8 ± 1.4% at 12 h and 38.4 ± 13.7% at 24 h ($p < 0.05$), following the hydrodynamic injection with PEG.

Time-course study on gene expression in the liver induced by the improved hydrodynamics-based procedure

The results described above indicate that our modified hydrodynamics-based procedure, i.e. a rapid injection of a large volume of 1% (w/v) PEG2000-containing pDNA solution, may enhance gene expression relative to that achieved by the regular hydrodynamics-based procedure. To examine this possibility, a time-course study on the expression of luciferase in the liver was carried out following the injection (Figure 6). In the regular hydrodynamic injection, luciferase activity reached a maximum level at 8–12 h. From then on the level gradually decreased by 72 h. Our modified hydrodynamics-based procedure moved the peak of maximum luciferase activity to 24 h and increased the maximum level nearly 2-fold compared to the regular procedure. From then on the expression was gradually decreased, but the level at 72 h was still much higher (approximately 300-fold) than that of the regular hydrodynamic injection. The area under the gene expression-time curve was enlarged approximately 2-fold by addition of 1% (w/v) PEG2000. Clearly, the addition of PEG2000 substantially enlarged the hepatic gene expression induced by the hydrodynamics-based procedure as we expected.

Discussion

The liver is an important target organ for gene transfer due to its large capacity for synthesizing serum proteins and its involvement in numerous genetic and acquired diseases. Thus, liver-targeted gene transfer is an important tool for expanding the treatment options for liver
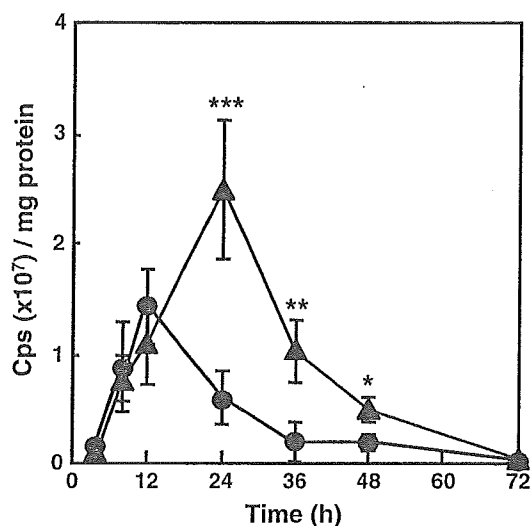
Figure 6. Profiles of gene expression in the liver following hydrodynamic injection. Mice received a large-volume intravenous injection of saline (40 μg pDNA in 2 ml/mouse/5 s) with or without 1% (w/v) PEG2000 and livers were processed for luciferase assay at the indicated times after the injection. ●; hydrodynamic injection without PEG (regular hydrodynamic injection), ▲; hydrodynamic injection with 1% PEG. Luciferase activities are expressed as mean ± S.D. (n = 6). Statistical significance against regular hydrodynamic injection was analyzed by Student's t-test. $*p < 0.05$; $**p < 0.01$; $***p < 0.005$

diseases and diseases affecting other organ systems, and for gene function studies. Among the various gene delivery systems available, naked pDNA-mediated gene transfer is the simplest, and techniques for introducing naked pDNA into hepatocytes have been among the most intensely studied methods to generate therapeutic amounts of gene product. Liu et al. [11] and Zhang et al. [5] have developed a technique that does not require local administration or surgical procedures, to produce extremely high expression levels of exogenous genes in mouse liver, using the systemic rapid administration with a large volume of naked pDNA solutions into the tail vein. However, in these reports, the induced gene expression was transient, probably because introduced pDNA was rapidly degraded in the hepatocytes and/or transfected hepatocytes were removed by the immune system. Our study indicates that enhanced gene expression levels in mouse liver can be achieved by addition of only 1% (w/v) PEG2000 to a large volume of pDNA solution and subsequent hydrodynamic injection. This modified simple gene transfer method would increase the potential of the regular hydrodynamics-based procedure for laboratory studies and gene therapy, although further studies will be required to understand the mechanism whereby enhancement of gene expression is achieved before application to other tissues or to humans can be considered.

Based on serum enzyme and histological examinations (Figures 2, 3, and 4), the hydrodynamic injection procedure clearly induces transient damage against the hepatocytes, irrespective of the presence of PEG2000.

