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Experimental and Clinical Therapeutic Uses of Low-Molecular-Weight Heparin/Protamine Micro/Nanoparticles

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Outline:

Introduction

Polyelectrolyte complexes (PECs) are generated by electrostatic interactions between oppositely charged polyelectrolytes. When this interaction occurs at non-equivalent ratios, nonstoichiometric PECs are produced, causing each PEC particle to carry an excess charge [1,2]. Proteins interact with both synthetic and natural PECs [3,4]. These binding characteristics, along with a simple preparation, allow PECs to be an excellent model for studying the *in vivo* behavior of charged biopolymers as well as having potential applications in medicine and biotechnology. Reported studies indicate that polyanions and polycations can bind to proteins below and above their isoelectric points, respectively. These interactions can result in soluble complexes, complex coacervation and/or the formation of amorphous precipitates. Main aspects studied by different authors are compositions of PECs obtained under various experimental conditions, such as the strength and position of ionic sites, charge density and rigidity of polymer chains as well as chemical properties such as solubility, pH, temperature and concentration [1,5-8].

Electrostatic interactions are also important because of their similarity to biological interactions. Interactions between proteins and nucleic acids, for example, play a role in the transcription process [3]. DNA/chitosan PECs [9], chitosan/chondroitin sulfate PECs and chitosan/hyaluronate PECs [10] function as gene and drug carriers. Moreover, PECs that are insoluble also have potential applications as membranes, microcapsules, micro/nanoparticles and scaffolds for tissue engineering [10].

Particulate drug delivery systems have become widely employed in both experimental and clinical setting to address a range of applications. Their popularity can be attributed to ease of application as a suspension and ease of manufacture. The size of the particles plays a substantial role in determining the properties of the final product and its potential applications. When injected into tissue, large particles (microparticles: approximately 1—5 μm in diameter) tend to stay where placed, while smaller particles (nanoparticles: about 50—200 nm in diameter) will circulate for a period of time determined by size, surface chemistry and other factors [11]. In general, microparticles will be useful if the particles are delivered locally. Examples include local anesthetic microparticles and microparticles including growth factor (GF), antibiotics and chemotherapeutics [12].

Heparin interacts with a variety of functional proteins, including heparin-GFs, cytokines, extracellular matrix components and adhesion molecules [13-15]. Thus, heparin may be useful as a therapeutic agent in various pathological conditions that involve functional proteins. However, high-dose heparin cannot be used because of the excessive risk of bleeding [16]. In contrast, low-molecular-weight heparin (LMW-H) has pharmacological and practical advantages compared with native heparin. The lower protein binding activity of LMW-H produces a low, stable and predictable anticoagulant response [16]. On the other hand, protamine neutralizes heparin and LMW-H by forming a stable complex that lacks anticoagulant activity [17]. Protamine is also in clinical use to reverse the anticoagulant activity of heparin following cardiopulmonary bypass as well as in cases of heparin-induced bleeding [18].

We previously prepared water-insoluble particles by mixing non-anticoagulant heparin with chitosan and by mixing fucoidan with chitosan, and investigated the ability of the resulting insoluble heparin/chitosan and fucoidan/chitosan microparticles to protect fibroblast growth factor-2 (FGF-2) activity [20, 21]. Water-insoluble micro/nano particles (100 nm—3 μm in diameter) were then prepared by mixing LMW-H with protamine, and reported the ability of the resulting injectable low-molecular-weight heparin/protamine micro/nano particles (LMW-H/P M/NPs) to protect and activate FGF-2 and hepatocyte growth factor (HGF) activity [21-23]. Furthermore, GFs released from platelets that were involved in cell proliferation, migration and angiogenesis could be adsorbed onto LMW-H/P M/NPs [24].

We also reported that LMW-H/P M/NPs bind to cell surfaces of adipose-derived stromal cells (ASCs) through specific interactions between LMW-H/P M/NPs and cell surface heparin-binding proteins such as some integrin. The interaction of the cells with LMW-H/P M/NPs resulted in cells/LMW-H/P N/MPs-aggregate formation. The ASCs/LMW-H/P M/NPs-aggregate formation substantially promoted cell viability *in vitro*. Furthermore, the aggregates induced vascularization and fibrous tissue formation *in vivo* [25,26]. The LMW-H/P M/NPs, in combination with ASCs, are a new convenient cell carrier and may be a promising novel therapy for inducing vascularization and fibrous tissue formation in ischemic disease by transplantation of the ASCs/LMW-H/P M/NPs-aggregates.
As a coating matrix, LMW-H/P M/NPs were efficiently bound to tissue culture plates. With the ability of LMW-H/P MPs to retain GFs, LMW-H/P M/NPs could be very useful in cell culture. Human microvascular endothelial cells (hMVECs) and human dermal fibroblast cells (hDFCs) adhered to LMW-H/P M/NPs-coated tissue culture plates [27,28] and grew optimally in low fetal bovine serum (FBS) (1－2%) medium supplemented with FGF-2 (5 ng/mL). This protocol could make it possible to use low autologous serum (1－2%) for the culturing of human bone marrow-derived mesenchymal stem cells (BMSCs) and ASCs [29,30]. Furthermore, CD34+ hematopoietic progenitor cells (CD34+ cells) derived from bone marrow exhibited a comparatively higher proliferation on LMW-H/P M/NPs-coated plates in hematopoietic progenitor growth medium (HPGM) supplemented with appropriate cytokines than those on uncoated plates [31]. Thus, we mainly describe in this Chapter on the LMW-H/P M/NPs which we originally prepared as PECs, its characterizations and its potential medical applications as protein carriers for GFs such as FGF-2, HGF and GFs in platelet-rich plasma (PRP), as a cell carrier for cell transplantation and as coating matrix for human cell cultures.

Preparation of LMW-H/P M/NPs

Heparinoids (heparin, heparin sulfate, low-molecular-weight heparin, and heparin-like polysaccharides) specifically interact with a variety of heparin-binding functional proteins with high affinity, including heparin-binding GFs, cytokines, extracellular matrix components and adhesion molecules [13-15]. Thus, heparin may be useful as a therapeutic agent in various pathological conditions that involve functional proteins. However, high-dose heparin cannot be used because of the excessive risk of bleeding [16]. In contrast, LMW-H (MW: approximately 5000 Da) has pharmacological and practical advantages compared with native heparin. The lower protein binding activity of LMW-H produces a low, stable and predictable anticoagulant response, thereby bypassing the need for laboratory monitoring of drug levels to adjust the dosage [16]. In addition, one or two subcutaneous injections per day are sufficient to maintain therapeutic concentrations because of its longer plasma half-life [16].
On the other hand, protamine, a purified mixture of proteins obtained from fish sperm, neutralizes heparin and LMW-H by forming a stable complex that lacks anticoagulant activity [17]. Protamine is also in clinical use to reverse the anticoagulant activity of heparin following cardiopulmonary bypass as well as in cases of heparin-induced bleeding [18]. Furthermore, protamine is used as a carrier for insulin (protamine Hagedorn (NPH) insulin) [32].
We previously prepared water-insoluble particles (2–10 μm in diameter) by mixing non-anticoagulant heparin with chitosan and by mixing fucoidan with chitosan. The abilities of resulting insoluble heparin/chitosan and fucoidan/chitosan microparticles were evaluated to protect and activated the activity of FGF-2 *in vitro* and *in vivo* [19,20]. Water-insoluble micro/nanoparticles (100 nm－3 μm in diameter) by mixing LMW-H (6.4 mg/ml) with protamine (10 mg/mL) at a ratio of 7:3 (vol:vol) (see Figure 6.1) was reported the ability of the resulting injectable LMW-H/P M/NPs (0.1－3 μm in

