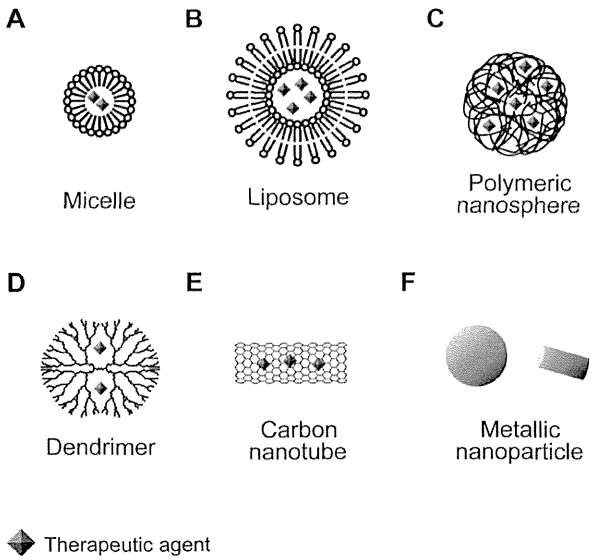

Figure 1. Schematic description of nanoparticle mediated drug delivery systems. **A:** Micelle self assembles from lipids or synthetic macromolecules with hydrophilic heads and hydrophobic tails in an aqueous solution. Placing hydrophobic tails inside, lipid micelles encapsulate hydrophobic therapeutic agents. **B:** Liposome is composed of lipid bilayer. Placing hydrophilic heads outside and inside, liposomes encapsulate hydrophilic solution and therapeutic agents inside. **C:** Polymeric nanosphere, formed from assembly of macromolecular polymers, contains hydrophilic and hydrophobic therapeutic agents. **D:** Dendrimer, composed of macromolecular polymer incorporating therapeutic agents within its structure. **E:** Carbon nanotubes, a cylindrical structure of covalently bonded carbon atoms, can carry therapeutic agent inside. **F:** Crystalline metals in nano-meter scale possess therapeutic or imaging function themselves.

cules with a controlled near monodisperse three-dimensional architecture emanating from a central core. Polymer growth starts from a central core molecule and growth occurs in an outward direction by a series of polymerization reactions, which determines the size of dendrimers starting from a few nanometers. Cavities in the core structure and folding of the branches create cages and channels for the incorporation of therapeutic agents (Figure 1D).[1,8] Carbon nanotubes belong to the family of fullerenes and consist of graphite sheets rolled up into a tubular form. The diameter and the length of single-walled nanotubes may vary between 0.5–3.0 nm and 20–1000 nm, respectively. Therapeutic agents are attached on either the inner or outer tube wall surfaces, which are the so-called filling or wrapping modes of binding, respectively (Figure 1E).[21] In contrast, metallic nanoparticles are functional themselves. Ion oxides are usually prepared as alkaline co-precipitation of Fe^{2+} and Fe^{3+} salts in water in the presence of a suitable hydrophilic polymer such as dextran or poly(ethyleneglycol). This yields an iron core of 4–5 nm in diameter, which is hexagonally shaped and surrounded by dextran or poly(ethyleneglycol) molecules to form superparamagnetic iron oxide particles (SPIO) (60-150 nm) as contrast agents for magnetic resonance imaging (MRI).[9,22,23] Gold nanoparticles possess the unique photodynamic properties of absorbing near-infrared light and emitting light and heat, and have been tested as a cancer photothermal therapy. Also, gold nanoparticles have been conjugated with various therapeutic agents and targeting moieties, and act as drug carriers (Figure 1F).[1]

Intravital kinetics of nano-DDS may be diverse; their behavior within the biological environment is affected not only by size, but also by their chemical makeup and morphology. However, the most important determinant of the physiological behaviors of nano-DDS is the size, as discussed below.

Physiological Behavior Of nano-DDS

Nano-sized materials (10–300 nm in diameter) tend to remain in circulation avoiding renal excretion, which is a primary feature of nano-DDS. While circulating in the blood stream, nano-DDS extravasates from the vasculature with enhanced permeability, such as angiogenic vessels in tumors, and vessels in organs after ischemia-reperfusion, which is an important mechanism that affects tissue distribution of nano-DDS.[2,5,24-26] The neovasculature in tumors lacks functional lymphatic vessels as well as enhanced vascular permeability, causing an accumulation of nano-DDS in the tumor microenvironment.[2] This phenomenon is referred to as enhanced permeability and retention (EPR) effects of nano-DDS.[27]

