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Laminin-derived peptides: Applications in drug delivery systems for targeting

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ABSTRACT

Recently, the development of drug delivery systems (DDSs) for clinical application of anticancer drugs and gene therapy has rapidly progressed. In particular, DDS carriers used for chemotherapy and gene therapy are required to selectively deliver drugs and genes to cancer cells. Both the carrier and the molecule must in combination be highly selective in most cases. Possible candidate targeting molecules are the laminins, major basement membrane proteins that interact with various cells through their multiple constituent active peptide sequences. Laminin-derived peptides bind to various cellular receptors and have been used for DDSs as a targeting moiety. Here, we review the progress in laminin-derived peptide-conjugated DDSs. Drug and gene carriers as well as ultrasound diagnostic contrast agents utilizing laminin-derived peptides for selective targeting are useful components of DDSs and play important roles in cancer and in the neovasculature.

Contents

1. Introduction

Recently advancement in drug discovery technology has been achieved. It is now possible to synthesize compounds with useful biological activities, such as anticancer agents in a shorter period of time. In the clinical practice, however, the use of anticancer agents is limited by their side effects. To overcome these adverse effects, there has been a focus on developing DDSs for new drug formulations. DDSs have a wide variety functions, such as extending blood circulation time using PEG(polyethylene glycol)ylation (Allen, Hansen, Martin, Redemann, & Yau-Young, 1991; Klibanov, Maruyama, Torchilin, & Huang, 1990; Maruyama et al., 1992) or controlled-release using pH (Oku, Shibamoto, Ito, Gondo, & Nango, 1987; Straubinger, Düzgünes, & Papahadjopoulos, 1985). Development of a selective targeting system for specific cells and tissues is important, as the targeting system helps reduce the side effects while enhancing the therapeutic effect by targeting to specific sites. For targeting cancer treatment, nano-sized carriers, such as liposomes and micelles, are often modified with targeting ligands (Allen, 2002), such as antibodies (Lukyanov, Elbayoumi, Chakilam, & Torchilin, 2004; Maruyama, Kennel, & Huang, 1990), folate (Gabizon, Shmeeda, Horowitz, & Zalipsky, 2004), sugars (Kawakami et al., 2000), and peptides (Xiong et al., 2005), to enhance their selectivity to tumor tissues. DDS carriers must also result in enhanced therapeutic effects.

Cell adhesive sequences have been identified from various ECM molecules, including fibronectin, collagen, and laminin, using synthetic

Abbreviations: DDS, drug delivery system; ECM, extracellular matrix; RES, reticuloendothelial system; PEG, polyethylene glycol; BL, Echo-contrast gas entrapping liposome; US, ultrasound; Dox, doxorubicin; Mal, maleimide.

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peptides (Yamada, 1991; Yamada & Kleinman, 1992). These active peptides interact with specific cellular receptors. The Arg-Gly-Asp (RGD) sequence was the first identified as a cell adhesive sequence from fibronectin that interacts with integrins (Yamada, 1991). The RGD peptides have been used in various therapeutic approaches, including therapeutic reagents, DDSs, and functional biomaterials, where their advantages have been demonstrated (Wang et al., 2013). Thus, active peptides derived from ECM molecules specifically interact with cellular receptors and are useful for developing therapeutic approaches.

Here, we focus on laminins and describe the applications for targeting DDS technologies using laminin-derived peptides.

2. Cell adhesive peptides from laminins

Laminins, a major component of the basement membrane, have various biological activities, such as promotion of cell adhesion, migration, neurite outgrowth, and angiogenesis (Miner & Yurchenco, 2004). Laminins interact with various cellular receptors, including integrins, syndecan, α-dystroglycan, and lutheran/basal cell adhesion molecule (Lu/B-CAM). Laminins are heterotrimeric glycoproteins consisting of α, β, and γ chains. Presently, five α chains, three β chains, and three γ chains have been identified that assemble into at least 19 laminin isoforms (Miner & Yurchenco, 2004). The laminin isoforms are specifically expressed in tissues and at specific developmental stages and promote various biological activities.