diameter) to protect and activated FGF-2 [21,22] and HGF activity [23]. Furthermore, GFs from PRP that were also involved in cell proliferation, migration and angiogenesis were able to adsorb onto LMW-H/P M/NPs. The studies indicate that LMW-H/P M/NPs may serve as an effective micro/nano-carrier for various GFs, particularly for the local application of GFs. GFs containing LMW-H/P M/NPs show a substantial effect to induce vascularization and fibrous tissue formation because of the gradual controlled release, protection and activation of GF molecules from GFs-containing LMW-H/P M/NPs [24]. In another study, we used diluted LMW-H (≤0.32 mg/mL) as an anion molecule and the diluted protamine (≤0.5 mg/mL) as a cation molecule to synthesize LMW-H/P NPs (approximately 100 nm in diameter) [22].

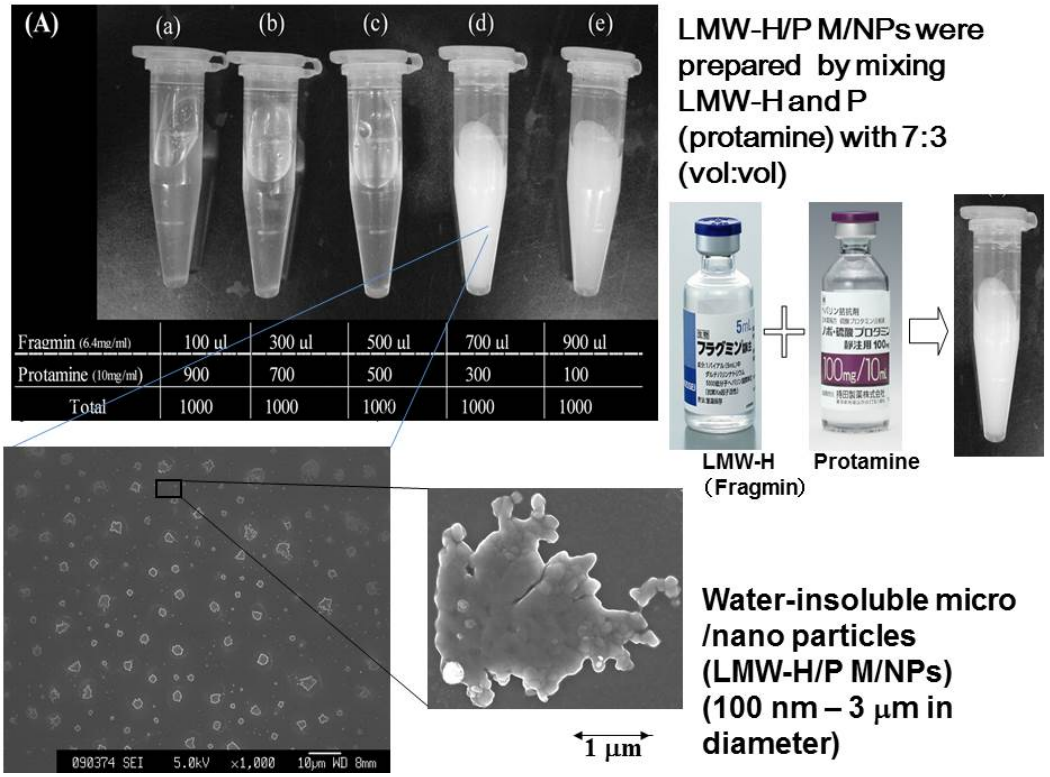

FIGURE 6.1
Preparation of LMW-H/P M/NPs

In order to produce of the nanoparticles, equally diluted LMW-H and protamine (100-fold, 50-fold and 20-fold diluted) were mixed in a ratio at 7:3 (vol:vol) (see Figure 6.2). The diameter of generated LMW-H/P NPs by mixing 100-fold, 50-fold and 20-fold diluted protamine to equally diluted LMW-H in the ratio of 3:7 (vol:vol) were 84.6 ± 26.8, 95.0 ± 27.0 and 112.5 ± 46.1 nm, respectively (see Figure 6.2) [22]. And no microparticles (>1 μm in diameter) were observed in the mixtures. In contrast, generations of small amount of microparticles (approximately 1 μm in diameter) were observed by mixing of 10-fold diluted protamine (1 mg/ml) to LMW-H (0.64 mg/ml) in the ratio of 3:7 (vol/vol). When non-diluted protamine (10 mg/ml) was added to non-diluted LMW-H (6.4 mg/ml) up to ratio of 3:7 (vol:vol), maximal LMW-H/P M/NPs (100 nm—3 μm in diameter) was produced and the high turbidity was observed. When 10-fold concentrated protamine (100 mg/ml) was added to the equally

10-fold concentrated LMW-H (64 mg/ml) up to ratio of 3:7 (vol:vol), mixtures of larger LMW-H/P MPs (3－10 μm in diameter) and larger cotton-like precipitates (>10 μm) were immediately generated and those products were insoluble [22]. Cotton-like compounds were generated after lyophilizations of both LMW-H/P M/NPs (100 nm－3 μm in diameter) and LMW-H/P NPs (approximately 100 nm in diameter) solutions without dextran, and they were hardly resoluble in water. However, both the freeze-dried LMW-H/P M/NPs were easily dessolved in water by adding 0.5% and 0.2% dextran, respectively, before their lyophilizations. In addition, aggregation of LMW-H/P NPs in solution to LMW-H/P MPs was prohibited in the presence of dextran [22]. Thus, the addition of dextran is effective to stabilize the LMW-H/P M/NPs and to prepare stable and resoluble freeze-dry LMW-H/P M/NPs. On the other hand, LMW-H/P NPs in suspension 20-fold diluted with saline was stable for at least 6 weeks at room temparature.