Recognition and incorporation by the mononuclear phagocyte system (MPS, also called the reticuloendothelial system), namely neutrophils, monocytes, and macrophages in the blood, liver, spleen, and lymph nodes is also a common physiological behavior for nano-DDS, which may affect the blood circulating time and tissue/cell distribution.[28-31] One of the first clinically approved nano-scale DDS therapies was a liposomal formulation of doxorubicin, a cytotoxic drug used for cancer chemotherapy. During its development, encapsulation of doxorubicin in liposomes prolonged its blood half-life compared to the free drug, but this was found to be unsatisfactory because of entrapment by MPS.[32] The addition of polyethylene glycol (PEG) to the surface of nano-DDS was shown to reduce the recognition of MPS, and in the case of doxorubicin liposomes, the addition of PEG reduced the clearance from the bloodstream, as well as cardiotoxicity, which is a major adverse effect.[32] On the other hand, incorporation into MPS itself is one of the mechanisms of drug delivery, especially when treating an inflammatory disease, such as atherosclerosis. Several studies employ this mechanism for the imaging and treatment of atherosclerosis, target-ing inflammatory monocytes/macrophages in atherosclerosis.[31]

The above-mentioned mechanisms underpin a 'passive-targeting' of nano-DDS on diseased organs or MPS. By contrast, an 'active-targeting' strategy employs a specific targeting structure on nano-DDS, which binds to the target molecule that is specific for a certain disease process. One good example is tumor-specific expression of folate receptor, which is targeted by addition of folate to the surface of nano-DDS.[33,34] For the treatment of cardiovascular disease, vascular endothelial cells can be targeted by an antibody for platelet endothelial cell adhesion molecule (PECAM-1). Liposomes loaded with a superoxide dismutase mimetic were conjugated with PECAM-1 antibody, which increased the delivery of liposomes to the pulmonary vasculature, and successfully enhanced the anti-inflammatory effects against endotoxin-induced acute lung injury.[35] Vascular cell adhesion molecule-1 (VCAM-1) is another molecule for targeting vascular endothelial cells.[36] There are still numerous opportunities for an 'active-targeting' strategy to find specific target molecules for a certain disease process, the effectiveness of which may be investigated in future studies.

Nano-DDS for Atherosclerosis

Atherosclerosis is one of the oldest diseases and was even present in ancient times, and atherosclerotic CVD, such as acute myocardial infarction (MI) and stroke, are still major causes of death and disability worldwide.[37] Atherogenesis begins as an endothelial dysfunction; when subjected to oxidative, hemodynamic, or biochemical stimuli (from smoking, hypertension, or dyslipidemia) and inflammatory factors, endothelial cells change their permeability and the expression of adhesion molecules to promote the recruitment of circulating monocytes and cholesterol-containing LDL particles. Inflammation and biochemical modifications ensue, causing endothelial and smooth-muscle cells to proliferate, produce extracellular matrix molecules, and form a fibrous cap over the developing atheromatous plaque.[38] Narrowing of the arteries by atheromatous plaque limits blood flow causing ischemic vascular diseases such as angina pectoris, whereas the rupture of a fibrous cap causes abrupt cessation of blood flow via thrombosis, resulting in end organ damage such as AMI. A series of pathological analyses in patients with sudden coronary deaths showed that ruptured coronary lesions typically have large necrotic cores and a disrupted fibrous cap infiltrated by macrophages with an expression of matrix metalloproteinases (MMP),[39,40] suggesting that inflammatory macrophages contribute to the destabilization and rupture of atherosclerotic plaques, resulting in a thrombotic occlusion of the coronary artery and AMI. Among the molecular and cellular mechanisms of long-term atherogenesis leading to plaque destabilization, (1) enhanced vascular permeability, (2) expression of adhesion molecules in endothelial cells, (3) accumulation of inflammatory monocytes ($Ly6C^{high}$ $CCR2^{+}$ in mice, $CD14^{high}CD16^{-}$ in humans)/macrophages, and (4) expression of proteases that facilitates plaque destabilization are potential mechanisms for drug delivery, and thereby targets for imaging and therapeutic intervention of atherosclerotic cardiovascular disease including coronary artery disease.[13,23]

Computed tomography (CT) is a modality which has been extensively used for the imaging of coronary arteries in clinical practice. Recent advances in multi-detector CT that can simultaneously acquire a volume of images have enabled us to acquire a complete coronary angiogram in less than a minute. Iodine-containing contrast agents are used to image coronary arterial lumens, identifying the narrowing of coronary lumens by atherosclerotic lesions. Recent clinical studies also describe 'unstable plaque' by coronary CT, as defined by a luminal narrowing that is associated with expansive or positive vessel remodeling (PR), and low-attenuation plaques (LAP), which suggests a high risk for plaque rupture and acute coronary syndrome.[40,41] It is conceivable that imaging of plaque macrophages using nano-DDS is a more specific approach to identify high-risk unstable plaques. N1177 is a crystalline iodinated aroyloxy ester covered with a polymer, with a mean diameter of 259 nm.[42] N1177 is incorporated into cultured macrophages, raising the iodine content by approximately 100-fold compared with conventional CT contrast, and delivery to lesion macrophages was histologically confirmed in an atherosclerotic rabbit model. Finally, intravenous use of N1177 resulted in the detection of macrophage-rich arterial walls in animal models by CT,[42] and is being tested in clinical trials.[43]