The liver damage was not the result of pDNA delivery, but rather a reaction to the vehicle or procedure, since injection of saline induced similar damage on the liver (data not shown). It is likely that the PEG2000 causes the liver damage to further escalate in a PEG-concentration-dependent manner, since the level of serum enzymes increased with an increase in the PEG concentration (Figure 4). This PEG-induced escalation of damage brings with it a concern that the PEG2000 increases the toxicity of the regular procedure. However, no significant additional liver damage was caused by addition of 1% (w/v) PEG2000 (Table 1, Figures 3 and 4), compared to that by the regular hydrodynamic injection. Liu et al. [11] found no indication of serious liver damage as assessed by animal growth and clinical biochemistry tests, which were all in normal range with the exception of a transient increase in serum concentration of alanine aminotransferase. Currently, there is an increasing interest in the usefulness of PEG for biomedical applications, particularly because of its relative low toxicity and rapid clearance by urinary excretion. Concentrations of PEG up to 40% (w/v) are considered safe and relatively nontoxic for use in animal studies [36]. This suggests that our procedure as described here, hydrodynamic injection with 1% (w/v) PEG2000, is safe for animals, and the procedure is acceptable and useful for analyzing regulation of gene expression in vivo, although further safety investigations will be required.

Interestingly, the PEG concentration was inversely related to the level of gene expression in the liver (Figure 1). It appears that the PEG plays a bifunctional role in gene expression following the hydrodynamic injection, i.e. it increases gene expression at lower concentration, whereas it diminishes it at higher concentration. Very similar results were reported on gene delivery in skeletal muscles with the block copolymers SP1017 and PE6400 [37,38]. At low concentrations (up to 0.01% (w/v) for SP1017 and 0.5% (w/v) for PE6400), gene expressions were increased in a dose-dependent manner. At higher concentrations, the efficacy of gene expression declined. Although the mechanism of the bifunctionality of block copolymers on gene transfer remains to be explored, that of PEG observed here probably results from the PEG-concentration-dependent liver damage observed in Figures 3 and 4. Death of a large number of hepatocytes would cause gene expression in very few labile hepatocytes, if the PEG enhanced the delivery of a large amount of pDNA to the hepatocytes relative to the regular hydrodynamics-based procedure.

Two mechanisms have been proposed to explain the entry of pDNA into hepatocytes following the hydrodynamic injection: endocytosis [39] or penetration by plasma membrane pores created by the hydrodynamic pressure [40]. According to recent reports [41,42], the second mechanism is more likely. PEG has a variety of effects on biological systems [23–26]. One of them is to increase membrane permeability due to osmotic effects [30]. We expected that the addition of PEG to the hydrodynamic injection solution would increase membrane permeability of hepatocytes and thereby

elevate the amount of pDNA transported intracellularly, resulting in enhanced gene expression in the hepatocytes. Our results showed that the PEG did enhance gene expression in the liver (Figures 1 and 6). However, this is not a result of an increased amount of pDNA transported to the hepatocytes, as revealed by Figure 5B. This figure suggests that the PEG does not affect the process facilitating the entry of pDNA into the hepatocytes, which is supported by our experiment with Evans Blue dye injection showing that the PEG did not affect the membrane permeability of hepatocytes due to the hydrodynamic injection (Figure 2).

Once the pDNA molecules have entered into the hepatocytes, they must be transported to the nucleus, where transcription takes place, to cause efficient gene expression. This process is considered one of the limiting steps governing the efficiency of gene expression. As mentioned above, both hydrodynamic procedures transported similar amounts of pDNA to the nuclei (Figure 5B). However, in terms of *intact* pDNA, the addition of PEG clearly increased the amount in the nuclei with increasing time after injection (the regular procedure, $10.8 \pm 3.7\%$ (intact pDNA to total pDNA, at 24 h) vs. our procedure, $38.4 \pm 13.7\%$, ($p < 0.05$), estimated from the results in Figures 5B and 5C). This efficient transport of *intact* pDNA to the nuclei may account for the enhancement of gene expression (Figures 1 and 6). The mechanism by which PEG elevates the amount of *intact* pDNA in the nuclei is unclear. It may involve in part cytosolic delivery of PEG via an influx along with the large volume of pDNA solution. Presumably then, the infused PEG prevents pDNA molecules from degradation by the intracellular DNases, resulting in retention of a large number of *intact* pDNA copies intracellularly. As a result, transport of intracellular *intact* pDNA to nuclei may be facilitated efficiently with increasing the time after the injection.

Recent observations by Andrianaivo *et al.* [42] may provide another possible mechanism by which PEG may account for the enhanced transport of *intact* pDNA to nuclei. They showed that a large amount of pDNA remains bound to the outside of the plasma membrane of hepatocytes for a relatively long time after a hydrodynamic injection. The authors hypothesized that the hydrodynamics-based transfection takes place in two phases. In the first one a few DNA molecules entering the hepatocytes very quickly lead to a strong expression in a relatively short time. In the second one the DNA molecules bound to the plasma membrane cause a low expression for a long time via the relatively slow endocytosis process. On the basis of their hypothesis, the second process may be reinforced with the PEG in our study. It is likely that the PEG protects the pDNA bound to the plasma membrane from degradation by DNase and consequently enhances delivery of a relatively large number of pDNA molecules inside the hepatocytes. As a consequence, the intracellular source of intact pDNA for intranuclear transport may be maintained for a relatively long time.

