International Journal of Pharmaceutics 423 (2012) 428–434

Contents lists available at SciVerse ScienceDirect

International Journal of Pharmaceutics

journal homepage: www.elsevier.com/locate/ijpharm

Pharmaceutical Nanotechnology

Collagenase-1 injection improved tumor distribution and gene expression of cationic lipoplex

Mako Kato, Yoshiyuki Hattori*, Manami Kubo, Yoshie Maitani

Institute of Medicinal Chemistry, Hoshi University, Ebara 2-4-41, Shinagawa-ku, Tokyo 142-8501, Japan

ARTICLE INFO

Article history:
Received 5 August 2011
Received in revised form 21 October 2011
Accepted 5 December 2011
Available online 17 December 2011

Keywords:
Collagenase
Collagen
Interstitial fluid pressure
Nonviral gene delivery
Lipoplex

ABSTRACT

Elevated interstitial fluid pressure (IFP) in a tumor is a barrier to tumor accumulation of systemic delivery of nanocarriers. In this study, we investigated whether intravenous injection of type I collagenase (collagenase-1) reduced IFP in tumors and increased the accumulation and gene expression of cationic liposome/plasmid DNA complex (lipoplex) in tumors after intravenous injection into mice bearing mouse lung carcinoma LLC tumors. Collagenase-1 reduced the amount of type I collagen in the tumor, and significantly decreased IFP by 65% at 1 h after injection. Therefore, collagenase-1 induced 1.5-fold higher accumulation and 2-fold higher gene expression of lipoplex in tumors after intravenous injection. These findings indicated that intravenous injection of collagenase-1 improved the accumulation of lipoplex by decreasing IFP in tumors. These results support the potential use of collagen digestion as a strategy to improve systemic gene delivery into tumors.

1. Introduction

Systemic delivery of anticancer drugs or therapeutic genes to tumors requires transport through the extracellular space in tumors, also called the tumor interstitium. Compounds with low molecular weight are mainly transported by diffusion, which is dependent on concentration differences. Meanwhile, macromolecules such as proteins and nanocarriers are mainly transported by convection flow, which is driven by differences between hydrostatic pressure in the vessel (microvascular pressure, MVP) and interstitial fluid pressure (IFP) in tumors (Bouzin and Feron, 2007; Rippe and Haraldsson, 1994). Therefore, transport of macromolecules and nanocarriers into tumors is impeded by imbalances between MVP and IFP. Generally, normal pressure from the capillaries into surrounding tissues is about 1–3 mm Hg; however, IFP in tumors is often increased (Young et al., 1950), and high IFP in tumors is an obstacle to the delivery of nanocarriers such as liposomes (Jain, 1987a,b). Many studies of human and rodent tumors have observed a relationship between decreased drug uptake and elevated IFP in tumors (Curti et al., 1993).

The mechanism for increased IFP in tumors involves the unique microenvironment of solid tumors. Tumor tissues contain leaky vessels which have deficient coverage by pericytes (Dvorak et al., 1995), and the leakiness of tumor vessels increases the outflow of osmotic proteins from the vessels. Furthermore, the lack of a lymphatic system (Alitalo and Carmeliet, 2002; DiResta et al., 2000; Leu et al., 2000; Padera et al., 2002; Ribatti et al., 2007) reduces the mechanism to drain excess proteins from the tumor interstitium, and these accumulated proteins elevate IFP in tumors. If osmotic proteins were accumulated in tissues, IFP in normal tissues are maintained by changes in the volume of the tissues such as edema in patients with inflammation or burn injuries; however, IFP in solid tumor could not be maintained, because the tumor interstitium has a denser network of connective-tissue molecules such as collagen, hyaluronan and proteoglycans. Consequently, increased IFP in tumors is persistent (Heldin et al., 2004). The tumor interstitium is enriched with tumor-associated fibroblasts (TAF), endothelial cells, pericytes, infiltrating inflammatory cells and extracellular matrix (ECM) (Fleming et al., 2010; Park, 2010). Platelet-derived growth factor (PDGF), which is released from tumor cells, stimulates the activity of TAF, which produces ECM (Desmouliere et al., 2004). Interstitial hydraulic conductivity has been found to correlate inversely with collagen and glycosaminoglycan content (Swabb et al., 1974; Weinberg et al., 1997), as well as with IFP (Jain and Baxter, 1988). The interstitial diffusion coefficient also correlated inversely with collagen (Netti et al., 2000).

Recently, the use of collagenase or hyaluronidase for modulation of ECM in tumors has proven useful in reducing IFP in animal models (Eikenes et al., 2004, 2005) and has succeeded in improving the accumulation of antibody (Eikenes et al., 2004) and liposomal doxorubicin in tumors (Eikenes et al., 2005; Zheng et al., 2011). In cancer gene therapy, cationic liposomes were used as a carrier for the delivery of therapeutic genes such as plasmid DNA (pDNA) into tumors; however, the application of these enzymes for gene

* Corresponding author. Tel.: +81 3 5498 5097; fax: +81 3 5498 5097.
E-mail address: yhattori@hoshi.ac.jp (Y. Hattori).

0378-5173/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.ijpharm.2011.12.015

delivery by cationic liposome has not been reported. Type I collagen is a major constituent of ECM in tumors and plays a central role in impeding solute transport within the tumor interstitium (Magzoub et al., 2008). The interstitial collagen network is heterogeneous, with a substantial amount of collagen surrounding the microvessels. Type I collagenase (collagenase-1), known as matrix metalloproteinase-1 (MMP-1), is a protease with substrate specificity for mainly type I and III collagens. In this study, we focused on collagenase-1 for the reduction of IFP in tumors, and investigated whether collagenase-1 treatment could improve accumulation and gene expression in tumors after intravenous injection of cationic liposome/pDNA complex (lipoplex).

