Nontoxic membrane translocation peptide from protamine, low molecular weight protamine (LMWP), for enhanced intracellular protein delivery: in vitro and in vivo study

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SPECIFIC AIM

The aim of this study is to demonstrate the feasibility of naturally derived low molecular weight protamine as a potential translocational domain for the intracellular delivery of molecular medicine including macromolecular drug (i.e., protein), therapeutic gene, or molecular diagnostic probes as well as nanoparticulates.

PRINCIPAL FINDINGS

1. Preparation of the low molecular weight protamine sequences and their translocational capacity through various types of cells as well as biocompatibility

LMWP fragments were derived from native protamine by thermolysine digestion, which provided 5 different fractions, denoted TDSP (thermolysin-digested segmented protamine) 1 to 5 (PRRRR, PRRRRSSSRP, RPVRRRRRPR, VSRRRRRRGGRRR, VSRRRRRRGGRRRR) depending on their elution order from a heparin affinity chromatography. Except for TDSP1, which possessed less arginine residues than those required for cell transduction, the cell internalization activity of TDSPs 2, 3, 4, and 5 were all examined. All of the studied LMWP peptides displayed sufficient cellular uptake at 30 min after incubation in accordance with the increase of the arginine content in these peptides. The TDSP5 peptide further demonstrated the enhanced cellular uptake as cells transfected with TDSP5 exhibited the highest fluorescence intensity and was largely localized in the cytoplasm of HeLa cells. Similar results were also obtained by using other cell line types including 293T, CT26, human MCF-7 cells, fibroblast, as well as MG 63 osteoblasts. To evaluate the biocompatibility of LMWP as a translocational carrier, the effect of serum on the transduction efficiency of LMWP was conducted in cell culture medium containing 10% fetal bovine serum. The efficiency of TDSP5 uptake was not affected by the presence of serum. In addition, these peptides showed no significant cytotoxicity (i.e., $<10\%$ reduction in cell viability) up to a concentration of 10 mM, whereas PEI, a common synthetic transfecting agent, showed a significant decrease (40%) of cell viability at or above a concentration of 5.0 mM.

2. Conjugation of LMWP to molecular probe and protein enhances biologic activities

To examine whether LMWP could be used as a carrier for intracellular delivery of cargo molecules for imaging or therapeutic purpose, phalloidin or gelonin was conjugated to LMWP. Each molecule was conjugated to LMWP by 1:1 molar ratio (**Fig. 1**). The heparin column elution profile of the reaction mixture provided 3 representative peaks: unreacted gelonin (1st peak), LMWP-gelonin conjugate (2nd peak), unconjugated LMWP, and the mixture of the conjugate with higher amount of LMWP attached (3rd peak). We used the sample from second peak (samples with 1:1 molar ratio of LMWP and gelonin) for further study. The LMWP-gelonin, TAT-gelonin, and gelonin had IC_{50} values of $\sim$23, 15, and 49 pM, respectively, indicating that conjugation of gelonin to both LMWP and TAT did not

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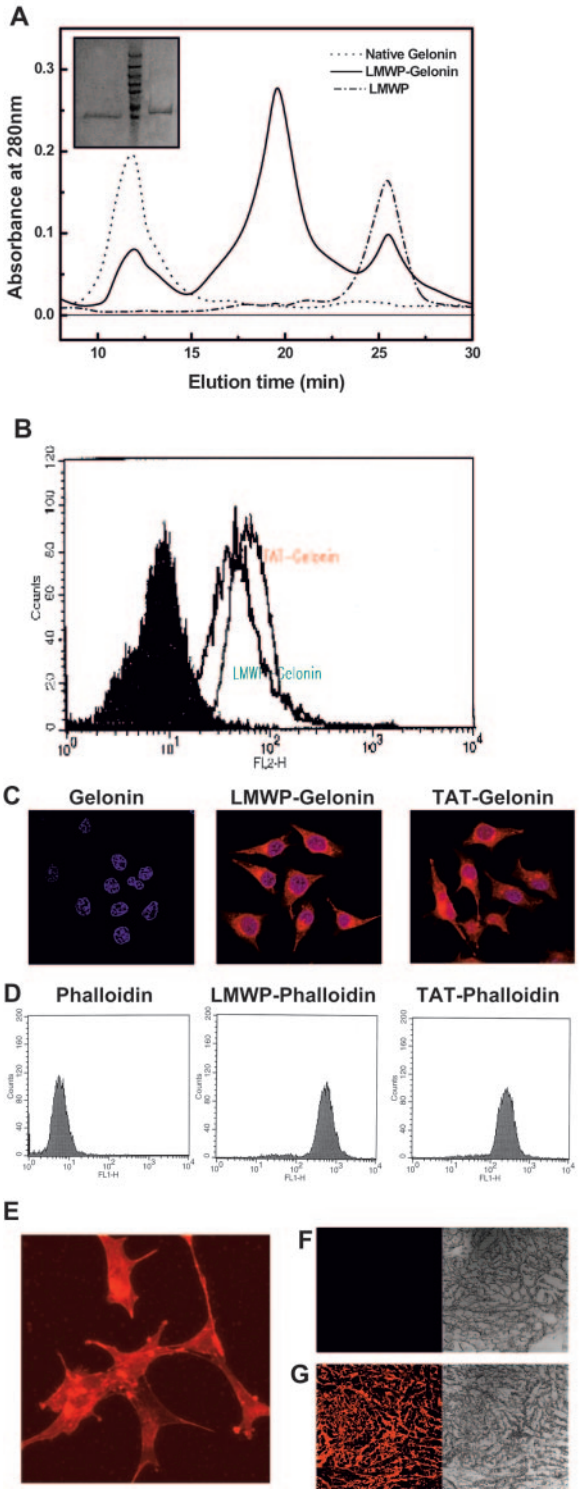