There are many active sequences in the laminin molecules. We developed a systematic peptide screening method for identification of cell adhesive peptides in proteins and have identified various active sequences in the laminin isoforms using laminin-derived synthetic peptides (Nomizu et al., 1995). We have screened for cell adhesive sequences in all the laminin isoforms using more than 3000 12-mer synthetic peptides covering all the sequences. The schematic process of screening of laminin-111 is shown in Fig. 1 as an example (Nomizu et al., 1995, 1997, 1998, 2000). Peptides were generally designed with a 12-amino acid length and overlapped with neighboring peptides by 4 amino acids. Any cysteine residues were omitted. All peptides were manually synthesized by the 9-fluorenylmethoxycarbonyl strategy with a C-terminal amide form and purified by high performance liquid chromatography. For the screening of cell attachment activity, peptide-coated plastic plates and peptide-conjugated Sepharose beads were prepared. This peptide-plate method can be used to analyze cell attachment activity quantitatively but conformation and coating efficiency of the peptides are variable. The peptide-bead method lacks quantitative capability but the conformation and binding efficiency of the peptides are maintained constant. Using these two methods, the cell attachment activity of the peptides was fully assessed. About 20 active peptides were identified from laminin-111 and 5 peptides showed strong cell attachment activity (Fig. 1) (Suzuki, Yokoyama, & Nomizu, 2005). The cell adhesive peptides were analyzed in detail and the functional sites of the laminins were identified. So far, more than 100 active peptides have been identified by screening over 3000 of the peptides from all of the laminin isoforms. These active peptides interact with cell surface receptors, such as integrins, syndecan, α-dystroglycan, and CD44, to promote various biological activities. The major active peptides are listed in Table 1. Peptide AG73 (RKRLQVQLSIRT, mouse laminin α1 chain, binds to syndecans) promoted strong cell attachment, neurite outgrowth, salivary gland acinilike differentiation, and angiogenesis (Hoffman et al., 2001). Peptides EF1 (DYATLQLQEGRLHFMFDLG, mouse laminin α1 chain, binds to integrin α2β1) and A2G10 (SYWYRIEASRTG, mouse laminin α2 chain, binds to integrin α6β1) showed cell attachment and cell spreading activity (Suzuki et al., 2003; Urushibata, Hozumi, Ishikawa, Katagiri, & Kikkawa, 2010). Peptide A13 (RQVFQVAYIIIKA, mouse laminin α 1 chain, binds to syndecan and to integrins) maintained primary hepatocyte functions (Kikkawa et al., 2011). Peptides A2G78 (GLLFYMARINHA, mouse laminin α2 chain) and A2G80 (VQLRNGFPYFSY, mouse laminin α2 chain) likely play functional roles as heparan sulfate- and α-dystroglycan-binding sites in the laminin α2 chain laminin G domain-like 4–5 modules (Suzuki et al., 2009). Peptide A3G756 (KNSFMALYLSKGRLVFALG, human laminin α3 chain, binds to syndecans) promoted wound healing *in-vivo* (Araki et al., 2009). Peptide A5G27 (RLVSYNGIIFFLK, mouse laminin α5 chain, binds to the CD44 receptor) inhibited B16-F10 melanoma cell migration, invasion, and angiogenesis in a dominant-negative manner (Hibino et al., 2005). These peptides are useful for the development of therapeutic reagents, biomaterials as an adhesiveness and functional regulator, and DDSs as a receptor-specific targeting moiety (Hozumi & Nomizu, 2018; Kikkawa et al., 2013; Yamada, Hozumi, & Nomizu, 2011). The