2. Materials and methods

2.1. Materials

N-[1-(2,3-dioleoyloxy) propyl]-*N*,*N*,*N*-trimethylammonium methyl-sulfate (DOTAP) was purchased from Avanti Polar Lipids Inc. (Alabaster, AL, USA). Cholesterol (Chol) and type I collagenase from *Clostridium histolyticum* (230 U/mg, EC 3.4.24.3) were purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan). Methoxy-poly (ethyleneglycol)–distearylphosphatidylethanolamine (PEG_{2000}–DSPE, PEG mean molecular weight, 2000) was purchased from NOF Inc. (Tokyo, Japan). Lissamine™ rhodamine B 1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine, triethylammonium salt (rhodamine-DHPE) was purchased from Invitrogen (Carlsbad, CA, USA). All other chemicals were of the finest grade available.

2.2. Plasmid DNA

pCpG free-Luc encoding the *firefly* luciferase gene under the control of human elongation factor 1 alpha promoter and mouse cytomegalovirus enhancer was constructed by inserting the *firefly* luciferase cDNA fragment from pMOD-LucSh (Invivogen, San Diego, CA, USA) into the *Bgl*II/*Nhe*I site of pCpG-mcs (Invivogen), and was then amplified in *Escherichia coli* GT115. A protein-free preparation of the plasmid DNA (pDNA) was purified following alkaline lysis using the EndoFree Plasmid Max Kit (Qiagen, Hilden, Germany).

2.3. Cell culture

Mouse Lewis lung carcinoma (LLC), mouse Colon 26, mouse sarcoma 180 (S180), mouse neuroblastoma Neuro2a and mouse melanoma B16 cells were supplied by the Cell Resource Center for Biomedical Research, Tohoku University (Miyagi, Japan). The cells were grown in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS) and kanamycin (100 μg/mL) at 37 °C in a 5% CO_2 humidified atmosphere.

2.4. Tumor model

All animal experiments were performed with approval from the Institutional Animal Care and Use Committee of Hoshi University. To generate tumors, 1×10^6 cells suspended in 50 μL RPMI-1640 medium were inoculated subcutaneously into the flank of mice (7 weeks of age). LLC and B16 cells were implanted into female C57BL/6NCrSlc mice (Sankyo Lab. Service Corp., Tokyo, Japan), Neuro2a into female A/J Jms Slc mice (Sankyo Lab. Service Corp.), Colon 26 into female CDF1 mice (Sankyo Lab. Service Corp.) and S180 into female ddY mice (Sankyo Lab. Service Corp.).

2.5. Toxicity by injection of collagenase

C57BL/6NCrSlc mice were intravenously injected with 100 μL of 0.3% collagenase-1 (0.3 mg) or 0.5% collagenase-1 (0.5 mg) in PBS. Twenty-four hours after injection, the lung was were immediately frozen, sectioned 16-μm thick and mounted. The sections were stained with hematoxylin and pure eosin (H&E staining) (Muto Pure Chemicals Co. Ltd., Tokyo, Japan).

2.6. Immunohistochemistry

When the average volume of LLC, B16, S180, Neuro2a and Colon 26 tumors reached 100–200 mm^3, the mice were sacrificed and the tumors were frozen on dry ice. The frozen tumors were embedded in O.C.T. compound and processed by frozen sectioning at 16 μm. Each frozen section was mounted on silane-coated slides. After protein blocking for 1 h at room temperature with PBS containing 5% rabbit serum (Sigma–Aldrich, St. Louis, MO, USA), the sections were incubated with goat polyclonal antibody against type I collagen (COL1A1 (D-13); Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), followed by incubation with Alexa 555-labeled rabbit anti-goat IgG (Invitrogen) as the secondary antibody. Fluorescence was examined microscopically using an ECLIPSE TS100 microscope (Nikon, Tokyo, Japan).

2.7. Western blotting

When the tumor volume reached approximately 100–200 mm^3, LLC tumor-bearing mice were intravenously injected with 100 μL of 0.3% collagenase-1 (0.3 mg) in PBS or intratumoraly injected with 50 μL of 0.6% collagenase-1 (0.3 mg) in PBS. One hour after injection, the tumors were excised and then homogenized in 10 mM Tris buffer (pH 8.0) containing 250 mM sucrose and phenylmethanesulfonylfluoride (PMSF). As a control, untreated LLC tumors were homogenized and then incubated with 0.3 mg collagenase-1 at 37 °C for 1 h. For the detection of type I collagen and β-actin proteins, 30 μg proteins were separated by 7.5 and 12.5% SDS-polyacrylamide gel, respectively, and then transferred to a polyvinylidene difluoride membrane (iBlot™ Gel Transfer Stacks PVDF, Regular®; Invitrogen) by a device (iBlot™ Gel Transfer Device®; Invitrogen). Type I collagen protein was identified using antibody against type I collagen (COL1A1 (D-13)) and rabbit anti-goat IgG horseradish peroxidase conjugate (Santa Cruz Biotechnology Inc.) as the secondary antibody. Expression of β-actin protein was identified using mouse anti-β-actin monoclonal antibody peroxidase conjugate (Santa Cruz Biotechnology). Type I collagen and β-actin proteins were detected with peroxidase-induced chemiluminescence (Super Signal West Pico Chemiluminescent Substrate; Pierce, Rockford, IL, USA).

