インジェクタブル自己硬化型水酸アパタイト／コラーゲン骨ペーストの作製

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博士学位請求論文

(要約)

Fabrication of injectable and self-setting hydroxyapatite/collagen bone paste

(インジェクタブル自己硬化型水酸アパタイト/コラーゲン骨ペーストの作製)

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Our bone has two roles; one is a structural function that supports our body from load and protects important organs, a brain, heart and lungs, against impact. Another is a biochemical function to maintain calcium homeostasis. To realize these two roles, bone has unique chemical composition and nanostructure. The mass composition of our bones is 60 to 70 % of inorganic substance, non-stoichiometric carbonate-containing hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$; HAp) of 20 to 40 nm in length, 20 to 25 mass% of organic substances, mainly type I collagen of approximately 300 nm in length, and approximately 10% of water; *i.e.*, simplified description of bone is a nanocomposite of HAp nanocrystals and collagen molecules, with a primary aligned nanostructure of *c*-axes of HAp nanocrystals and elongation direction of collagen fibers. Secondary structure of bone is a bundle formed by the five nanocomposite fibers. Tertiary structure is found in cortical bone, as an osteon, an annual-ring like structure. Each ring is nanocomposite membrane composed of aligned bundles and direction of bundles is different from the next rings. Bone is constantly metabolized by the bone remodeling process that old and/or cracked parts of bones are resorbed by osteoclasts, bone resorbing cells, followed by formation of new bone by osteoblasts, bone forming cells. A bone resorption or bone formation is also play a important role on the calcium homeostasis.

The bone remodeling process allows bone to self-repair small defects due to a disease or injury with appropriate treatments. However, defects exceeding the critical size of self-repair induce invasion of the scar tissue before bone formation and do not regenerate completely. Therefore, grafting of bone void fillers, to prevent invasion of scar tissue and to aid in regeneration of bones, is generally applied to repair large bone defects. Autologous bone grafting using the patient's own ilium and fibula is a gold standard due to its high effectiveness, because it transplants not only bone matrix but also cells, cytokines and other effective substances to support bone regeneration. Further, it does not show rejection and is eventually incorporated into bone remodeling process to substitute with new bone. Even it seems to have no bad points, it still has several problems, *i.e.*, the need for secondary surgery in healthy parts to collect bones, restriction on the amount, size and shape of the collected bone, and side effects, mainly pain at the harvesting site after the surgery. Therefore, artificial bone void fillers have been being studied and applied to support autologous bone transplantation.

Conventionally, sintered HAp ceramics and bioactive glasses are widely used

because of their high osteoconductivity. However, even HAp nanocrystals in bone are resorbed rapidly by osteoclasts, absorption/resorption of these materials are very slow, and they remain for life-long of patients. Consequently, the remaining materials become a latent risk as a cause of secondary bone fracture in the implantation site, due to the difference in mechanical properties with bone. Therefore, the artificial bone void fillers using bioabsorbable materials to be replaced with living bone have been developed to the latent risk.

To have biological reactions closer to autologous bone, researches on composite of HAp and collagen have been conducted to develop artificial bones, which have bone similar nanostructure and chemical composition. In 2001, prior to above mentioned apatite/collagen composites, Kikuchi *et al*. synthesized a hydroxyapatite/collagen bone-like nanocomposite (HAp/Col) by a simultaneous titration method *via* self-organization between HAp nanocrystals and collagen molecules. The simultaneous titration method is a simple method as written, that starting solutions, $Ca(OH)_2$ aqueous suspension and collagen-added phosphoric acid solution, are simultaneously titrated to a reaction vessel with a maintenance of the reaction temperature at the body temperature, *i.e.* approximately 37 ºC, and controlling pH at 9 in the vessel by addition of the starting solutions through tube pumps with an on/off control. The HAp/Col synthesized by these conditions have the target mass ratio of collagen/(HAp and collagen) from the ratio of starting materials, and its compact shows the highest three-point bending strength among various conditions. The temperature is generally considered as good for collagen fibrillogenesis, and the pH is for HAp, basic calcium phosphate, stable formation as well as a point of zero charge of collagen. Accordingly, collagen fiber formation and HAp nucleation on carboxyl groups on the collagen fibers are simultaneously occurred. Directional relation between the HAp nanocrystals and carboxyl group on the collagen fibers is restricted by their chemical bonding, *i.e.*, *c*-axis of HAp is perpendicular to COO^- face of carboxyl group. Carboxyl groups on the collagen molecule can rotate to any direction but when two or more carboxyl groups bonded to surface calciu3m of one HAp nanocrystal, *c*-axis of the HAp nanocrystal and long axis of collagen molecule become almost parallel. In addition to that, HAp works as adhesive for collagen molecules, because ion concentration of the reaction solution is too low to occur collagen fibrillogenesis. From another point of view, the carboxyl group in collagen induces nucleation and precipitation of HAp, even though the optimal ion concentration