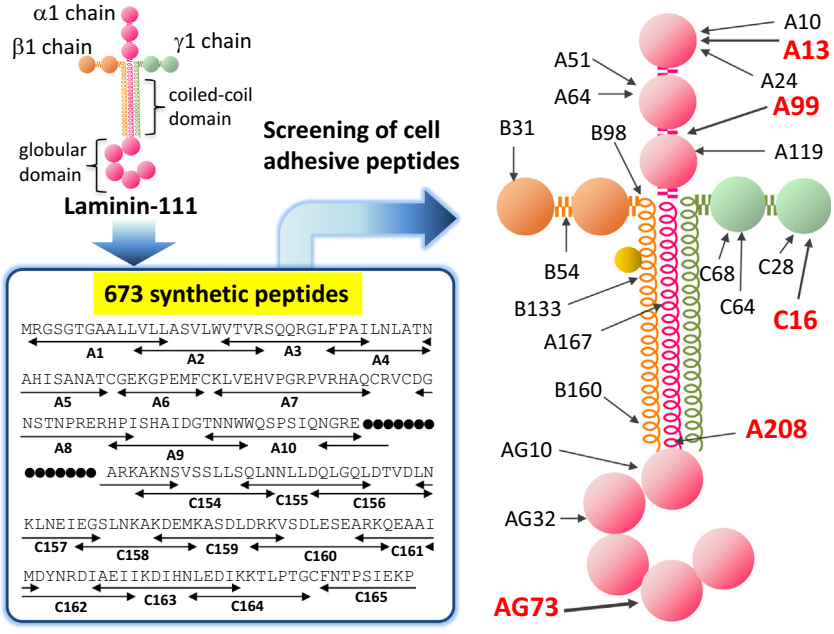

Fig. 1. Screening of cell adhesive sequences in laminin-111. Cell adhesive peptides are indicated by peptide names with arrows in the diagram on the right. Major cell adhesive peptides are shown in bold red colors.

Table 1
Major biologically active peptides from laminins and their receptors.

Peptide	Sequence *Chain (Residues)*[1]	Receptor	Activity
A13	RQVFQVAYIIIKA *mouse laminin α1 chain (121−133)*	syndecan integrin β1	hepatocyte attachment angiogenesis
A99	AGTFAL**RGD**NPQG *mouse laminin α1 chain (1141–1153)*	integrin αvβ3	cell spreading neurite outgrowth metastasis suppression
A208	AAS**IKVAV**SADR *mouse laminin α1 chain (2121−2132)*	110-kDa protein	fibril formation neurite outgrowth MMP↑[2]
AG73	RKR**LQVQLSIR**T *mouse laminin α1 chain (2719–2730)*	syndecan	cell differentiation neurite outgrowth wound healing
EF1	DYATLQLQEGRLHFMFDLG *mouse laminin α1 chain (2747–2765)*	integrin α2β1	cell spreading
C16	KAFDITYVRLKF *mouse laminin γ1 chain (139–150)*	integrin β1	MMP↑ angiogenesis
A2G10	SYWYRIEASRTG *mouse laminin α2 chain (2223–2234)*	integrin α6β1	cell spreading
A2G78	GLLFYMARINHA *mouse laminin α2 chain (2796–2807)*	α-dystroglycan	cell attachment
A2G80	VQLRNGFPYFSY *mouse laminin α2 chain (2812–2823)*	α-dystroglycan	not determined
A3G756	KNSFMALYLSKGRLVFALG *human laminin α3 chain (1411–1429)*	syndecans	wound healing
A5G27	RLVSYNGIIFFLK *mouse laminin α5 chain (2892–2904)*	CD44	metastasis suppression

[1] Active core sequence is indicated by bold.
[2] MMP↑: matrix metalloproteinase promotion.

applications of biologically active laminin peptides for DDSs are unique and widely applicable.

3. Applications of laminin-derived peptides

3.1. Application of laminin-derived peptides for gene delivery systems

The success of human gene therapy depends on the development of delivery vehicles or vectors capable of safely, selectively, and effectively delivering therapeutic genes to target cells. There are two main approaches to gene delivery: viral gene delivery and non-viral gene delivery. Virus-mediated gene delivery methods using either retroviruses or adenoviruses have high delivery efficiencies and have been used in clinical trials (Giacca and Zacchigna, 2012). However, there is often concern about inflammatory responses and oncogenic effects.