It has been shown that the osmotic pressure of PEG affects the regulation of a number of physiological processes of cells. Both hypo- as well as hyperosmotic shocks induce changes in plant cell metabolism [43,44]. Cells have specific mechanisms for the restoration of their volume after osmotic shocks, involving the activation of tyrosine kinases [45]. Cell volume changes have been suggested to be of importance in the regulation of protein degradation and synthesis [46]. The increased activity of ornithine decarboxylase in osmotically swollen cells is at least partially related to an enhanced synthesis of the enzyme protein in the absence of a change in its mRNA production [47]. Transcription of specific genes in mammalian cells is enhanced by hypersmotic shock [48,49]. Taken together, the PEG in the large-volume pDNA solution may affect the physiological processes of hepatocytes after the hydrodynamic injection. The physiological change of the hepatocytes, along with a large number of *intact* pDNA molecules in the nuclei, may result in enhancement of gene expression in the liver, following the injection.

Recently, it was demonstrated that Pluronic block copolymers can increase regional expression of the naked pDNA after its injection in the skeletal and cardiac muscles [37,38,50]. The mechanism by which Pluronic block copolymer acted on the naked pDNA expression has not been established; however, the following speculations were made: (1) Interactions between the block copolymer and membrane accelerate cellular uptake of naked pDNA; (2) the block copolymer enhances pDNA distribution through muscle tissues; (3) the block copolymer may increase the transport of free pDNA from the cytoplasm to the nucleus of muscle cells; and (4) the block copolymer acts as synthetic biological response modifier, resulting in increased gene expression as a consequence of activating transcription of the gene. Taken together with these speculations and our results described here, the PEG may increase the transport of free pDNA from the cytoplasm to the nucleus of hepatocytes as well as activate the transcription of the transgene expression. However, at present, we cannot exclude the possibility that the infused PEG may also protect pDNA-derived mRNA or expressed exogenous protein (luciferase) from digestion by intracellular DNase or proteinases.

It is noteworthy that the vascular and/or hepatocyte-membrane permeability increased with increasing the time interval (up to 30 min) between hydrodynamic injection and Evans Blue injection (conventional injection) (Figure 2). This indicates that the enlargement of the liver endothelial fenestrae and hepatocyte-membrane defects remain in existence for at least 30 min after the hydrodynamic injection. In contrast, Zhang *et al.* [31] recently showed that most of the defects of the hepatocyte membranes resealed within 10 min after hydrodynamic injection. In addition, these defects have to disappear rapidly, as living cells have to reseal within seconds to a minute in order to survive [51]. This discrepancy may be due to the difference in experimental conditions. We

determined the level of Evans Blue in the liver by extraction of the dye directly from the liver tissue, while Zhang *et al.* assessed the microdistribution of the dye in the liver section by fluorescence microscopy. Under our conditions, all Evans Blue dye in the liver was quantitatively determined. This amount includes not only the dye entering the hepatocytes through the pores generated by the elevated pressure, but also that accumulating in Disse's space or Kupffer cells because the dye associates with serum albumin (Figure 3).

Despite many desirable features associated with our procedure, such as simplicity, convenience and high efficiency, the level and time of gene expression driven by our method may be yet too low and short. Recently, Herweijer *et al.* [52] reported the effect of a liver-specific promoter on longevity of transgene expression: the albumin promoter (a 300 bp promoter in conjunction with a 2 kb upstream enhancer region) sustained expression of luciferase in the liver following hydrodynamics-based transfection. Miao *et al.* [53] also reported a similar result that long-term expression of human factor IX in mice was achieved by tail vein injection of a pDNA employing an α 1-antitrypsin promoter in conjunction with a hepatic control region. Therefore, we are now testing whether high-level, sustained transgene expression in the liver can be achieved by using a pDNA containing liver-specific promoters with our method.