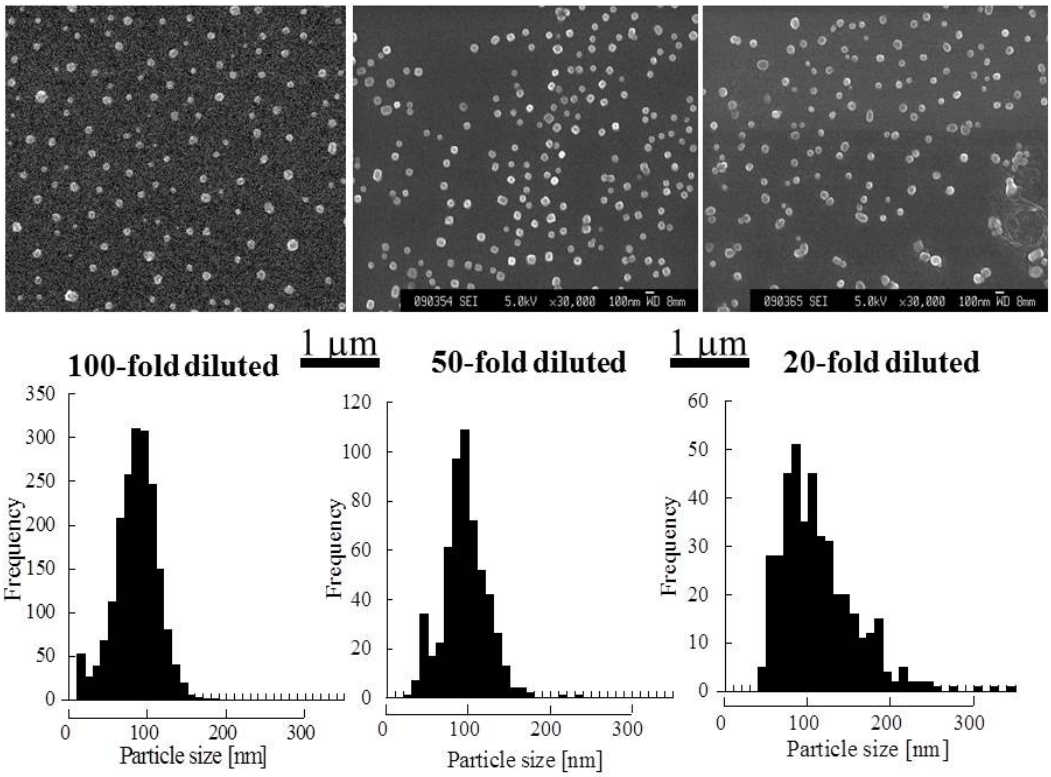

FIGURE 6.2
LMW-H/P M/NPs generated by mixing diluted LMW-H and Protamine

Protein-Delivery Micro /Nano Particles

We previously reported the ability of the injectable LMW-H/P M/NPs to adsorb and to protect and activate FGF-2 [21,22], HGF [23] and GFs in PRP [24] that were also involved in cell proliferation, migration and angiogenesis. The studies suggested that LMW-H/P M/NPs serve as an effective

micro/nano carrier for various GFs, particularly for the local application of GFs. Thus, GFs containing LMW-H/P M/NPs show a substantial effect to induce vascularization and fibrous tissue formation because of the gradual controlled release, protection and activation of GF molecules from GFs-containing LMW-H/P M/NPs [24] (See Figure 6.3).

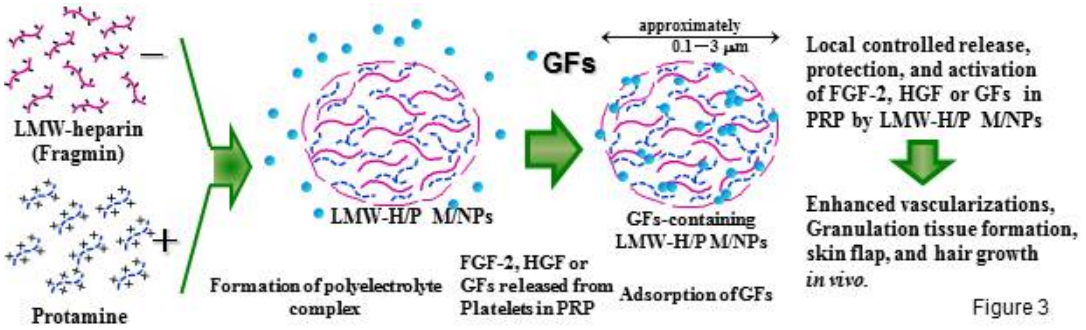

FIGURE 6.3

FGF-2-Containing LMW-H/P M/NPs

FGF-2 binds heparin with high affinity (Kd of 8.6×10^{-9} M). The polysaccharides can prolong the biological half-life of FGF-2 as well as protect FGF-2 from heat, acid and proteolytic inactivation [21]. Similarly, the LMW- H/P M/NPs have high affinity for FGF-2 (Kd = 2.4×10^{-9} M) [21], and this interaction of FGF-2 with the LMW-H/P M/NPs can substantially prolong the biological half-life time of FGF-2. The protection of FGF-2 against heat inactivation and trypsin degradation by the LMW-H/P MPs was effective in a concentration-dependent manner [21]. FGF-2 molecules were released *in vitro* from the FGF-2-containing LMW-H/P M/NPs with half-releasing time of about 6 days. Those results demonstrated that FGF-2 molecules are bound and stabilized on the LMW-H/P M/NPs, and that the FGF-2 molecules incorporated into the LMW-H/P M/NPs will be gradually released upon biodegradation of the hydrogel *in vivo*.

When the FGF-2-containing LMW-H/P M/NPs were subcutaneously injected into the backs of mice, neovascularization was induced near the injection site after 3 days. Neovascularization induced by the FGF-2-containing LMW-H/P M/NPs reached a maximum at 1 week, after which a slight decrease in the neovascularization rate occurred. No significant vascularization was observed after either the injection of FGF-2 alone or the LMW-H/P M/NPs alone [21]. Figure 6.4 shows prevension of limb loss in hindlimb ishchemic model (nude mice) by FGF-2-containing LMW-H/P M/NPs. Five ishchemic hindlimbs out of 8 treated with FGF-2-containing LMW-H/P M/NPs were normally recovered after 2 weeks and two of 8 were maintained normal hindlimbs for at least 3 months [33]. Thus the intramuscular injection of FGF-2-containing LMW-H/P M/NPs into ischemic hindlimbs promotes vascularization and reduces limb loss associated with local ischemia. FGF-2-containing LMW-H/P M/NPs thus provide an excellent biomaterial to immobilize, retain and gradually release FGF-2 for the optimal induction of vascularization and granulation tissue formation. The present approach of using FGF-2-containing LMW-H/P M/NPs would be a valuable option for therapeutic angiogenesis that is targeted for tissue regeneration and for treating ischemic disease.