Non-viral vectors, which are generally delivered as a complex with chemicals and/or biochemical vectors, such as cationic lipids (Audouy, de Leij, Hoekstra, & Molema, 2002; Hirko, Tang, & Hughes, 2003), polymers (Quader & Kataoka, 2017), cell-penetrating peptides (Nakase, Takeuchi, Tanaka, & Futaki, 2008), or gold nanoparticles (Pissuwan, Niidome, & Cortie, 2011) have emerged as a promising alternative to viral vectors due to their safety, versatility, ease of preparation, and convenience of scaling-up. Although the vector formulations for delivering genes have been improved repeatedly, they generally suffer from relatively low transfection efficiencies. Therefore, there is still a need for novel and more efficient delivery systems.

To achieve efficient cancer gene therapy, A promising strategy, such as tumor-targeted gene delivery is needed for efficient cancer gene therapy. Some studies using tumor targeting moieties, such as transferrin, folate, anisamide, RGD-peptides, and antibodies have shown promise (Leamon, Weigl, & Hendren, 1999; Li and Huang, 2006; Li, Chen, Hackett, & Huang, 2008; Merdan et al., 2003; Pirollo et al., 2008; Suk et al., 2006).

In the present study, we focused on peptide AG73, which is a 12 amino acid synthetic peptide derived from the globular domain of the laminin α1 chain. AG73 is a ligand for syndecans, a transmembrane heparan sulfate proteoglycan (Carey, 1997; Hoffman et al., 2001).

Syndecan-2 is highly expressed in various cancer cell lines and plays a role in angiogenesis (Essner, Chen, & Ekker, 2006; Fears and Woods, 2006; Noguer, Villena, Lorita, Vilaró, & Reina, 2009; Tkachenko, Rhodes, & Simons, 2005). We hypothesized that novel delivery systems could be developed using AG73 as a moiety to target cancer cells (Figs. 2, 3). We therefore prepared liposomes modified with AG73 that selectively interacted with syndecan-2, known to be highly expressed in cancer cells. When considering *in vivo* adaptation, there is concern that many nanoparticles, like liposomes, can easily be sequestered by the reticuloendothelial system (RES), such as in the liver and spleen. To avoid capture by the RES, liposomes are modified with PEG, leading to improvement in the stability of the liposome itself in the blood and increased retention after systemic administration. This method is important for enhancing accumulation into the target site and for obtaining anti-cancer therapeutic effects by the included genes (Allen & Cullis, 2013; Klibanov et al., 1990; Lee, Hong, & Papahadjopoulos, 1992; Petros and DeSimone, 2010).

Therefore, AG73 was modified onto the PEG chain of a PEGylated liposome to create AG73-PEG liposomes. Liposomes containing phospholipids, such as 1,2-dioleoyl-*sn*-glycero-3-phospho-*rac*-1-glycerol (DOPG), 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE), and 1,2-distearoyl-*sn*-glycero-3-phosphatidylethanolamine-N-[methoxy (polyethylene glycol)-2000] (DSPE-PEG_{2000}-OMe), were prepared by a rehydration method (Shew & Deamer, 1985). For coupling of AG73 and PEG, AG73 containing a reactive cysteine at the N-terminus was conjugated with the maleimide (Mal) of the PEG liposomes using a thioether bond (Negishi, Omata, Iijima, Hamano, et al., 2010). Polycation (poly-L-lysine, PLL) was also used for encapsulating plasmid DNA into the liposome. Electron microscopy analysis showed that monodisperse AG73-labeled liposomes were present (Negishi, Omata, Iijima, Takabayashi, et al., 2010). In the transfection experiment using AG73-PEG liposomes, gene transfection efficiency was enhanced by 100-fold in syndecan-2 overexpressing cancer cells compared to that in low syndecan-2 expressing cancer cells, suggesting that AG73-PEG liposomes can be a useful targeted gene carrier for syndecan-2 overexpressing cancer cells.