for HAp/Col self-organization is lower supersaturated environment to the homogeneous HAp nucleation. In other words, HAp and collagen in the HAp/Col complement each other in self-organization. The obtained HAp/Col has, therefore, a bone-like nanostructure and chemical composition and is resorbed by osteoclasts followed by new bone formation by osteoblasts, *i.e*. the HAp/Col is completely incorporated into the bone remodeling process. The HAp/Col has attracted attention from the viewpoint of tissue regeneration and biomimesis, and its applied research has been conducted. Among them, the porous body of the HAp/Col is commercially available as Refit® in Japan.

Bone void fillers have variety of shapes to correspond to variety of cases, *i.e*, dense body, porous body, granule and paste. Dense bodies are applied to comparatively load-bearing site and porous bodies are applied to introduce cells and tissues in its body. Granules are filled in various void shapes easily. Paste type fillers are most interesting materials and reduce invasion of surgical operation site as well as completely fit to bone defect. In other words, the paste is an important form enabling effective treatment that cannot be implemented with other material form. Utilization of suitable hardening agents and the HAp/Col as raw material of paste is considered to be one effective solution to respond to the surgeons' demand. Although the HAp/Col is reported as a dense body, a porous body, a granule, and a sheet, a practical self-setting injectable HAp/Col paste has not been reported at present. A HAp/Col paste cannot apply self-setting mechanism of the hydration reaction used in conventional bone cement; therefore, hardening agent is required for preparing it. For example, a method of hardening (gelling) HAp/Col fibers by gelling the added collagen has already been reported. However, this gelling method takes several hours at 37 °C to gelation of collagen, *i.e.* it does not solidify for several hours after injection into the body. Moreover, in order to fabricate the injectable HAp/Col by this method, cross-linking agent besides HAp/Col and collagen solution are required, and it has little strength after gelling; therefore, this approach is considered to be poor in practical use. As another approaches, sodium alginate (Na-Alg) which reacts with calcium ion or acid to gel and (3-glycidoxypropyl)trimethoxysilane (GPTMS) which is one type of silane coupling agents will be considered for hardening agent to prepare a HAp/Col paste.

Aiming at fabrication of a paste type bone filling using HAp/Col, preparation of paste raw materials, condition of the hardening agents and mixing ratio of HAp/Col

and hardening agent were examined, and then the properties of the prepared HAp/Col pastes were investigated. In chapter 2, the optimum mixing conditions of HAp/Col paste using Na-Alg were determined. Furthermore, the influences of additives of organic acid or Ca compound were investigated. In chapter 3, in order to obtain anti-washout property for the HAp/Col–Na-Alg paste, the influence of Ca compound addition was examined in further detail. In chapter 4, preparation of paste using GPTMS was performed and its physical properties were investigated. In chapter 5, a biological investigation of HAp/Col-GPTMS paste was conducted. Finally, conclusion of this thesis was described in chapter 6.