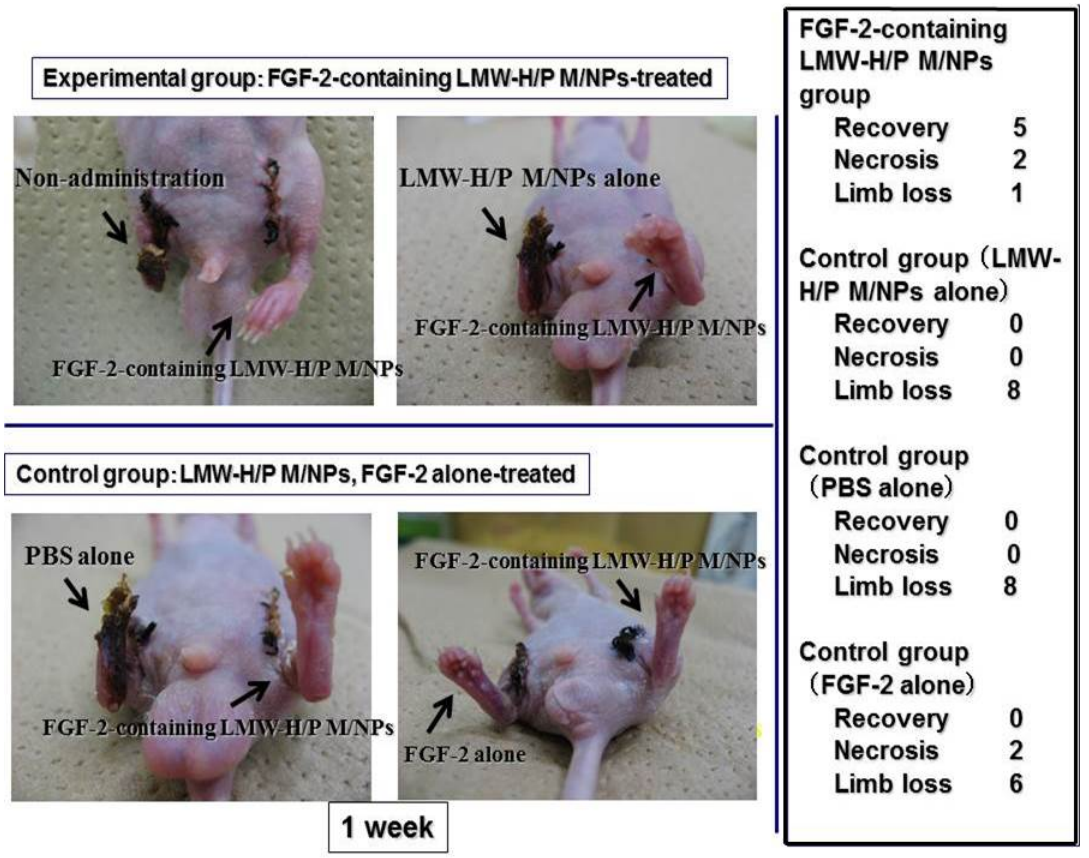

FIGURE 6.4
Prevention of limb loss in hindlimb ishchemic model by FGF-2^containing LMW-H/P M/NPs

Another study demonstrated advanced fat survival and capillary formation in FGF-2-containing LMW-H/P M/NPs-assist subdivided free fat-grafting groups in rats [34]. Furthermore, our study demonstrated the ability of FGF-2-containing LMW-H/P M/NPs to induce both arteriogenesis and angiogenesis in rabbit models of ischemic limbs [35] (see Figure 6.5). The primary conclusion is that FGF-2-containing LMW-H/P M/NPs-treatment effectively induces the development of collateral vessels, which can provide sufficient blood flow to the pre-existing vascular network in ischemic tissue. Since all components used in the FGF-2-containing LMW-H/P M/NPs are also used clinically, we feel safety in a clinical setting is probable [35].

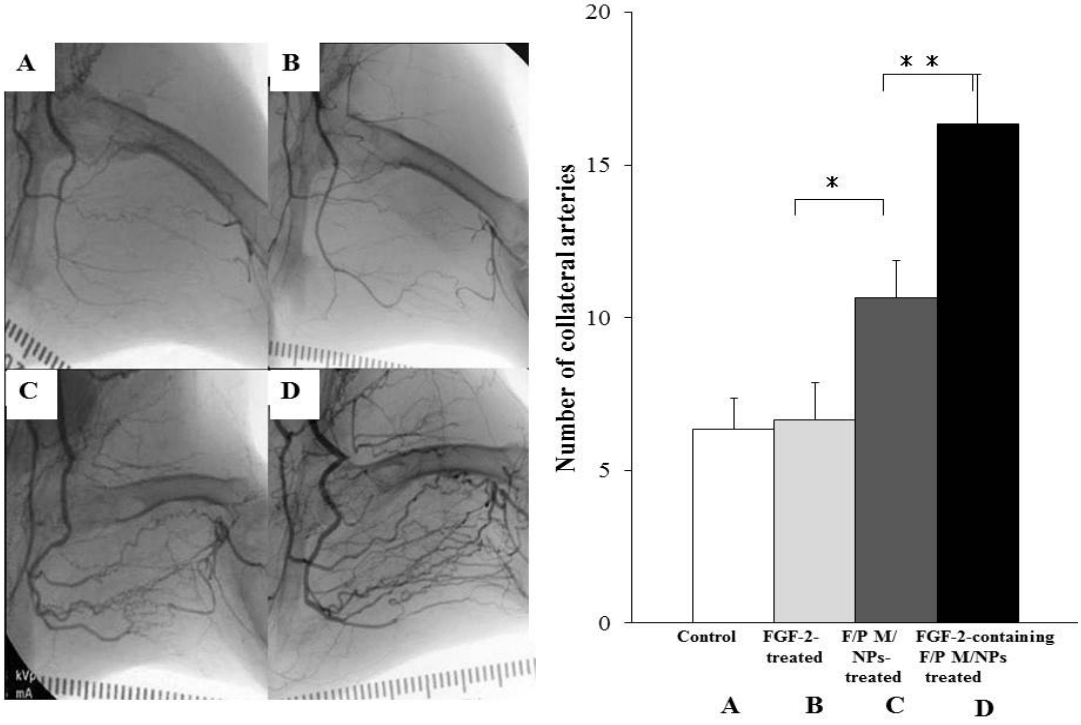

FIGURE 6.5
Quantification of visible collateral arteries under angiographic viewing on day 28

HGF-Containing LMW-H/P M/NPs

HGF can accelerate the regeneration of damaged tissue in animal models of hepatic and renal failure, but the local biological activity of exogenous HGF may be limited *in vivo* without an adequate protein delivery system that acts to maintain HGF at the target site and prevents rapid clearance by the liver [36,37]. Without such a system, very large doses are usually required to exert a significant regenerative effect [38]. To enhance the repetitive efficacy of HGF, we developed LMW-H/P M/NPs for high-affinity absorption and controlled release of HGF. LMW-H /P M/NPs prolonged the biological activity of HGF and protected HGF against heat and proteolytic inactivation. Furthermore, these HGF-containing LMW-H /P M/NPs were injectable and biocompatible, and triggered significant angiogenesis at the site of injection. Thus, the LMW-H /P M/NPs are a reliable, efficient and safe protein delivery system to enhance and stabilize HGF activity at local administration sites for subcutaneous or muscular injection [23].