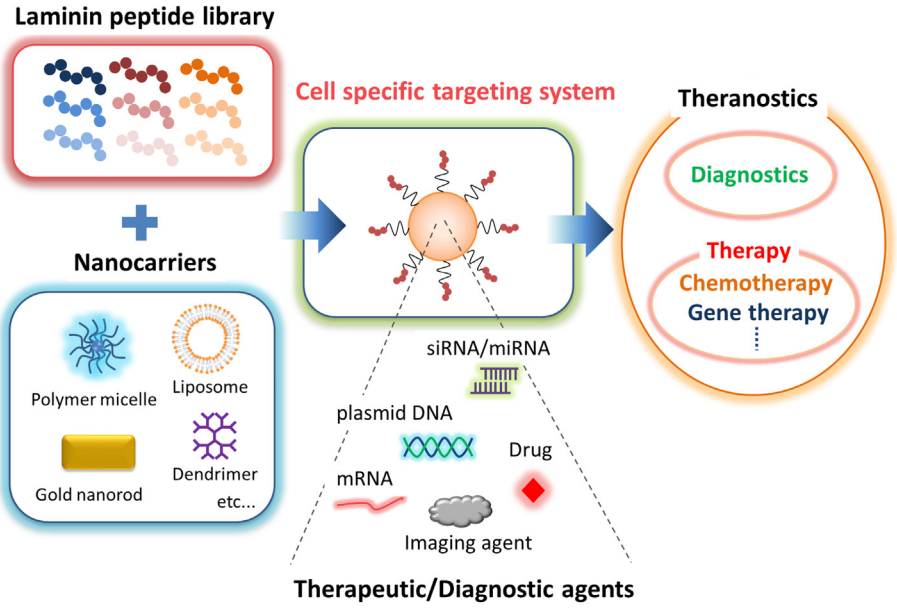

Fig. 2. Application of laminin peptide library in drug delivery system for diagnostics and/or therapy.

Although AG73-PEG liposomes could transfer genes into the cells *via* syndecan-2, it has been reported that the PEG-modification of liposomes affects cellular uptake and endosomal escape of liposomes, resulting in reduced transfection efficiency (Hatakeyama et al., 2007; Shin, Shum, & Thompson, 2003; Walker et al., 2005; Zalipsky et al., 1999). Therefore, when using AG73-PEG liposomes in the presence of chloroquine, which is recognized as an endosomolytic agent (Cheng et al., 2006; Sonawane, Szoka, & Verkman, 2003; Wibo & Poole, 1974), the transfection efficiency increased 10-fold compared to that with AG73-PEG liposomes in the absence of chloroquine. From these results, it was thought that gene transfer efficiency can be further enhanced by promoting escape of the AG73-PEG liposomes from the endosomes.

Echo-contrast gas entrapping liposomes (BL)s and ultrasound (US) exposure can temporarily enhance the permeability of tissue cell membranes (Negishi et al., 2008; Suzuki et al., 2007). We hypothesized that the combination of BLs and US exposure may affect not only the cell membrane but also intracellular vesicles, leading to a boost in escape of plasmid DNA from endosomes into the cytoplasm. For this reason, we assessed whether AG73-mediated liposomal gene transfection could be enhanced by BLs and US exposure.

