主論文の要旨

Atelocollagen-mediated intravenous siRNA delivery specific to tumor tissues orthotopically xenografted in prostates of nude mice, and its anticancer effects

（ヌードマウス前立腺に同所移植した癌組織に対する
アテロコラーゲンを介した siRNA の特異的送達とその抗癌効果）

名古屋大学大学院医学系研究科　分子総合医学専攻
生物化学講座　分子生物学分野
（指導：門松 健治　教授）
袁 媛

Introduction

Successful siRNA-based therapy for cancers depends on functional siRNA delivery specific to tumors. We have shown atelocollagen-mediated siRNA systemic delivery specific to 'PC-3 subcutaneous tumors' in nude mice. Atelocollagen, a type I collagen derivative from calf dermis, binds with siRNA via electrostatic interaction. Atelocollagen contributes to tumor specific delivery and uptake of the siRNA as well as maintenance on stability and bioavailability of the siRNA in vivo. We used a siRNA for human Bcl-xL, an anti-apoptotic factor which is overexpressed in prostate cancers, as a model target. In this study, we examined the therapeutic effect of the complex of Bcl-xL siRNA and atelocollagen on 'PC-3 orthotopic tumors' in nude mice, as these tumors resemble the human clinical situation. We found that the systemic i.v. injection of the siRNA complex significantly reduced tumor enlargement and metastasis in the orthotopic tumors. We also showed the tumor-specific accumulation of the siRNA. Our siRNA delivery method significantly depended on the tumor permeability enhanced by vascular endothelial growth factorA (VEGFA) in the tumors.

Materials and Methods

Cell culture. PC-3 cells, a human prostate cancer cell line, and PC-3-Luc (JCRB1406) cells, the PC-3 cells with stable luciferase expression, were cultured in F-12K medium with 10% FBS.

Atelocollagen. Atelocollagen was prepared by removing telopeptides on the both terminals of a calf dermal collagen via pepsin digestion.

Formulation procedure of the siRNA and atelocollagen complex. An equal volume of siRNA in PBS and atelocollagen solution (0.1%) were mixed by gently rotating for 20 min at 4℃. The final concentration of atelocollagen for i.v. injection became 0.05%.

PC-3 orthotopic tumor model. Male BALB/c nude mice were anesthetized with somnopentyl (i.p., 5 mg/kg). Following a lower midline abdominal incision, PC-3 cells (5×10^5 cells in 20 μl of F-12K medium without any serum) were injected into the ventral lobe of the prostate gland. The incision was then closed, and the mouse was placed on a heat pad until awake.

Examination of distant metastasis from the PC-3 orthotopic primary tumors. We inoculated PC-3-luc cells into the prostate gland of nude mice. Four weeks later, the liver and lung were removed and homogenized. The lysates were centrifuged and the supernatant was measured by the Dual-luciferase reporter assay. The values of luciferase activity were normalized to the total protein concentration.

Cancer therapy via the systemic intravenous administration of the siRNA complex. We intravenously injected the tumor-bearing mice with the Bcl-xL siRNA complex (siRNA 50 μg in 200 μl, three consecutive injections per week) for four weeks. Cisplatin (CDDP)

administration (2 mg/kg, single injection per week), was performed via an intraperitoneal (i.p.) injection one day after the last injection of the siRNA for four weeks. The therapeutic effect was judged by determining the actual tumor weights.

Enzyme-linked immunosorbent assay (ELISA) specific to human Bcl-xL protein. Lysates from the excised tumor tissues were prepared. Bcl-xL protein levels in the lysates were determined with the human Total Bcl-xL DuoSet IC ELISA Kit. The total protein concentration was used for normalization.

Measurement of tumor permeability. A tumor-bearing mouse was injected i.p. with 100 μl anti-human VEGFA neutralizing antibody (100 μg) or isotype-matched control IgG (mouse IgG1, 100 μg). After the two injections over two days, we directly measured the resistance (Ohm) and current (Ampere) in the tumors when we loaded the voltage (70 V) in the tumors by using needle-type electrodes. The tumor resistance and current in the tumors when electronically pulsed are a reliable index for measuring the tumor permeability.

Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay. We performed TUNEL staining of the frozen section of the excised tumors using a MEBSTAIN® Apoptosis Kit II. Nuclei were stained with DAPI to determine the total cell numbers. TUNEL-positive cells were counted under a fluorescent microscope.

Quantitation of Bcl-xL siRNA delivered in PC-3 orthotopic tumors and several other organs. The tumor-bearing mice were i.v.-injected with Bcl-xL siRNA/atelocollagen complex (siRNA, 50 μg/mouse). The tumors and several organs were harvested at 15 min or 30 min post-injection, and lysed by homogenization. The lysates were hybridized with a fluorescence-labeled oligoribonucleotide probe, which is complementary to the antisense strand of Bcl-xL siRNA, and the siRNA was quantified with reverse-phase HPLC.

Administration of Cy3-labeled siRNA. We i.v.-injected tumor-bearing mice with Cy3-labled Bcl-xL siRNA/atelocollagen complex (siRNA, 50 μg/mouse). The tumors were harvested 24 h after the injection, embedded in OCT compound, and frozen in liquid nitrogen. The frozen sections were cut and nuclear staining was performed with DAPI. Images of the sections were obtained with fluorescent microscopy.

Statistical analysis. The statistical significance was examined by Mann-Whitney U-test. P-values <0.05 were accepted as significant.

Results

PC-3 orthotopic tumor model was successfully established by inoculating PC-3 cells into the prostate of BALB/c nude mice and human Bcl-xL gene was constantly expressed in the tumors (Fig. 1). Bcl-xL expression was significantly reduced by systemic i.v. injection of Bcl-xL siRNA complex in a dose-dependent manner and the optimal dose of

the siRNA was 50μg/injection over three consecutive days (Fig. 2). The four sets of the three consecutive injections of the complex significantly suppressed tumor growth as well as liver metastasis (Fig. 3) and induced apoptosis in the tumors (Fig. 4). Combinational injections of a low dose of CDDP increased these therapeutic effects. We successfully showed tumor-specific accumulation of the complex by Cy3-labeled siRNA (Fig. 5), and by the direct quantification of the siRNA (Fig. 6). On the other hand, no accumulation in normal tissues was observed (Fig. 6). We further investigated the mechanism of siRNA delivery. We inhibited the activity of VEGFA, a major factor to maintain EPR effect, by anti-VEGFA neutralizing antibody. After the injection of the antibody, tumor permeability was reduced, and Bcl-xL siRNA-mediated RNAi effect was impaired (Fig 7). It was suggested that our tumor-specific delivery of the siRNA complex was achieved by the EPR effect.

Discussion

In this study, using the siRNA for targeting human Bcl-xL gene and atelocollagen systemic siRNA delivery techniques, we observed significant antitumor effects and antimetastatic effects against PC-3 orthotopic tumors (Fig. 3). Combinational treatment of the siRNA/atelocollagen complex and CDDP enhanced these therapeutic effects. What`s more, additional adverse effects, including liver and renal damage, were not observed (data not shown). Thus, our atelocollagen-mediated siRNA therapy showed superior potency from four different viewpoints; antitumor effect, antimetastatic effect, safety and potential for combination with chemotherapeutics. Next, we showed the tumor-specific accumulation of siRNA (Figs. 5, 6). We further investigated the mechanism of the atelocollagen- mediated siRNA delivery and found that the EPR effect was essential to achieve the tumor-specific siRNA delivery. The abundant VEGFA expression in the orthotopic tumors contributed to the upregulation of tumor permeability, and to the EPR effect. Indeed, the i.p. administration of anti-human VEGFA-neutralizing antibody impaired the RNAi efficacy via the atelocollagen-mediated siRNA i. v. delivery (Fig. 7). However, the EPR effect is not enough to explain the delivery mechanism. We have been especially focusing on Endo180, which binds with type I collagen, and takes the collagen into the cells. Further, we found Endo 180 is also overexpressed in prostate cancers (unpublished results).

Conclusion

In conclusion, our atelocollagen-mediated siRNA delivery is specific to the PC-3 orthotopic tumors in nude mice, and is feasible for the treatment of tumors.