More than 5 μg of HGF bound to 1 mg of LMW-H /P M/NPs and was gradually released from HGF-containing LMW-H /P M/NPs *in vitro*. HGF-containing LMW-H /P M/NPs appeared to be bioactive since they stimulated human micro-vascular endothelial cells (hMVECs) proliferation. In fact, HGF-containing LMW-H /P M/NPs was more mitogenic than HGF alone, reducing the cell doubling time from 45 to 25 h, possibly because bound HGF is more resistant to biodegradation and inactivation under physiological conditions. Indeed, proliferation of MVECs was substantially stimulated by preloaded LMW-H /P M/NPs even after 10 days at 37°C, while HGF alone did not stimulate MVEC proliferation at all after 7 days of

pre-incubation at 37°C. Furthermore, LMW-H F/P M/NPs could effectively protect HGF against heat inactivation and trypsin degradation [23].
When HGF-containing LMW-H /P M/NPs were subcutaneously injected into the back of mice, large and medium vessels were induced near the injection site after 8 days. The number of small vessels induced by the HGF-containing LMW-H /P M/NPs reached a maximum on days 8–11, after which a slight decrease in the neovascularization rate occurred. No significant induction in large vessels was observed after injection of HGF or LMW-H /P M/NPs alone, although a minor induction of medium and small vessels was observed. We suggest that free HGF diffused away too rapidly to induce arteriogenesis and that inactivation of HGF remaining at the injection site within a few days also led to less efficient vascularization. The modest vascularization (mainly small and medium vessels) induced by LMW-H /P M/NPs alone result from the binding of various endogenous angiogenic growth factors around the injection site, leading to local accumulation and controlled release [23].

PRP Containing LMW-H/P M/NPs

PRP contains a high concentration of thrombocytes (platelets). When the platelets are activated, various GFs and other bioactive proteins in α-granules of platelets are released and those proteins augment tissue repair and regeneration processes [24,39-41]. Platelets contain over 20 GFs, including platelet-derived growth factors (PDGFs), FGFs, HGF, transforming growth factors (TGFs), and vascular endothelial growth factors (VEGFs), almost all of which are known to bind to heparin and to LMW-H/P M/NPs. Recent studies suggest that GFs in PRP not only influence the viability of transferred cells but may also play bioactive roles in the regulation of proliferation and differentiation in various types of cells [24]. Any treatment aiming to mimic the critical aspects of the natural biological process should not be limited to the provision of a single GF, but rather should release multiple GFs at an optimized ratio, at a physiological dose and in a specific spatiotemporal pattern. Those results indicated that the LMW-H/P M/NPs also activate the platelets to release the GFs, and that in turn the released GFs from the platelets can be immobilized, be stabilized and be activated on the LMW-H/P M/NPs [24].
The GFs in PRP are stably bound to LMW-H/P M/NPs *in vivo*. The GFs adsorbed onto LMW-H/P M/NPs may be gradually diffused and released upon biodegradation of LMW-H/P M/NPs. When PRP-containing LMW-H/P M/NPs were subcutaneously injected into the backs of mice, significantly higher neovascularization and granulation tissue with enhanced filtration of inflammatory cells were observed compared with the mouse groups injected with PRP alone, LMW-H/P M/NPs alone and the control [24]. Compared to either PRP alone or LMW-H/P M/NPs alone, locally administered PRP-containing LMW-H/P M/NPs augmented the wound bed and substantially increased viability of rat dorsal paired pedicle skin flaps [42]. The improved flap survival was noted if PRP-containing LMW-H/P M/NPs was administered 2 days before the flap elevation [42]. PRP-containing LMW-H/P M/NPs thus represent a promising new biomaterial for improving skin flaps, particularly in the field of reconstructive surgery. Clinical research was performed using autologous PRP-containing LMW-H/P M/NPs and PRP alone in 26 patients with thin hair (including 10 women) [43]. Hair growth and thickening following administration of both PRP-containing LMW-H/P M/NPs and PRP alone was observed in all patients compared with the control, but PRP-containing LMW-H/P M/NPs appeared to provide the most substantial change in the hair (see Figures 6.6 and 6.7) [43]. Because of the use of autologous materials, this method using PRP-containing LMW-H/P M/NPs is simpler and cheaper, and has no side effect compared with conventional methods.