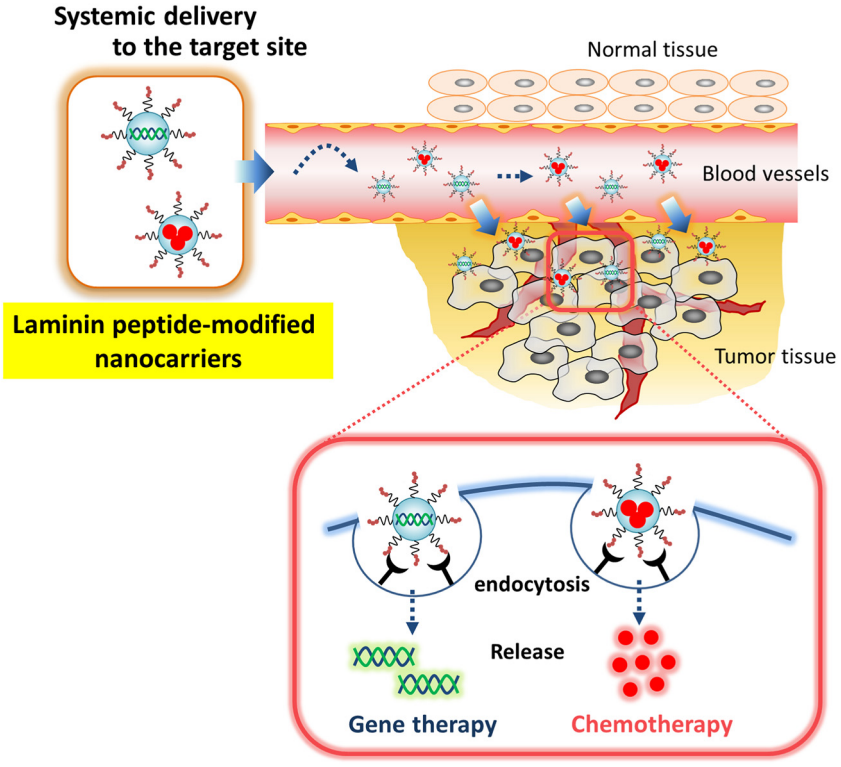

Fig. 3. Schematic representation of a nanocarrier modified with a laminin-derived peptide for cancer targeting.

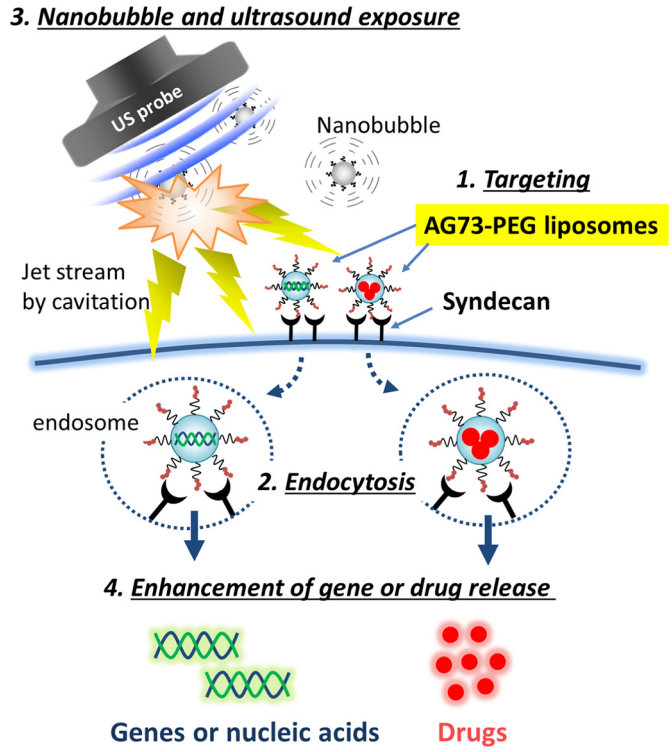

Fig. 4. Gene or drug delivery by AG73-PEG liposomes with nanobubbles and ultrasound exposure.

Treatment using BLs and US affected the intracellular trafficking of AG73-PEG liposomes, thereby enhancing the delivery efficiency of genes into both the cytoplasm and nucleus (Fig. 4) (Negishi, Omata, Iijima, Hamano, et al., 2010; Omata et al., 2012). Thus, AG73-PEG liposomes combined with BLs and US may be a promising method to achieve selective and efficient gene delivery for cancer gene therapy *via* systemic administration.