Excellent biological functions of the HAp/Col derive from its nanostructure and chemical composition; thus, conventional hardening systems of HAp bone pastes, based upon *in situ* formation of HAp, are not suit for the HAp/Col because its nanostructure cannot be formed under the hardening systems. Consequently, gradual crosslink of collagen in the HAp/Col with a biocompatible crosslinker and/or fix the HAp/Col powder in a biocompatible and biodegradable hydrogel are possible candidates for preparation of the HAp/Col paste. Collagen is one of the best hydrogel in biological properties but its handling and long gelation time are big demerits for clinical applications. Gelatin, a denatured product of collagen, also has high biocompatibility and gelation abilities; however, gelatin gel cannot be used because it changed into sol at body temperature and temperature control of gelatin sol not to denature collagen molecules in the HAp/Col is also difficult at bed side. Contrarily, Na-Alg has rapid gelation in appropriate calcium salt solution and used for scaffold of regenerative medicine in many researches. The Na-Alg is an acidic polysaccharide contained in marine algae such as kelp, has biocompatibility and biodegradability An alginic acid has a linear structure and is composed of randomly aligned mannuronic acid (M) and guluronic acid (G) monomers. The blocks where G are arranged consecutively form a chelate so called “egg-box structure” by capturing multivalent cations including Ca ions to induce gelation of Alg. This gelation improved anti-decay property of apatite cement in the blood and would give the same effect on the HAp/Col paste. In addition, the Na-Alg has another gelation property via precipitation of alginic acid gel by extrusion of Na^+ in an acidic environment. Further, Alg gel has a biodegradability by exchange of multivalent ions in gel and Na^+ ions in living tissue. In chapter 2, the optimal conditions of preparing HAp/Col–Na-Alg paste was investigated for injection,

and the HAp/Col–Na-Alg paste of optimal conditions was added several kinds of Ca-salt or organic acid as hardening (gelling) accelerator for improving pastes' anti-washout property. The HAp/Col powder of 100-212 μm was prepared from the HAp and collagen mass ratio of 80:20 that was fabricated by the simultaneous titration method, and the HAp/Col paste was then prepared by mixing of the HAp/Col powder and the Na-Alg aqueous solution at several powder/liquid ratios. The paste optimal conditions for injection were determined by results of viscosity test, hardening behavior test and compressive strength test; however, the HAp/Col–Na-Alg paste with optimal conditions immersed in phosphate buffered saline was decayed within few hours. Therefore, Ca compound or organic acid was supplemented to the paste of optimal conditions in order to improve the paste anti-washout property, and properties of the additive supplemented pastes were investigated. The optimal preparation conditions of the HAp/Col paste are that the P/L ratio is 0.6 with the 90:10 mass ratio of HAp/Col powder treated with $CaCl_2$ and low viscous Na-Alg. The prepared paste formed viscoelastic solid by direct injection into $CaCl_2$ aqueous solution. As an additive, organic acid increased paste viscosity very rapidly and Ca compound accelerate formation of egg-box cross-link in comparison to the non-additive paste. The pastes before hardened were decayed by soaking in D-MEM or PBS, some additive increased time to completely decay. In conclusion, the HAp/Col based paste prepared in this chapter would be applied in rapid prototyping with $CaCl_2$ aqueous solution soaking and could be a good candidate for injectable artificial bone with a property to completely incorporate into bone remodeling process after improvement of anti-decay property.

The chapter 2 describes preparation and characterization of injectable HAp/Col pastes utilizing Na-Alg as a lubricant and hardening agent, and the supplementation of small amount of any calcium compounds or organic acids did not improve the fast-setting and anti-washout properties sufficiently. In these calcium compound, insufficient but dose-dependent improvements were observed by supplementation of calcium compounds with a low solubility, *i.e.*, calcium carbonate and calcium citrate tetrahydrate (Ca-Cit). These results suggested that excess supplementation of these calcium compounds or combined supplementation of calcium compound and organic acid would be candidates of the HAp/Col paste which can be applied for practical use. In chapter 3, the influence of large amounts of supplementation of low soluble calcium compounds on the physical properties of the HAp/Col paste utilizing Na-Alg was

investigated. Furthermore, the cytocompatibility of the HAp/Col pastes with large amount of Ca-Cit which obtained anti-washout was evaluated by a proliferation of human osteoblastic cell line, MG-63, cultured with the pastes. The pastes with excess supplementation of calcium compounds showed a gradual viscosity increase and injectability through a syringe with a 1.8 mm inner diameter to apply as injectable bone filler. The HAp/Col pastes showed a washout ratio at less than 10 % in mass by the addition of Ca-Cit at 8 or more times the equivalent reaction amount of Ca^{2+} ion to Na-Alg. All the anti-washout HAp/Col pastes showed a sufficient cytocompatibility; the MG-63 cells proliferated the same as the clinically available HAp/Col. Consequently, the HAp/Col injectable anti-washout pastes might be good candidates for bioresorbable bone filler pastes.