Figure 1. *A)* Formation of LMWP-gelonin conjugates. Prepared LMWP (TDSP5) -gelonin conjugate was purified from unconjugated gelonin and LMWP by eluting through a heparin-affinity chromatography column. Elution profiles were followed by absorbance at 280nm. The Inset shows molecular weight of LMWP-gelonin conjugates by SDS-PAGE analysis. LMWP-gelonin conjugate and native gelonin were examined by SDS-PAGE on a 12% gel, stained with Coomassie brilliant blue. The left lane of the molecular marker indicates the molecular weight of gelonin (~29,000 Da), whereas the right indicates LMWP-gelonin (~31,000 Da). The molecular weight marker ranges from 29,000 to 700,000. *B)* FACS analysis of cell uptake by rhodamine-labeled LMWP-gelonin conjugate and rhodamine-labeled TAT-gelonin conjugate in CT-26 cell line (1×10^6 cells/well) in the presence of 10% serum. *C)* Cellular localization of rhodamine-labeled LMWP-gelonin conjugates in CT-26 colon adenocarcinoma cells. Rhodamine-labeled gelonin, LMWP-gelonin conjugate, and TAT-gelonin conjugate were overlaid onto cultured CT-26 cells in the presence of 10% FBS. Cellular localization was monitored by confocal microscopy. *D)* FACS analysis of cell uptake by Alexa Fluoro488-labeled LMWP-phalloidin conjugate and TAT-phalloidin conjugate in MG63 osteoblast cells (1×10^6 cells/well) in the presence of 10% serum. Alexa Fluoro488-labeld phalloidin was served as control. *E)* Cellular localization of rhodamine-labeled LMWP-gelonin conjugates in MG63 osteoblast cells. Rhodamine-labeled LMWP-phalloidin conjugate was overlaid onto cultured MG63 cells in the presence of 10% FBS. Cellular localization was monitored by confocal microscopy. *F)* Tumor penetration of rhodamine-labeled free gelonin into the colon cancer and (*G*) rhodamine-labeled LMWP-gelonin. The mice were then analyzed for penetration extent with LMWP-gelonin conjugate through the tumor. Tumors were isolated at 10 h after injection of either rhodamine-labeled gelonin or rhodamine-labeled LMNP-gelonin conjugate, cryosectioned, and photographed by confocal microscopy. Left panel: fluorescent image of rhodamine labeled protein transduced in the tumor tissue. Right panel: identical DIC mode.

reduce the bioactivity of the toxin component of gelonin.

To compare the translocation activity of LMWP with other known protein transduction domains (PTDs), HIV-TAT peptide was selected as a control and conjugated to gelonin. As shown by the FACS results (Fig. 1), the ability to translocate gelonin by LMWP and TAT was closely comparable. In both cases, rhodamine labels were clearly detected after 30 min of incubation of these peptides with CT-26 cells. Data from confocal microscopic studies were also in agreement with the FACS results, displaying a cytoplasmic localization of the TDSP5-gelonin conjugates instead of adsorption to the cell surfaces (Fig. 1). Cells treated with LMWP-phalloidin conjugates exhibited quick and obvious cytoskeleton labeling without any permeabilization step of the cell membranes (Fig. 1), while treating with phalloidin could not label cells without permeabilization (data not shown). To further confirm the LMWP-gelonin conjugates actually penetrated into the tumor, rhodamine-labeled gelonin was applied during in vivo studies. The tumor tissues from LMWP-gelonin-treated mice displayed a strong and uniform red staining from rhodamine on the labeled LMWP-gelonin conjugates (Fig. 1), implicitly confirming the penetration of the LMWP-gelonin conjugate into the tumor tissues. Conversely, mice injected with rhodamine-labeled free gelonin displayed virtually no or at most, a sporadic, weak staining of rhodamine (Fig. 1). Therefore, antitumor effect of LMWP-gelonin was anticipated by the results from the enhanced distribution of the conjugates into the tumor tissues.