2.8. IFP measurement in tumors

When the tumor volume reached approximately 200 mm^3, LLC, Colon 26 and Neuro2a tumor-bearing mice were intravenously injected with 100 μL of 0.3% collagenase-1 in PBS. One and twenty-four hours after injection, mice were anesthetized with isoflurane, and then interstitial fluid pressure (IFP) of tumors was measured with a needle probe pressure monitor, fitted with an 18-gauge side-ported needle (Intra-Compartmental Pressure Monitor System; Stryker, Kalamazoo, MI, USA) connected to a syringe filled with 0.9% saline, as previously reported (Tailor et al., 2010). The needle probe was inserted into the center of the tumor or normal muscle, and IFP was recorded. The IFP in tumors was normalized to that in muscle (normalized IFP = IFP (mm Hg) of tumor/IFP (mm Hg) of muscle).

2.9. Preparation of liposomes and lipoplex

Liposomes were prepared from DOTAP/Chol at a molar ratio of 1/1 by a dry-film method. Briefly, all lipids were dissolved in chloroform, which was removed by evaporation. For preparation of rhodamine-labeled liposomes, rhodamine-DHPE was incorporated into the liposome formulation at 0.5 mol% in the total lipid. The thin film was hydrated with water at 60 °C by vortex mixing and sonication. For intravenous injection, the liposome/pDNA complex (lipoplex) was prepared by mixing 50 μg pDNA with the liposomes at a charge ratio (+/−) of 4/1 with gentle shaking and standing for 15 min at room temperature. For modification of the surface of the lipoplex by PEG-lipid, the lipoplex was further incubated with PEG_{2000}–DSPE at 5 mol% total lipid at 50 °C for 15 min, and the resulting formulation was allowed to cool to room temperature. The particle size distributions and ζ-potentials were determined by the dynamic light scattering method (ELS-Z2; Otsuka Electronics, Osaka, Japan) at 25 °C after diluting the dispersion to an appropriate volume with water. Size and ζ-potential of lipoplex after PEGylation were 152 nm and +38.2 mV, respectively.

2.10. Biodistribution of lipoplex

The accumulated amount of rhodamine-labeled liposome in tissues was estimated by determining the fluorescent intensity of rhodamine-DHPE extracted from tissues as previously reported (Morimoto et al., 2007). When the average volume of LLC tumors reached 100–200 mm^3, rhodamine-labeled lipoplex was intravenously administered 1 h after intravenous injection of 100 μL of 0.3% collagenase-1. For quantification of the amount of rhodamine-DHPE in tissues, tissues were excised 3 h after injection of the lipoplex, and then homogenized in PBS. To extract rhodamine-DHPE from the homogenate, 200 μL homogenate was added to 480 μL methanol, 500 μL chloroform and 200 μL saturated aqueous sodium chloride, and then the mixture was vigorously shaken for 5 min, following by centrifugation at 15,000 × *g* for 10 min. The amount of rhodamine-DHPE in the lower layer was estimated by determining the fluorescence intensity at excitation and emission wavelengths of 544 and 590 nm, respectively.

For observation of the distribution of liposomes by fluorescent microscopy, 3 h after injection of the lipoplex into LLC tumor-bearing mice, tissues were frozen immediately on dry ice, and sliced at 16 μm. The localizations of lipoplex in the tissues were examined microscopically using an Eclipse TS100 microscope.

2.11. Luciferase activity

When the average volume of LLC and Neuro2a tumors reached approximately 100–200 mm^3, lipoplex of pCpG free-Luc was intravenously administered 1 h after intravenous injection of 100 μL of 0.3% collagenase-1. At 24 h post-injection, mice were sacrificed by cervical dislocation, and tissues and tumors were removed for analysis. Three microliters of ice-cold reporter lysis buffer (Promega) per 1 mg tissues or tumors were added, and then immediately homogenized. The homogenates were centrifuged for 10 min at 4 °C and the supernatants were stored at −70 °C until the assays. Luciferase expression was measured according to the luciferase assay system (Pica gene luciferase assay kit; Toyo Ink Mfg. Co. Ltd.). Aliquots of 10 μL of the supernatants were mixed with 50 μL luciferase assay system (Pica gene) and counts per second (cps) were measured with a chemoluminometer (Wallac ARVO SX 1420 multilabel counter; PerkinElmer Life Science, Japan). The protein concentration of the supernatants was determined with a bicinchoninic acid (BCA) protein assay (Microplate BCA Protein Assay Kit – Reducing Agent Compatible; Pierce) using bovine serum albumin as the standard, and luciferase activity was calculated as cps/μg protein.

2.12. Statistical analysis

Significant differences in the mean values were evaluated by Student's unpaired *t*-test. A *p* value of 0.05 or less was considered significant.

3. Results and discussion

3.1. Assessment of type I collagen in various tumor sections

First, we evaluated the amount of type I collagen in various tumors by immunostaining (Fig. 1). Type I collagen was detected widely in LLC tumors, but locally in S180, B16, Neuro2a and Colon 26 tumors.