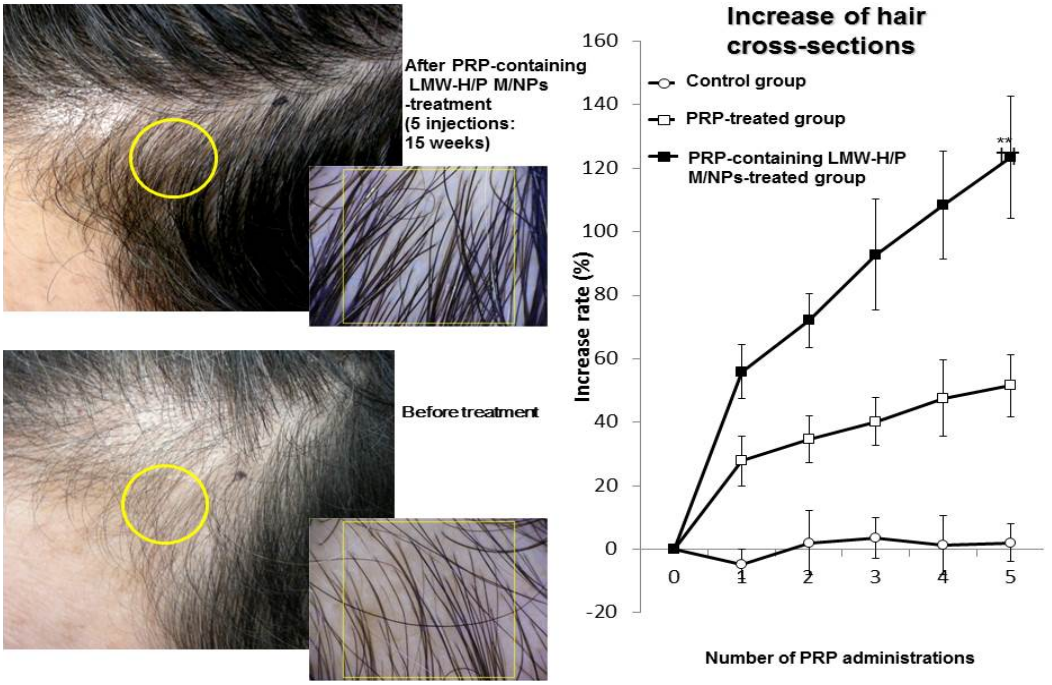

FIGURE 6.6
PRP-containing LMW-H/P M/NPs treatment for Alopecia Areata

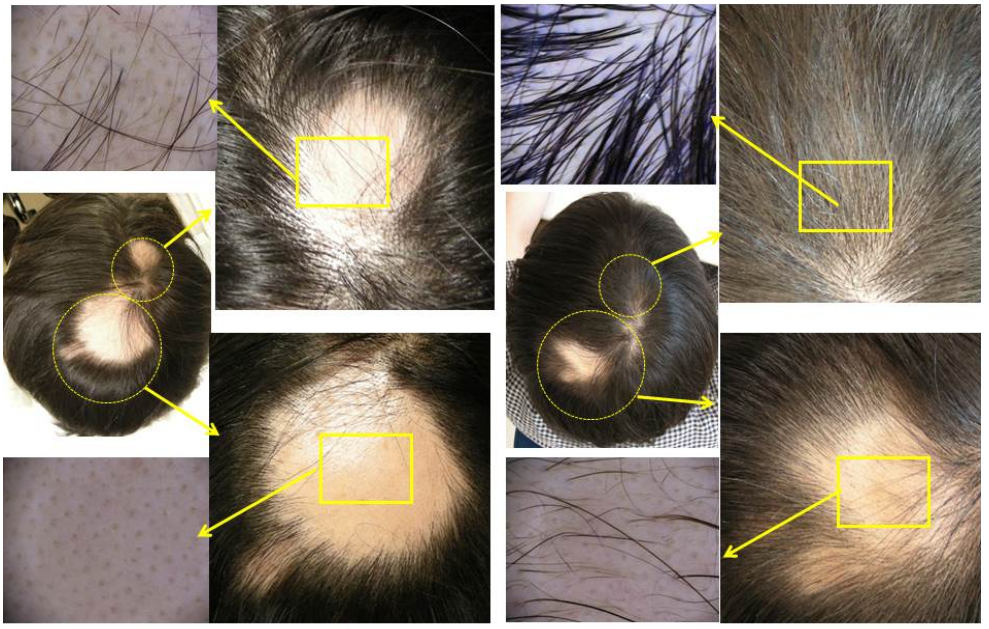

FIGURE 6.7
PRP-containing LMW-H/P M/NPs-treatment for Alopecia Areata

Cell-Delivery Micro/Nano Particles

LMW-H/P M/NPs as Stromal Cell Carriers

LMW-H/P MPs as cell carriers can enhance cell viability as well as control the release of GFs. LMW-H/P MPs could substantially enhance the cellular viability of various suspension cultures, including hMVECs, hDFCs and ASCs [27,28]. In particular, ASCs have the potential to differentiate into skin, bone, cartilage, fat, myocardium, skeletal muscle and neurons [44]. Several reports indicate that the transplantation of human ASCs-cultured constructs significantly stimulates angiogenesis, wound repair and re-epithelialization in athymic mice when compared with the corresponding human fibroblast-cultured constructs [45]. ASCs can be easily harvested with lower donor site morbidity compared with other pluripotent stem cell sources. Furthermore, ASCs can easily attach and proliferate in culture, and therefore, are available on a large scale even for autologous grafting in small animals such as rodents [46]. However, an application of ASCs for therapeutic angiogenesis and vasculogenesis requires microcarriers to act as injectable vehicles necessary for transplantation of ASCs. It was observed that LMW-H/P M/NPs could bind to the surface of the cells. The interaction of these cells with LMW-H/P M/NPs induced ASCs/LMW-H/P M/NPs-aggregate formation, and substantially promoted cell viability for at least 3 days in cell suspensions (see Figure 6.8) [25]. The ASCs/LMW-H/P M/NPs-aggregates adhered and grew on suspension culture plates, and the aggregates similarly grew on type I collagen-coated plates. Furthermore, cultured ASCs secreted a significant amount of angiogenic GFs such as FGF-2, HGF, PDGF and VEGF. These secreted GFs could have been retained within the ASCs/LMW-H/P M/NPs-aggregates. When the ASC/LMW-H/P M/NPs-aggregates were subcutaneously injected into the back of nude mice, a significant increase in neovascularization and fibrous tissue formation was observed near the injected site from 3 days to 2 weeks [25]. Taken together, these data indicate that ASCs/LMW-H/P M/NPs-aggregates are an useful and convenient biomaterial for angiogenesis and wound repair cellular therapy. The interaction of adhesive cells with LMW-H/P M/NPs induced ASCs/LMW-H/P M/NPs-aggregate formation, and substantially promoted cell viability in cell suspensions *in vitro,* and injections of the aggregates significantly enhanced vascularization and fibrous tissue formations *in vivo.*

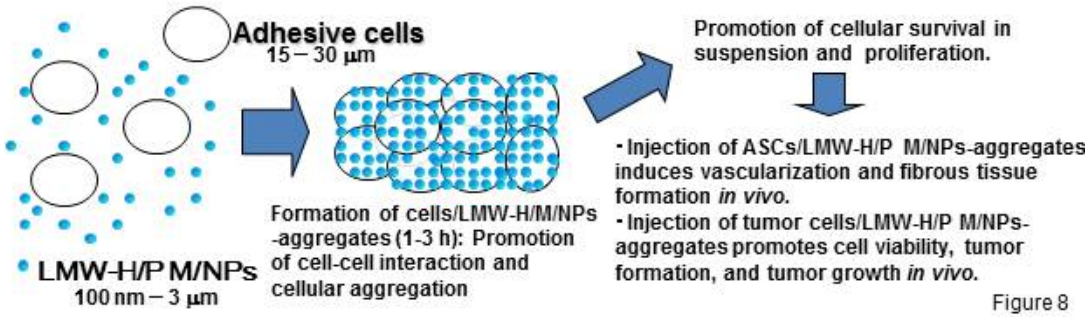

FIGURE 6.8

LMW-H/P M/NPs as Tumor Cell Carriers

Tumor cell transplantation models offer great strategy for cancer research. They include allograft transplantation and xenograft transplantation models. Allograft mouse tumor systems, also known as a

syngeneic model, consist of tumor tissues derived from the same genetic background as a given mouse strain. The xenograft transplantation method involves actual human cancer cells or solid tumors which are transplanted into a host mouse [47]. Therefore, to effectively utilize tumor cell transplantation, the host mouse must possess an impaired immune system similar to nude mice, thereby allowing foreign tumor cells to survive and not be rejected by the host. In both cases of tumor cell transplantation, effective tumor cell carrier systems are required to improve cellular viability and growth as well as decreasing tumor cell rejection by the animals [47].

The LMW-H/P M/NPs also bind to the surface of tumor cells such as Lewis lung cancer cells (3LL), B16 melanoma cells (B16) and human hepatoma cells (Huh7). They promote cell-to-cell interaction, and increase the aggregation of the tumor cells with the LM-H/P M/NPs. These tumor cells/LMW-H/P M/NPs-aggregates substantially promote cell survival and proliferation of the tumor cells *in vitro* as well as reliably induce tumor formation and rapid tumor growth *in vivo* (see Figure 6.9) [48]. Taken together, these data indicate that LMW-H/P M/NPs constitute a new, convenient and effective biomaterial that functions as a tumor cell carrier *in vivo*. The application of LMW-H/P M/NPs as a tumor cell carrier offers a more reliable model in both allograft and xenograft transplantation for cancer research [48].

LMW-H/P M/NPs bind to the surface of Huh7 cells, promote cell-to-cell interaction, and increase the aggregation of the tumor cells with the LMW/P M/NPs. These tumor cells/LMW-H/P M/NPs-aggregates substantially promote cell survival and proliferation of the tumor cells *in vitro* as well as reliably induce tumor formation and rapid tumor growth *in vivo* (see Figure 6.9).