Biological consequences of treating subcutaneous solid tumors with LMWP-gelonin were assayed after peritumoral injection of test samples. Mice treated with

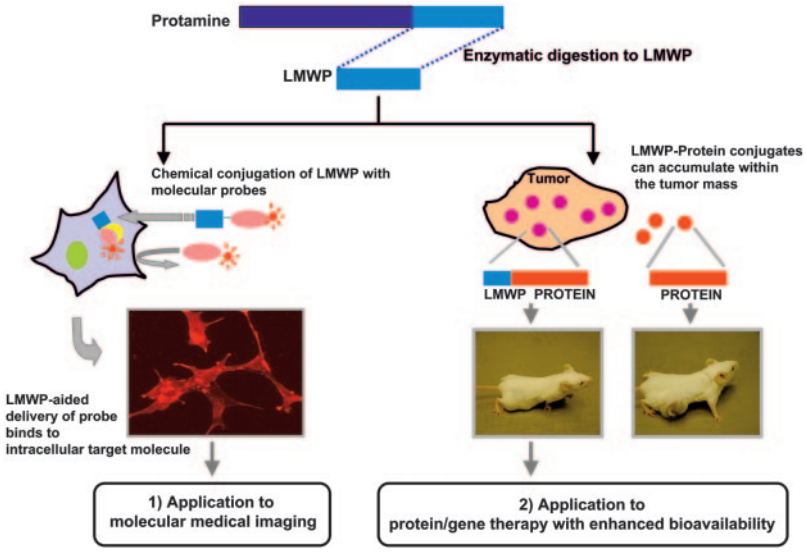

Figure 2. Possible application strategy of prepared LMWP: *1)* to enhance the molecular medical imaging efficiency by directly delivering probe molecules into the cells or organs; and *2)* to improve bioavailability of poorly penetrating/absorbed protein or genetic drug by conjugating with LMWP. Pictures were taken from mouse administered with LMWP-gelonin conjugate or gelonin 2 wk after injection. Note the significant marginal tumor mass of the mouse treated with LMWP-gelonin conjugate while the mouse treated with gelonin showed larger mass.

free gelonin did not display regression on tumor growth, since gelonin could not penetrate the tumor. An average tumor weight of 2.63 ± 0.5 g was observed 4 wk after injection of gelonin. In a sharp contrast, mice treated with the LMWP-gelonin conjugate displayed a significant regression in tumor growth, as the tumor weight was reduced to an insignificant value of 0.33 ± 0.12 g. Addition of free LMWP to the gelonin solution did not elicit any effect on tumor regression, as an average tumor mass of 2.74 ± 0.68 g was observed 4 wk after treatment, clearly indicating that only covalent conjugation of LMWP was able to transduce gelonin and present the anti-tumor effect with the same extent to that by TAT conjugate (0.21±0.04 g).

CONCLUSIONS AND SIGNIFICANCE

Although the currently used protein transduction domain (PTD) peptides, including TAT, has great potential as a universal carrier for the intracellular delivery of biomolecules, clinical applications of PTDs are hindered by two major shortcomings. One is that many of PTDs are primarily derived from highly infective viral proteins, yet the toxicity and immunogeneicity profiles of these peptides have not been established. The other limitation is that PTDs are not naturally occurring peptides and thus must be produced by synthetic routes. Synthetic preparation of arginine rich peptides has a shortcoming of low yields rendering it practically difficult to meet with the demand for numerous clinical applications. To this regard, the most significant, novel finding of this study is that the freely modifiable potential translocation domain peptide can be derived from natural protamine by enzymatic digestion. The in vitro and in vivo results have demonstrated that LMWP, especially TDSP5, is as potent a cell transduction peptide as any existing PTD including TAT. Yet, the presence of thoroughly established toxicological and immunological profiles, the derivation from a natural resource, and the suitability and ease for manufacturing mass quantities would render LMWP to become the most preferable choice among current PTDs for important clinical applications including molecular probes, protein, gene, and virtually all types of medical imaging and drug therapies. Aside from these direct clinical utilities, the universal applicability of LMWP to transduce all types of compounds into cells regardless their physical and chemical attributes (i.e., small or large, hydrophilic or hydrophobic) could enable the discovery and/or development of new therapeutic or imaging agents that are initially considered unusable due to their inability to enter the cells (**Fig. 2**). Two major potential application of LMWP are described in Fig. 2. One is for molecular medical imaging of probes by conjugating with LMWP. The labeled probes can interact with intracellular target when they are conjugated with transduction carrier, LMWP. LMWP-probe conjugates can translocalize through the target organ, which enables real-time in vivo imaging without sacrifice of animal. The other possible application of LMWP is as a carrier for therapeutic proteins or genetic materials including DNA and RNA, which by themselves cannot cross the cell membranes or disease site such as solid tumor mass. The anticancer protein drug, as an example, by itself could not provide with such significant anticancer efficacy, however, can be transduced only after conjugation with LMWP, which presents marked anticancer activity. The insensitivity of cell uptake against the presence of serum and cell viability could also allow LMWP to be used in the screening of potential therapeutic proteins, investigating protein–protein interaction in the cells, screening of potential therapeutic proteins and elucidating the role of proteins in certain specific disease development. Taken together, naturally derived nontoxic LMWP was suggested as a potential tool for carrying any biologic molecules to enhance the therapeutic or diagnostic efficacy. FJ