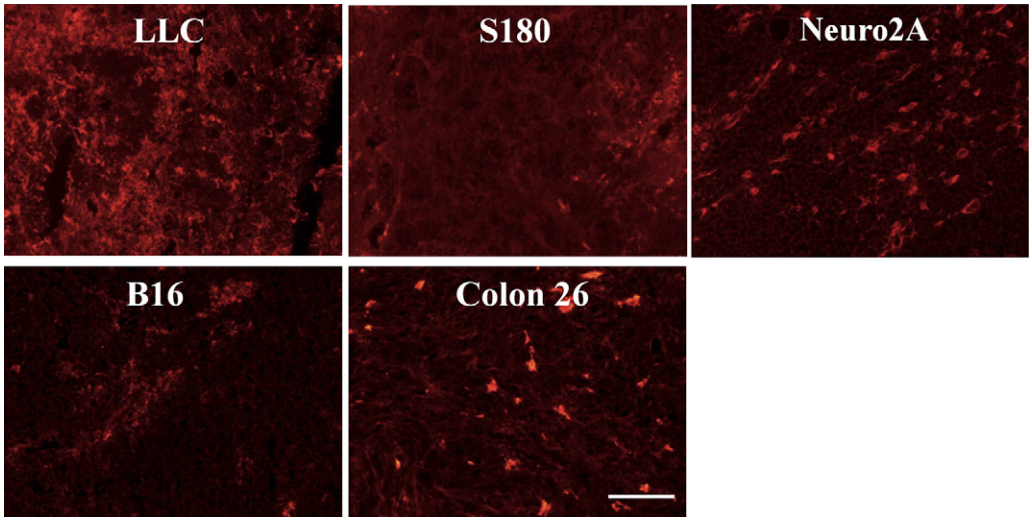

Fig. 1. Assessment of type I collagen in LLC, B16, S180, Neuro2a and Colon 26 tumors by immunostaining. Red signals show the localization of type I collagen. Scale bar = 100 μm.

3.2. Reduction of the amount of collagen and change of IFP

Next, we determined whether injection of collagenase-1 could digest type I collagen and reduce IFP in tumors. The U.S. Food and Drug Administration (FDA) has approved Xiaflex (collagenase from *C. histolyticum*) as the first drug to treat a progressive hand disease known as Dupuytren's contracture (Thomas and Bayat, 2010). Although safety by local injection of Xiaflex has been established in human, that by intravenous injection has not been. In a preliminary study, we intravenously injected 100 μl of 0.3, 0.5, 0.8 and 1.0% collagenase-1 solutions (0.3, 0.5, 0.8 and 1.0 mg collagenase-1, respectively) into mice. With dose above 0.8 mg, death occurred within a few hours (data not shown). Other researchers also reported that the lethal dose of collagenase-1 was between 0.5 and 1% (Eikenes et al., 2004). In mice, the main signs of toxicity by intravenous injection of excess collagenase-1 are hemorrhages and focal necroses in lung (Diener et al., 1973). Therefore, we examined whether collagenase-1 induced pulmonary damages 24 h after intravenous injection. Histologically, mild pulmonary damages (necrosis) were observed at some area in lung by injection of 0.5% collagenase-1 solution, but not 0.3% collagenase-1 solution (supplemental Fig. 1S). From these findings, we decided to use 100 μl of 0.3% collaganase-1 (0.3 mg) for digestion of collagen by intravenous injection into mice bearing tumors. For intratumoral injection of collagenase-1, 50 μl of 0.6% type I collagenase (0.3 mg) was directly injected into tumors because of a limited injection volume.

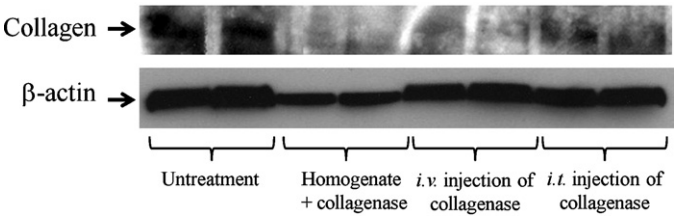

Fig. 2. Reduction of the amount of collagen in LLC tumors after injection of collagenase-1. Type I collagen in tumors was detected by Western blotting 1 h after intravenous or intratumoral injection into mice bearing tumors. As the control, homogenate of untreated tumors was incubated with collagenase-1 for 1 h at 37 °C. β-actin was used as a loading control.

When total homogenate of untreated LLC tumors was incubated with collaganase-1 (0.3 mg) for 1 h at 37 °C, type I collagen was not detected (Fig. 2A). The amount of type I collagen in LLC tumors 1 h after intravenous injection of collagenase-1 was strongly decreased, but that after intratumoral injection was moderately decreased (Fig. 2A). Intravenous injection seemed to be a better administration route of collagenase-1 than intratumoral injection. This is probably due to the large distribution of collagenase-1 *via*