Acknowledgements

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Pharmaceutical Nanotechnology

Applicability of anti-neovascular therapy to drug-resistant tumor: Suppression of drug-resistant P388 tumor growth with neovessel-targeted liposomal adriamycin

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Abstract

Anti-neovascular therapy, one of the effective anti-angiogenic chemotherapy, damages new blood vessels by cytotoxic agents delivered to angiogenic endothelial cells and results in indirect eradication of tumor cells. We previously reported that liposomes-modified with a pentapeptide, Ala-Pro-Arg-Pro-Gly (APRPG-Lip) homing to angiogenic site, highly accumulated in tumor tissue, and APRPG-Lip encapsulating adriamycin (APRPG-LipADM) effectively suppressed tumor growth in tumor-bearing mice. In the present study, we examined the topological distribution of fluorescence-labeled APRPG-LipADM as well as TUNEL-stained cells in an actual tumor specimen obtained from Colon 26 NL-17 carcinoma-bearing mice. The fluorescence-labeled APRPG-Lip dominantly localized to vessel-like structure: A part of which was also stained with anti-CD31 antibody. Furthermore, TUNEL-stained cells were co-localized to the same structure. These data indicated that APRPG-LipADM bound to angiogenic endothelial cells and induced apoptosis of them. We also investigated the applicability of anti-neovascular therapy using APRPG-LipADM to ADM-resistant P388 solid tumor. As a result, APRPG-LipADM significantly suppressed tumor growth in mice bearing the ADM-resistant tumor. These data suggest that APRPG-LipADM is applicable to various kinds of tumor including drug-resistant

tumor since it targets angiogenic endothelial cells instead of tumor cells, and eradicates tumor cells through damaging the neovessels.

1. Introduction

Tumor angiogenesis, construction of new blood vessels in tumor tissue, is critical for tumor growth, since the supply of oxygen and nutrients is essential for many tumors. Angiogenesis is also related to blood-borne metastasis to distal organs, since it is initiated through this angiogenic vasculature (Folkman, 1971). A number of previous studies on angiogenesis have elucidated the functions of pro-angiogenic factors such as vascular endothelial growth factor (VEGF) and the biological process of angiogenesis (Asahara et al., 1999; Ferrara, 2002). Based on these findings, cancer therapy targeted to angiogenesis has been considered and various inhibitors for angiogenic process have been developed (Kerbel and Folkman, 2002). These agents often prevent pro-angiogenic factors from binding to their receptors or inhibit signal transduction in angiogenesis. This therapy is generally called as anti-angiogenic therapy. For example, the treatment of antibody against VEGF receptor, KDR/flk-1 suppressed tumor growth by inhibition of angiogenesis (Brekken et al., 2000). We previously suggested that disruption of angiogenic vasculature by allowing cytotoxic agent to angiogenic endothelial cells could effectively inhibited tumor growth (Oku et al., 2002a). Since angiogenic endothelial cells have acquired enhanced growth ability, cytotoxic anti-cancer agents are able to damage angiogenic endothelial cells as well as tumor cells. This anti-neovascular therapy is expected to overcome several but critical problems in traditional cancer chemotherapy. For example, anti-neovascular therapy can reduce injected dose, since the angiogenic endothelial cells, which should be eradicated by this therapy, are fewer than the tumor cells in a tumor tissue. In addition, this therapy is promising to apply to various kinds of solid tumors including drug-resistant tumors since the nature of angiogenic vessels may be the same or quite similar among tumors despite the tumor cells acquire the drug-resistance (Browder et al., 2000).

In the previous study, we isolated peptides specific for tumor angiogenic vasculature by in vivo biopanning of a phage-displayed peptide library (Oku et al., 2002b). Obtained Ala-Pro-Arg-Pro-Ala (APRPG) peptide was used for an active targeting tool for angiogenic vessels. In fact, APRPG-modified liposomes (APRPG-Lip) highly accumulated in tumor tissue and adriamycin (ADM)-encapsulated APRPG-Lip (APRPG-LipADM) effectively suppressed tumor growth in Meth A sarcoma- and Colon 26 NL-17 carcinoma-bearing model mice. Furthermore, APRPG-Lip bound specifically to VEGF-stimulated human umbilical vein endothelial cells (HUVECs) compared with unmodified ones (Oku et al., 2002b). These findings suggested that APRPG-LipADM shows potent anti-tumor effect in order to eradicate angiogenic vasculature and, therefore, is expected to apply to drug-resistant tumor.

In the present study, to confirm that APRPG-LipADM really damages angiogenic endothelial cells, intratumoral distribution of APRPG-Lip and vessel damage in tumor tissue after the treatment of APRPG-LipADM were examined. Furthermore, to investigate whether APRPG-modified liposomal agent can be applied to drug-resistant cancer, we performed therapeutic experiment using ADM-resistant P388 solid tumor-bearing mice.

2. Materials and methods

2.1. Materials

Stearoyl-APRPG derivative was synthesized as previously described (Asai et al., 2002). Distearoylphosphatidylcholine (DSPC) was a gift from Nippon Fine Chemical Co. Ltd. (Takasago, Hyogo, Japan). Cholesterol was purchased from Sigma Chemical Co. (St. Louis, MO, USA). Balb/c and DBA/2 male mice were purchased from Japan SLC, Inc.