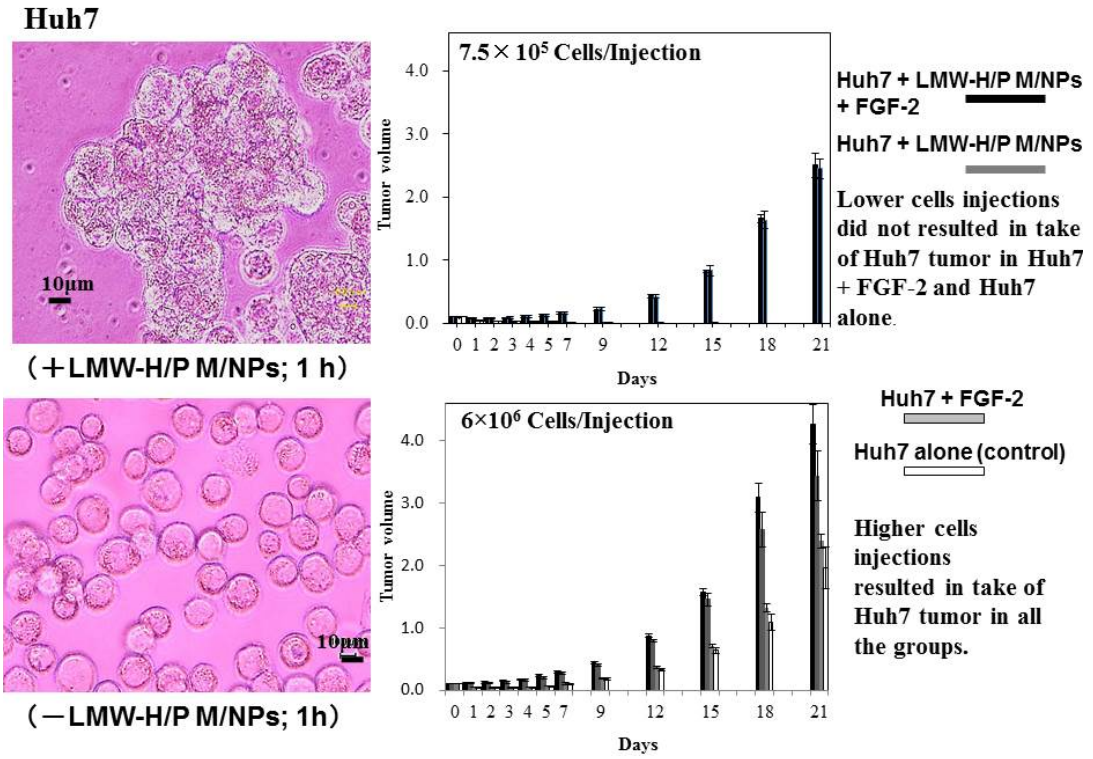

FIGURE 6.9
Huh7/LMW-H/P M/NPs aggregates and tumor formation and growth in nude mice

A Cell Culture System Using LMW-H/P M/NPs-Coated Plates

Various Types of Cell Cultures Using LMW-H/P M/NPs-Coated Plates

The LMW-H/P M/NPs are able to attach to polymeric surfaces such as plastic and glass. The LMW-H/P M/NPs generate a stable paste-like coating through complete drying. It is probable that polypeptides, such as FGF-2, interleukin (IL)-3 and granulocyte/macrophage-colony stimulating factor (GM-CSF), once bound to the LMW-H/P M/NPs-coated plates, are gradually released from the coated surface *in vitro* with a half-life of 4－6 days [27]. Furthermore, LMW-H/P M/NPs-coating could optimally stimulate growth of hMVECs and hDFCs in low FBS (1%) containing culture medium with FGF-2 and growth of hematopoietic cell line (TF-1) with IL-3 and GM-CSF [27] (see Figure 6.10).
Heparinoids bind various GFs and cytokines including FGFs, HGF, VEGF, heparin-binding epidermal growth factor (HBEGF), PDGF, TGF-*β*, GM-CSF, interleukins (*i.e.*, IL-1, IL-2, IL-3, IL-4, IL-6, IL-7 and IL-8), interferon *γ* and macrophage inflammatory protein-1 [13-15]. These GFs and cytokines can potentially be immobilized on the LMW-H/P M/NPs-coated plates. Actually, in addition to FGF-2, IL-3 and GM-CSF described above, we have already observed that FGF-1, HGF, HBEGF, TGF-*β*, human stem cell factor (SCF), thrombopoietin (Tpo) and Flt-3 ligand (Flt-3) could be efficiently immobilized on the LMW-H/P M/NPs-coated plates [31]. Furthermore, the bound GFs to the LMW-H/P M/NPs-coated plates appeared to enhance and to stabilize those biological activities. Thus, LMW-H/P M/NPs-coating provides an excellent biomaterial to immobilize and retain GFs and cytokines for optimal growth of various types of cells with low (no) serum medium (see Figure 6.10).

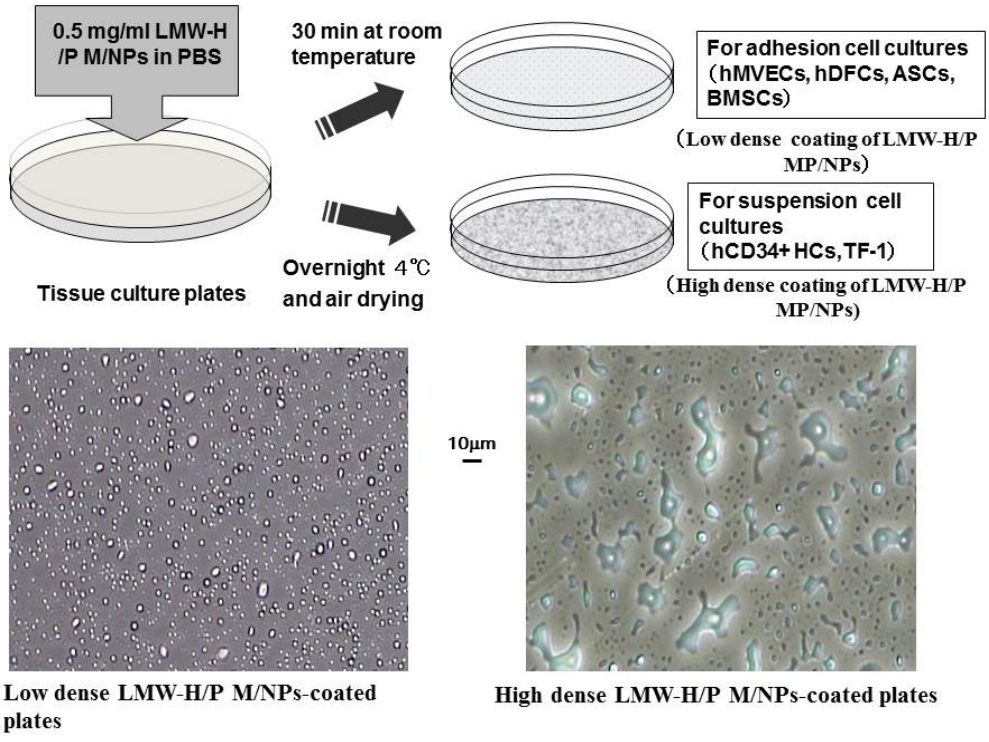

FIGURE 6.10
Preparation of LMW-H/P M/NPs-coated plates

Proliferation of bone marrow-derived mesenchymal stem cells (BMSCs) and ASCs on LMW-H/P M/NPs-Coated Plates

Cell-based therapies such as tissue engineering will benefit from a source of autologous multipotent stem cells, including BMSCs and ASCs. There are two stem cell lineages in bone marrow cell populations, *i.e.*, hematopoietic progenitor cells (HCs) and BMSCs. The BMSCs and ASCs are multipotential, indicating that in culture [49,50] or after *in vivo* implantation these cells can differentiate into a variety of cell types including osteoblasts, chondrocytes, adipocytes, myoblasts [51] and neuronal cells [52]. Furthermore, cultured ASCs secreted significant amounts of angiogenic growth factors such as FGF-2, HGF, PDGF and VEGF at levels that are bioactive [53]. Thus, LMW-H/P M/NPs serve as an effective matrix for cultures of BMSCs and ASCs. The safe and effective expansions of BMSCs and ASCs represent a promising option for tissue engineering strategies.