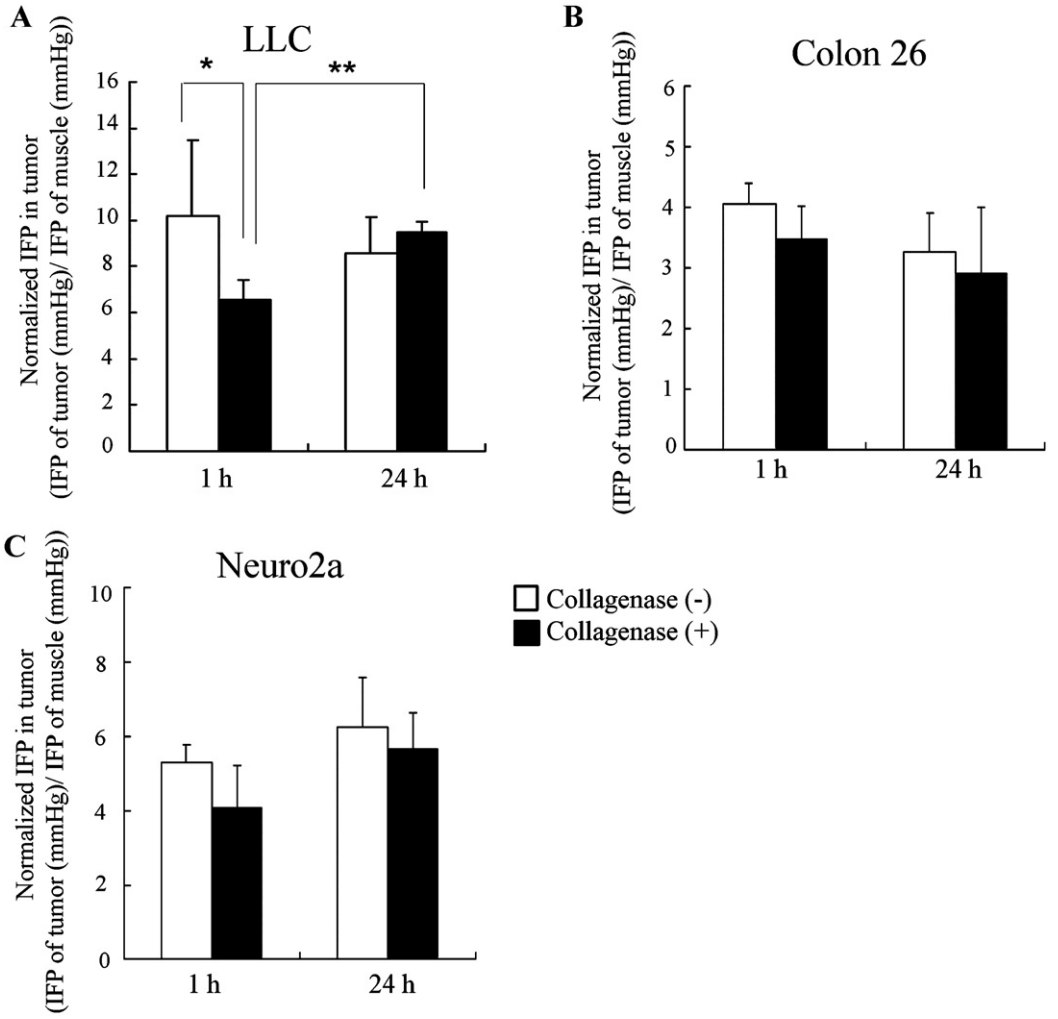

Fig. 3. Reduction of IFP in LLC, Colon 26 and Neuro2a tumors after intravenous injection of collagenase-1. IFP in tumors was measured 1 and 24 h after intravenous injection of collagenase-1 into mice bearing tumors. Normalized IFP of tumors was calculated by dividing IFP of tumors (mm Hg) by IFP of normal muscle (mm Hg). Error bars represent the mean ± one standard deviation of four to nine samples. Asterisk denotes statistical significance ($^{*}p < 0.05$, $^{**}p < 0.01$).

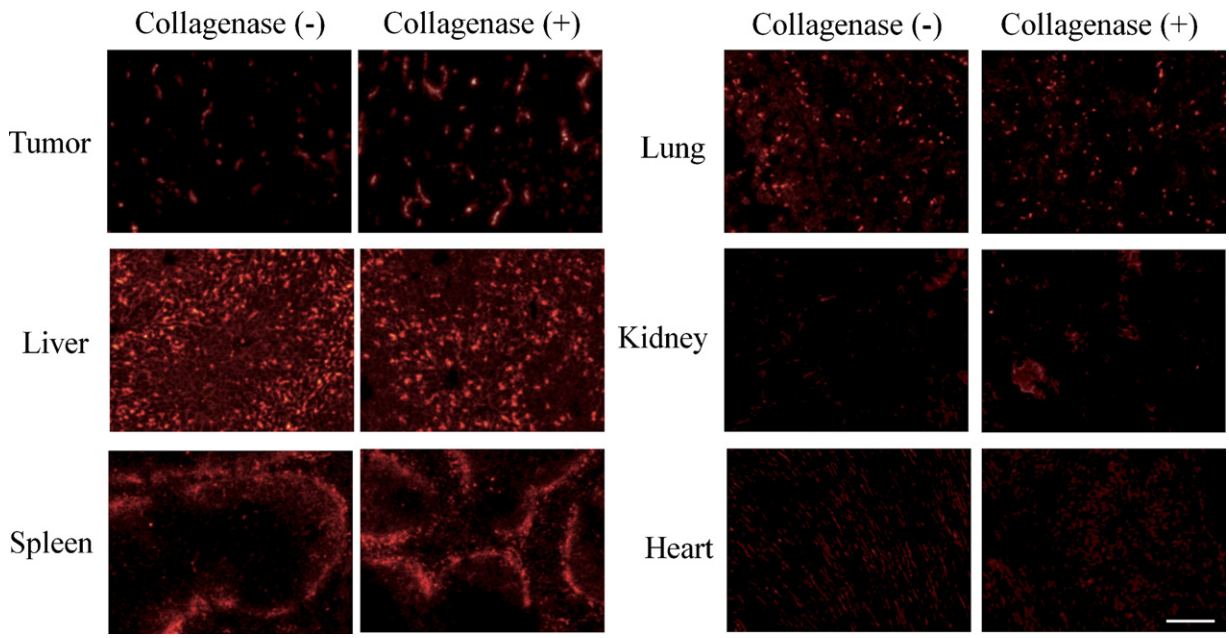

Fig. 4. Biodistribution of rhodamine-labeled lipoplex by collagenase-1 injection. Lipoplex was intravenously injected 1 h after intravenous injection of collagenase-1 into mice bearing LLC tumors. Sections of tumors and tissues 3 h after injection of lipoplex were examined by fluorescent microscope. Red signals show the localization of lipoplex. Scale bar = 100 μm.

vascular vessels in tumors. It has been reported that 3-h incubation is needed for digestion of collagen by direct injection of collagenase (Magzoub et al., 2008). In intratumoral injection, the collagenase might be not widely diffused through the tumor; therefore, we decided to use intravenous injection of collagenase-1 in subsequent experiments.