3.2. Application of laminin-derived peptides for drug delivery systems

AG73-PEG liposomes also have the potential as DDS carriers that selectively deliver anticancer drugs to cancer cells (Fig. 3). Therefore, we sought to prepare AG73-modified liposomes encapsulated with doxorubicin (Dox) (AG73–Dox), a representative anticancer drug. Dox-encapsulated liposomes (Dox–PEG) containing phospholipids, such as 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC), DSPE–PEG_{2000}–OMe, and 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[maleimide(polyethylene glycol)-2000] (DSPE–PEG_{2000}–Mal), were prepared using both a rehydration method (Shew and Deamer, 1985) and a remote loading method with a pH gradient (Dos Santos et al., 2004). For coupling, AG73 was added to the Dox-encapsulated liposomes, and the cysteine of the Cys–AG73 was conjugated with the Mal of the Dox–PEG using a thioether bond (Negishi et al., 2011). The selective cellular uptake of Dox–PEG *via* the syndecan-2 receptor was assessed by flow cytometry analysis. The results showed that the cellular uptake of Dox–PEG on cancer cells, including syndecan-2 overexpressing cells, was enhanced by the modification of AG73 and in a sequence specific manner. This led to higher cytotoxicity against cancer cells than that of Dox–PEG alone. The cytotoxicity of AG73-Dox showed about 2-times higher than Dox-PEG alone. Thus, AG73-PEG liposomes function sufficiently as a DDS carrier of anticancer drugs.

After intracellular uptake of AG73–Dox, BLs and US also boosted drug release of Dox in the cytoplasm, leading to an enhancement of cytotoxicity (Fig. 4) (Hamano et al., 2013). The cytotoxicity of AG73-Dox with BLs and US showed about 2-times higher than Dox-PEG alone. This enhancement by BLs and US is similar to that seen with the AG73-PEG liposomes discussed in Section 3.1. In addition, evaluation of the biodistribution of liposomes showed that AG73-PEG liposomes tended to bind to intratumoral vessels and extravasated in the tumor tissue. AG73-Dox also showed antitumor activity *in vivo* (Fig. 3) (Negishi et al., 2011). AG73-Dox accumulated not only in the tumor tissue due to the enhanced permeability and retention effect (Fang, Nakamura, & Maeda, 2011; Matsumura and Maeda, 1986) but also in blood vessels in the tumor tissue, leading to suppression of tumor growth by reducing proliferation of the neovasculature. Thus, further optimization of AG73-PEG liposomes towards tumor targeting may enable development of a useful DDS carrier for cancer chemotherapy.

3.3. Application of laminin-derived peptides for diagnosis

In Sections 3.1 and 3.2, AG73 liposomes demonstrated potential as useful DDS carriers, especially in cancer therapy. It is also critical to develop diagnostic imaging methods for detecting cancer lesions as an important part cancer treatment. Among various diagnostic methods, ultrasonography has been widely used in clinical practice because of its safety. Based on this wide and safe use, we developed AG73 liposome encapsulating diagnostic ultrasound contrast gas (AG73-BLs) as a neovascular-targeting agent to enhance contrast imaging (Negishi et al., 2013). After administrating AG73-BLs to tumor bearing mice, AG73-BLs enabled the enhancement of ultrasound diagnostic imaging by accumulating at neovascular vessels in the cancer tissue, suggesting that AG73-BLs may be a useful ultrasound contrast agent for cancer targeting diagnostics.

To date, integrin $\alpha_v\beta_3$-targeting liposomes (C16Y liposomes: C16Y-L) have been developed (Hamano et al., 2012). The C16Y peptide is a 12-amino acid synthetic peptide, which is a modified C16 peptide, derived from the globular domain of the laminin γ1 chain that binds to endothelial cell integrins, $\alpha_v\beta_3$ and $\alpha_5\beta_1$. Recently, we have successfully developed a screening system for targeting carriers using a laminin-peptide library (Negishi et al., 2018). It is expected that the use of this laminin-peptide library will lead to the development of useful carriers for gene or drug delivery against various diseases other than cancer.

Conflicts of interest

The authors declare that they have no conflicts of interest associated with this work.

Acknowledgment

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