Most protocols for the expansion of BMSCs and ASCs include high concentrations (10%－20%) of animal serum such as FBS as a nutritional supplement. In some cell cultures, this involves multiple doses of FBS, which raises concerns over possible contamination as well as immunological reactions caused by medium-derived FBS proteins, sialic acid derivatives, etc. [54,55]. Patients may experience problems when undergoing autologous cell-based therapies if a serum other than an autologous serum is used during the culturing of the cells. However, it would be difficult to obtain large amounts of autologous serum from the patient for large-scale autologous cell culture [27-30]. It should be noted that the growth of cultured BMSCs or ASCs on LMW-H/P M/NP-coated plates in combination with FGF-2 and FBS (1－2%) was significantly stimulated, and similar stimulation was coated plates with FGF-2 and 1－2% human serum (HS) prepared from adult bloods instead of FBS [27-30]. Thus, LMW-H/P M/NPs may serve as an effective matrix for cultures of BMSCs or ASCs. The safe and effective expansions of BMSCs or ASCs represent a promising option for tissue engineering strategies.

Proliferation of CD34+ Hematopoietic Progenitor Cells (CD34+ HCs) on LMW-H/P M/NPs-Coated Plates

HCs proliferate and mature in semi-solid media when stimulated by exogenous hematopoietic cell growth factors (HCGFs) such as SCF, Tpo, Flt-3, IL-3 and GM-CSF [31,56,57]. These cells also proliferate in association with BMSCs [29,58,59], although biologically active amounts of HCGFs cannot be detected in stromal culture supernatants [59]. It is possible that HCGFs are synthesized by the stromal cells but remain bound to the stromal cells and/or their extracellular matrix. In fact, it was demonstrated that both natural and recombinant HCGFs, such as IL-3 and GM-CSF, could be adsorbed by heparan sulfate, which is the major sulfated glycosaminoglycan of bone marrow stroma [29,58,59]. Serum-free medium supplemented with large amounts of SCF, Tpo and Flt-3 was reported for expansion of CD34+ HCs [31,60,61]. Although such medium is commercially available (HPGM, Lonza Japan Corp., Tokyo, Japan), it is prohibitively expensive. We demonstrated that recombinant HCGFs such as SCF, Tpo, and Flt-3 were immobilized onto LMW-H/P M/NPs-coated plates, and the immobilized cytokines were stabilized, were activated, and were gradually released into the medium. Those cytokines, once bound, can be presented in the biologically active form to HCs [58,59]. Furthermore, only one-fourth of the concentration of the cytokines recommended by the manufacture was required for maximal expansion of CD34+ HCs on the LMW-H/P M/NPs-coated plates [31,60,61]. These findings have important implications for the use of heparinoid as an artificial matrix for *ex vivo* expansion of HCs with adequate cytokines. The LMW-H/P M/NPs-coating matrix in the presence of lower concentrations of SCF, Tpo and Flt-3 is a convenient and safe material for stable expansion of CD34+ HCs using HPGM without any animal serum [31].

Conclusions

It is recognized in polymer chemistry that positively and negatively charged polymers interact ionically [1,3]. Through these ionic interactions, basic protamine molecules can bind with acidic molecules (LMW-H) to form micro/nano particle complexes. We previously reported that GF-containing LMW-H/P M/NPs, which are 100 nm－3 μm in diameter, can be easily injected [20-22]. Furthermore, the LMW-H/P M/NPs were observed on the protection of FGF-2 and GFs in PRP activity from heat and proteolytic inactivation. These results indicate that LMW-H/P M/NPs serve as an effective microcarrier for various GFs, particularly for the local application of GFs. GFs-containing LMW-H/P M/NPs show a substantial effect to induce vascularization and fibrous tissue formation because of stabilization, activation and gradual release of GF molecules from GFs-containing LMW-H/P M/NPs [20-22].

LMW-H/P M/NPs rapidly bound to adhesive cell surfaces such as ASCs through specific interactions of LMW-H/P M/NPs and various cell surface heparin-binding proteins, can promote cell-to-cell interaction and increase cellular aggregation. The cells/LMW-H/P M/NPs-aggregate formation substantially promoted cell viability *in vitro*. The ASCs/LMW-H/P M/NPs-aggregates induced vascularization and fibrous tissue formation *in vivo* [25]. The LMW-H/P M/NPs, in combination with ASCs, are a new convenient cell carrier and may be a promising novel therapy for inducing vascularization and fibrous tissue formation in ischemic disease (Figure 6.5). LMW-H/P M/NPs also bind to the surface of various adhesive tumor cells, promoting cell-to-cell interaction and increase cellular aggregation with the LMW-H/P M/NPs. The tumor cells/LMW-H/P M/NPs-aggregates substantially promote cell survival and proliferation of those tumor cells *in vitro*, and reliably induce tumor formation and rapid tumor growth *in vivo* [25,48]. Taken together, LMW-H/P M/NPs constitute a new convenient and effective biomaterial that function as a tumor cell carrier *in vivo*. The application of LMW-H/P M/NPs as tumor cell carrier offers a more reliable model in both allograft and xenograft transplantation for cancer research.

The presented method for the optimal proliferation and differentiation of ASCs and BMSCs on LMW-H/P M/NPs-coated plates in low concentration HS (1－2%) supplemented with FGF-2 (5 ng/ml). No animal serum is required in the culture of those cell types. The bound GFs to the LMW-H/P M/NPs-coated plates appeared to enhance and to stabilize those biological activities. The proliferated cells maintained their potential to differentiate into adipocytes and osteoblasts [25,29,30]. Furthermore, the LMW-H/P M/NPs-coating matrix in the presence of lower concentrations of SCF, Tpo and Flt-3 were convenient materials for stable expansion of CD34+ HCs using HPGM without any animal serum [31]. These results suggest a promising cell source, particularly for the preparation of large amounts of ASCs, BMSCs or CD34+ HCs required for cell-based therapies in several clinical fields.

LMW-H, protamine, several GFs and cytokines, and autologous PRP are already in clinical use. Since autologous ASCs, BMSCs or CD34+ HCs are available, the clinical safety of LMW-H/P M/NPs as protein-carrier and as cell-carrier is possible. Furthermore, ASCs, BMSCs or CD34+ HCs can be efficiently expanded as cell sources for regenerative medicines with the use of LMW-H/P M/NPs-coated plates as a matrix without animal serum or feeder cells.

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