To examine the effect of collagenase-1 on IFP in tumors, we measured IFP of tumors and muscles 1 and 24 h after intravenous injection of collagenase-1, and normalized the IFP of tumors by that of muscles. As a result, normalized IFP in LLC tumors 1 h after injection of collagenase-1 was significantly decreased compared with that after injection of saline (Fig. 3A), and reached a mean of 65% of its initial value; however, IFP in tumors had returned to the initial levels in mice by 24 h after intravenous injection of collagenase-1. Our results were similar to a previous report that IFP of tumors was reduced by 45% at 60–100 min after intravenous injection of collagenase-1 and had returned to the initial levels by 24 h (Eikenes et al., 2004). Furthermore, we examined distribution of type I collagen in LLC tumor at 1 and 24 h after intravenous injection of collagenase-1. The amount of type I collagen in tumors was strongly decreased at 1 h after injection of collagenase-1 and seemed to be increased to the initial levels at 24 h (supplemental Fig. 2S). In contrast to LLC tumor, normalized IFP in Colon 26 and Neruo2a tumors was not affected by intravenous injection of collagenase-1 (Fig. 3B and C). Regarding normalized IFP in tumors, LLC tumors showed about 2-fold higher IFP compared with Neuro2a and Colon 26 tumors, indicating that widely distribution of collagen in tumor might induce high IFP in tumor. As the results, the decrease of IFP in tumor by collagenase-1 injection could be induced for LLC tumor having high IFP, but not for Colon 26 and Neuro2a. From these findings, we decided to use LLC tumors in subsequent experiments.

3.3. Biodistribution of rhodamine-labeled lipoplex

To determine whether collagenase-1 treatment increased the accumulation of lipoplex in tumors, rhodamine-labeled lipoplex was intravenously injected into mice bearing LLC tumors 1 h after intravenous injection of collagenase-1, and biodistribution of lipoplex was observed by fluorescent microscopy 3 h after injection of lipoplex (Fig. 4). The increased accumulation of lipoplex by collagenase-1 injection was observed in tumors, but not in the liver, lung, spleen, kidney and heart. Moreover, we quantified the amount of rhodamine-labeled lipoplex by extracting rhodamine-DHPE from tumors and tissues 3 h after injection of lipoplex. Collagenase-1 treatment increased rhodamine-DHPE levels 1.5-fold in tumors than without treatment (Fig. 5A and B). This may be because collagenase-1 could digest type I collagen in the tumor interstitium surrounding the microvessels (Eikenes et al., 2004), followed by increased lipoplex leakage from the tumor vessels. Furthermore, the digestion of collagen in tumors may increase the available interstitial volume for the accumulation of lipoplex. In other tissues, collagenase-1 treatment did not affect the accumulation of lipoplex (Fig. 5A). The vascular basement membrane is mainly composed of type IV collagen (LeBleu et al., 2007) which is a substrate for MMP-2, -3, -9, -10 and -13, not for collagenase-1 (MMP-1); therefore, collagenase-1 might not affect the distribution of lipoplex in normal tissues. These results suggested that collagenase-1 treatment could decrease IFP in tumors by degradation of type I collagen and increased accumulation of lipoplex in tumors.

3.4. Luciferase activities in tumors and tissues

Finally, we evaluated the effect of collagenase-1 on transfection efficiency by assaying luciferase activity 24 h after injection of lipoplex. LLC tumor was used as a model having high collagen content (Fig. 1) and high IFP (Fig. 3A) in tumor, and Neuro2a tumor as a model having low collagen content (Fig. 1) and low IFP (Fig. 3C). Collagenase-1 treatment increased luciferase activity 2-fold in LLC tumors compared with no treatment (Fig. 6A). In other tissues, collagenase-1 treatment did not affect activity. In contrast, collagenase-1 treatment did not affect luciferase activity in Neuro2a tumors compared with no treatment (Fig. 6B). High IFP has been correlated with poor prognosis (Curti et al., 1993; Milosevic et al., 2001) and is assumed to form a barrier to transport and obstacle in treatment of the tumors. These findings suggested

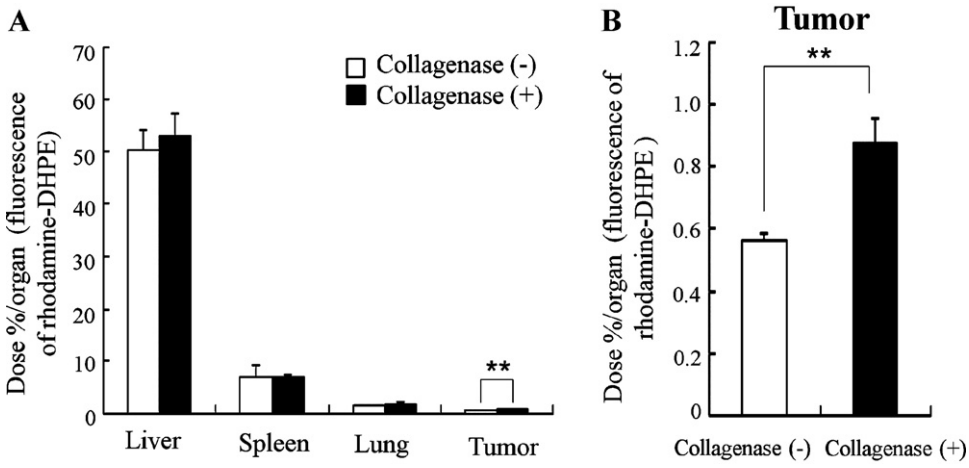

Fig. 5. Quantification of the amount of lipoplex in organs 3 h after intravenous injection of collagenase-1 into mice bearing LLC tumors. In A, rhodamine-labeled lipoplex was intravenously injected 1 h after intravenous injection of collagenase-1 into mice. Rhodamine-DHPE was extracted from the tumors and tissues 3 h after injection of lipoplex, and fluorescence intensity was measured by fluorescence spectrophotometer. In B, the amount of rhodamine-DHPE in tumors in A was enlarged. Error bars represent the mean ± one standard deviation of samples from three mice. Asterisk denotes statistical significance ($p < 0.01$).