In chapter 3, anti-decay property of the HAp/Col paste prepared with Na-Alg was sufficiently improved by adding Ca-Cit. Meanwhile, Ca-Cit needs 30 mass% to the HAp/Col amount to acquire an anti-decay property, it may cause a decrement of HAp/Col advantages. Accordingly, preparation of HAp/Col paste with GPTMS, which is hardening agent substituting Na-Alg was researched. A GPTMS is one of the silane coupling agents which are commonly reported as functional materials to prepare biomaterials composed of inorganic or organic materials or their composites. The silane coupling agents generally have the property of generating silanol groups by hydrolysis, and then they bond covalently to inorganic substances, and moreover, they form siloxane networks by their self-condensation. In this study, GPTMS had been chosen because epoxy groups in GPTMS bond to amino groups in collagen. In addition, the pH of the GPTMS aqueous solution at 0.1 - 10 vol% is around 4, which is the most stable region without requiring pH adjustment, so this property seems to be advantageous as a liquid component of paste. In chapter 4, HAp/Col-GPTMS pastes were prepared by mixing of HAp/Col powder and GPTMS aqueous solution, and the influences of the GPTMS concentrations and the powder to liquid (P/L) ratios on the physical properties of the pastes were investigated. The pastes with P/L ratios between 0.33 and 1.50 showed good kneading performance and they could be injected via syringe with 1.8 mm inner diameter. Moreover, the pastes with P/L ratio of 0.33 could be injected via 18G needle. The pastes using HAp/Col powder with non-dehydrothermal cross-link showed anti-washout property. Particularly, the G0.1- and G1.0- pastes showed minimum anti-washout ratios with a P/L ratio of 1.00. The HAp/Col-GPTMS pastes after

hardening showed lower young's moduli than conventional bone cements, and they demonstrated viscoelasticity.

The chapter 4 describes excellent physical properties as a bone void filler paste of the HAp/Col-GPTMS paste. The HAp/Col itself is already confirmed its safety and excellent bone tissue reactions; however, biological properties including safety and bone tissue reactions of combination of the HAp/Col and GPTMS have not been investigated. In chapter5, biological properties of the HAp/Col-GPTMS pastes were evaluated by cell culture tests and animal experiments using SD rats and a pig. In detail, initial resorption behavior of the paste was investigated by implantations of the paste into SD rats' tibia. In addition, long-term resorption was studied by a direct injection of the paste into pig tibia. Although increasing the amount of GPTMS inhibited cell proliferation in a cell culture environment, no systemic or local symptoms except for the local inflammation generally observed after surgery were observed during the implantation test. Furthermore, the pastes were resolved by osteoclasts, and were bioresorbable and completely substituted by newly formed bone. Hence, HAp/Col-GPTMS pastes are good candidates for use as novel bioresorbable bone void filler.

Calcium phosphate cements, of which the main component after setting is HAp, have been widely used due to their biocompatibility, injectability and ability to fit irregularly shaped bone defects as well as these self-setting ability. Since the rather low resorption rate of HAp causes a decrease in the new bone formation rate; however, injectable bone void fillers composed mainly of higher bioresorbable materials are being prepared to achieve acceleration in bone formation. The HAp/Col proposed by Kikuchi has a bone-like nanostructure, and it is the first material that is completely incorporated into the bone remodeling process and whose resorption rate can be controlled by the crosslink ratio of its collagen content. Sponge-like porous HAp/Col, sold as Refit® in Japan. Hence, HAp/Col is a promising candidate for use as a bioresorbale bone substitute for other materials besides porous materials. The purpose of this study was to prepare a self-setting injectable HAp/Col paste. For that purpose, preparation of raw materials, kind of hardening agent, raw material mixing ratio and addition of hardening reaction accelerator were studied.

This thesis concluded based on above results that HAp/Col pastes are promising candidates for use as bioresorbable injectable pastes.