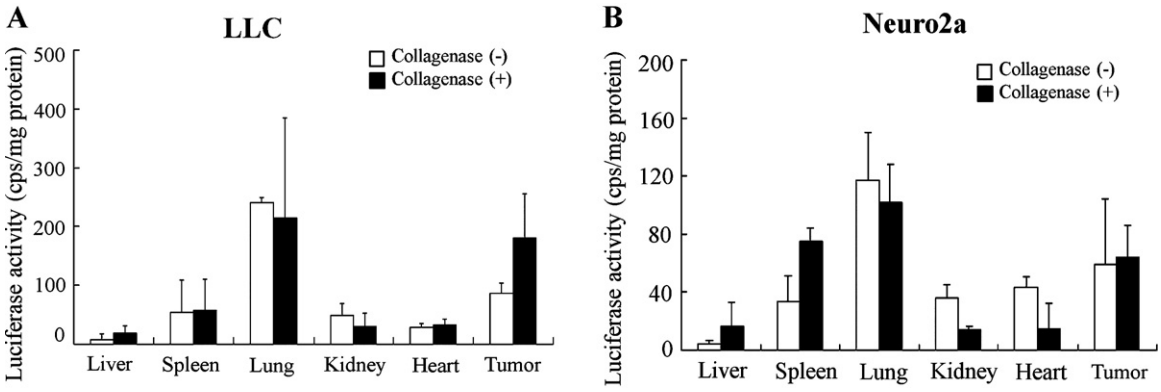

Fig. 6. Luciferase activities in tumors and tissues 24 h after intravenous injection of lipoplex into mice bearing LLC (A) and Neuro2a (B) tumors. Lipoplex was intravenously injected 1 h after intravenous injection of collagenase-1 into mice bearing tumors, and luciferase activity was measured 24 h after injection of lipoplex. Error bars represent the mean ± one standard deviation of samples from three mice.

that collagenase-1 treatment might be effective for improvement of liposomal delivery into malignant tumors which has high IFP by a dense collagen network. However, overall luciferase activities in all tissues were relatively low, because it has been reported that PEG modification of lipoplex increased lipoplex accumulation in tumors, but inhibited interaction with the cell surface and reduced transfection activities (Harvie et al., 2000; Hyvonen et al., 2004). In this study, we used PEGylated lipoplex to investigate the effect of collagenase-1 on tumor accumulation because rhodamine-labeled lipoplex without PEGylation could not be detected in LLC tumors after intravenous injection (data not shown). To evaluate the effect of collagenase-1 treatment on gene expression, it might be necessary to develop long circulating lipoplex without PEG modification, which could induce high gene expression.

4. Conclusion

In this study, we investigated whether intravenous injection of collagenase-1 reduced IFP in tumors and increased accumulation and gene expression of cationic lipoplex in tumors after intravenous injection into mice bearing tumors. The present results showed that intravenous injection of collagenase-1 could transiently reduce IFP by digestion of collagen in tumors and increase tumor accumulation and gene expression of lipoplex. These results support the potential use of collagen digestion as a strategy to enhance gene delivery in tumors.

Acknowledgements

This project was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science, and Technology of Japan, and by the Open Research Center Project.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ijpharm.2011.12.015.

References

Alitalo, K., Carmeliet, P., 2002. Molecular mechanisms of lymphangiogenesis in health and disease. Cancer Cell 1, 219–227.
Bouzin, C., Feron, O., 2007. Targeting tumor stroma and exploiting mature tumor vasculature to improve anti-cancer drug delivery. Drug Resist. Updat. 10, 109–120.
Curti, B.D., Urba, W.J., Alvord, W.G., Janik, J.E., Smith, J.W., Madara, K., Longo, D.L., 1993. Interstitial pressure of subcutaneous nodules in melanoma and lymphoma patients: changes during treatment. Cancer Res. 53, 2204–2207.
Desmouliere, A., Guyot, C., Gabbiani, G., 2004. The stroma reaction myofibroblast: a key player in the control of tumor cell behavior. Int. J. Dev. Biol. 48, 509–517.

Diener, B., Carrick Jr., L., Berk, R.S., 1973. In vivo studies with collagenase from *Pseudomonas aeruginosa*. Infect. Immun. 7, 212–217.
DiResta, G.R., Lee, J., Healey, J.H., Levchenko, A., Larson, S.M., Arbit, E., 2000. Artificial lymphatic system: a new approach to reduce interstitial hypertension and increase blood flow, pH and pO2 in solid tumors. Ann. Biomed. Eng. 28, 543–555.
Dvorak, H.F., Brown, L.F., Detmar, M., Dvorak, A.M., 1995. Vascular permeability factor/vascular endothelial growth factor, microvascular hyperpermeability, and angiogenesis. Am. J. Pathol. 146, 1029–1039.
Eikenes, L., Bruland, O.S., Brekken, C., Davies, C.L., 2004. Collagenase increases the transcapillary pressure gradient and improves the uptake and distribution of monoclonal antibodies in human osteosarcoma xenografts. Cancer Res. 64, 4768–4773.
Eikenes, L., Tari, M., Tufto, I., Bruland, O.S., de Lange, D.C., 2005. Hyaluronidase induces a transcapillary pressure gradient and improves the distribution and uptake of liposomal doxorubicin (Caelyx) in human osteosarcoma xenografts. Br. J. Cancer 93, 81–88.
Fleming, J.M., Miller, T.C., Quinones, M., Xiao, Z., Xu, X., Meyer, M.J., Ginsburg, E., Veenstra, T.D., Vonderhaar, B.K., 2010. The normal breast microenvironment of premenopausal women differentially influences the behavior of breast cancer cells in vitro and in vivo. BMC Med. 8, 27.
Harvie, P., Wong, F.M., Bally, M.B., 2000. Use of poly(ethylene glycol)-lipid conjugates to regulate the surface attributes and transfection activity of lipid-DNA particles. J. Pharm. Sci. 89, 652–663.
Heldin, C.H., Rubin, K., Pietras, K., Ostman, A., 2004. High interstitial fluid pressure—an obstacle in cancer therapy. Nat. Rev. Cancer 4, 806–813.
Hyvonen, Z., Ronkko, S., Toppinen, M.R., Jaaskelainen, I., Plotniece, A., Urtti, A., 2004. Dioleoyl phosphatidylethanolamine and PEG-lipid conjugates modify DNA delivery mediated by 1,4-dihydropyridine amphiphiles. J. Control Release 99, 177–190.
Jain, R.K., 1987a. Transport of molecules across tumor vasculature. Cancer Metastasis Rev. 6, 559–593.
Jain, R.K., 1987b. Transport of molecules in the tumor interstitium: a review. Cancer Res. 47, 3039–3051.
Jain, R.K., Baxter, L.T., 1988. Mechanisms of heterogeneous distribution of monoclonal antibodies and other macromolecules in tumors: significance of elevated interstitial pressure. Cancer Res. 48, 7022–7032.
LeBleu, V.S., Macdonald, B., Kalluri, R., 2007. Structure and function of basement membranes. Exp. Biol. Med. (Maywood) 232, 1121–1129.
Leu, A.J., Berk, D.A., Lymboussaki, A., Alitalo, K., Jain, R.K., 2000. Absence of functional lymphatics within a murine sarcoma: a molecular and functional evaluation. Cancer Res. 60, 4324–4327.
Magzoub, M., Jin, S., Verkman, A.S., 2008. Enhanced macromolecule diffusion deep in tumors after enzymatic digestion of extracellular matrix collagen and its associated proteoglycan decorin. FASEB J. 22, 276–284.
Milosevic, M., Fyles, A., Hedley, D., Pintilie, M., Levin, W., Manchul, L., Hill, R., 2001. Interstitial fluid pressure predicts survival in patients with cervix cancer independent of clinical prognostic factors and tumor oxygen measurements. Cancer Res. 61, 6400–6405.
Morimoto, K., Kondo, M., Kawahara, K., Ushijima, H., Tomino, Y., Miyajima, M., Kimura, J., 2007. Advances in targeting drug delivery to glomerular mesangial cells by long circulating cationic liposomes for the treatment of glomerulonephritis. Pharm. Res. 24, 946–954.
Netti, P.A., Berk, D.A., Swartz, M.A., Grodzinsky, A.J., Jain, R.K., 2000. Role of extracellular matrix assembly in interstitial transport in solid tumors. Cancer Res. 60, 2497–2503.
Padera, T.P., Kadambi, A., di, T.E., Carreira, C.M., Brown, E.B., Boucher, Y., Choi, N.C., Mathisen, D., Wain, J., Mark, E.J., Munn, L.L., Jain, R.K., 2002. Lymphatic metastasis in the absence of functional intratumor lymphatics. Science 296, 1883–1886.
Park, K., 2010. A new ligand for targeted drug delivery to tumor stromal cells. J. Control Release 145, 75.
Ribatti, D., Nico, B., Crivellato, E., Vacca, A., 2007. The structure of the vascular network of tumors. Cancer Lett. 248, 18–23.
Rippe, B., Haraldsson, B., 1994. Transport of macromolecules across microvascular walls: the two-pore theory. Physiol. Rev. 74, 163–219.
Swabb, E.A., Wei, J., Gullino, P.M., 1974. Diffusion and convection in normal and neoplastic tissues. Cancer Res. 34, 2814–2822.
Tailor, T.D., Hanna, G., Yarmolenko, P.S., Dreher, M.R., Betof, A.S., Nixon, A.B., Spasojevic, I., Dewhirst, M.W., 2010. Effect of pazopanib on tumor microenvironment and liposome delivery. Mol. Cancer Ther. 9, 1798–1808.
Thomas, A., Bayat, A., 2010. The emerging role of *Clostridium histolyticum* collagenase in the treatment of Dupuytren disease. Ther. Clin. Risk Manag. 6, 557–572.
Weinberg, P.D., Carney, S.L., Winlove, C.P., Parker, K.H., 1997. The contributions of glycosaminoglycans, collagen and other interstitial components to the hydraulic resistivity of porcine aortic wall. Connect. Tissue Res. 36, 297–308.
Young, J.S., Lumsden, C.E., Stalker, A.L., 1950. The significance of the tissue pressure of normal testicular and of neoplastic (Brown–Pearce carcinoma) tissue in the rabbit. J. Pathol. Bacteriol. 62, 313–333.
Zheng, X., Goins, B.A., Cameron, I.L., Santoyo, C., Bao, A., Frohlich, V.C., Fullerton, G.D., 2011. Ultrasound-guided intratumoral administration of collagenase-2 improved liposome drug accumulation in solid tumor xenografts. Cancer Chemother. Pharmacol. 67, 173–182.