MRI produces tomographic images with high soft-tissue contrast and spatial resolution, and is another important imaging modality for cardiovascular disease. SPIO are strong nanoscale contrast enhancers for MRI, and because of their remarkable biocompatible and biodegradable properties, several SPIO particles with diverse sizes, coatings, and targeting abilities have been applied for imaging of the inflammatory process of atherosclerosis and myocardial infarction.[22,23] Macrophages internalize SPIO, altering the local magnetic field and thus producing the T2 shortening effect as visualized by signal reduction. Monocrystalline iron oxide nanoparticles (MION)-47 have an approximate 5-nm diameter core of SPIO coated with an approximate 10-nm-thick dextran layer and have a long blood half-life, which facilitates their accumulation in macrophages of atherosclerotic plaques. Incorporation of MION-47 in macrophages in atherosclerotic lesions was confirmed histologically in a rabbit model. Increased Fe content causes loss in T2 signals, which enables negative imaging of macrophage-rich lesions.[44] Targeting VCAM-1 was also tested for the imaging of atherosclerotic lesions in a mouse model, and showed enhanced delivery of SPIO by VCAM-1 targeting.[36]

Our group has tested polymeric PLGA nanoparticles as a nano-DDS for the treatment of atherosclerotic plaque destabilization.[13] PLGA is a biodegradable material and is approved by the FDA and the European Medicine Agency for various DDS in human clinical use.[5,6] In atherosclerotic ApoE-deficient mice fed a high fat diet and infused with angiotensin II, neutrophils and monocytes in the peripheral blood and the aorta incorporated FITC-loaded PLGA nanoparticles (FITC-NP) 2 hours after injection, determined by flow cytometric (FCM) analysis (Figure 2A). FITC-NP accumulated in atherosclerotic lesions in the aortic arch (Figure 2B), and fluorescence microscopy analysis revealed that FITC signals are observed mainly in the macrophages of atherosclerotic plaques, which is partially blocked by depletion of monocytes by clodronate, suggesting that PLGA nanoparticles are delivered to atherosclerotic lesions partly through monocyte/macrophage phagocytosis, and directly via enhanced permeability in the lesions. Weekly intravenous treatment with PLGA nanoparticles containing the HMG-CoA reductase inhibitor pitavastatin reduced circulating inflammatory Ly6Chigh monocytes, macrophage infiltration to the atherosclerotic lesions in the aortic root, and plaque destabilization in the brachiocephalic arteries (Figure 2C, D).[13] Another group tested liposome-dependent delivery of siRNA against chemokine receptor CCR2, and showed successful delivery to spleen, bone marrow, and liver, and the inhibition of monocyte/macrophage recruitment to the aorta and atherosclerotic lesions.[31]

The above-mentioned studies consistently showed successful delivery of nano-DDSs including crystalline metal, polymers, and liposomes to monocytes/macrophages, mainly through physiological entrapment by MPS, and these nano-DDSs can be applied to both the imaging and treatment of atherosclerosis.

Nano-DDS for Acute Myocardial Infarction and Ischemia-Reperfusion Injury

Acute MI is a major cause of death and heart failure worldwide.[37,45,46] In patients with ST-segment elevation acute MI (STEMI), early reperfusion therapy is a standard strategy to limit MI size; however, recent cohort studies suggest that the

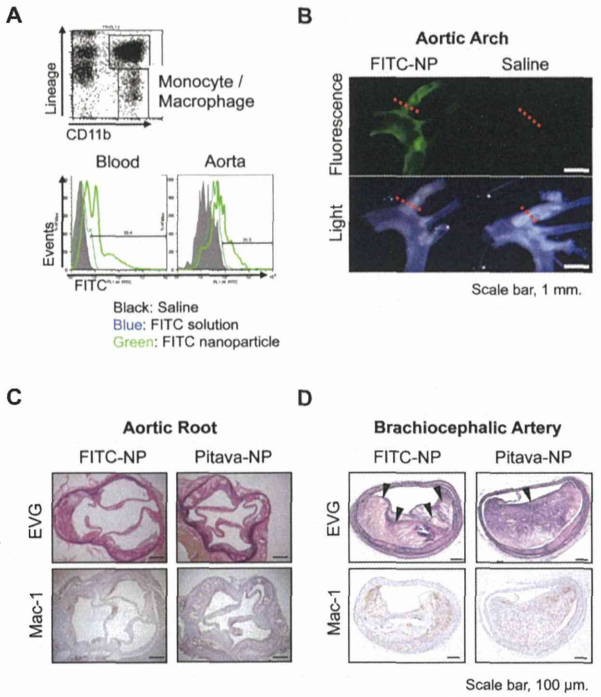

Figure 2. Nano-DDS for atherosclerosis. **A:** Flow cytometry of circulating leukocytes 2 hours after intravenous injection of PLGA nanoparticles encapsulated with FITC (FITC-NP). The histograms demonstrate FITC uptake by monocytes in the blood and the aorta. **B:** Fluorescent and light micrographs of isolated aortic arch 24 hours after intravenous injection of FITC-NP or saline. **C, D:** Photomicrographs of atherosclerotic plaques in aortic root (**C**) and brachiocephalic arteries (**D**) stained with EVG or Mac3. Arrows indicate disrupted/buried fibrous caps.

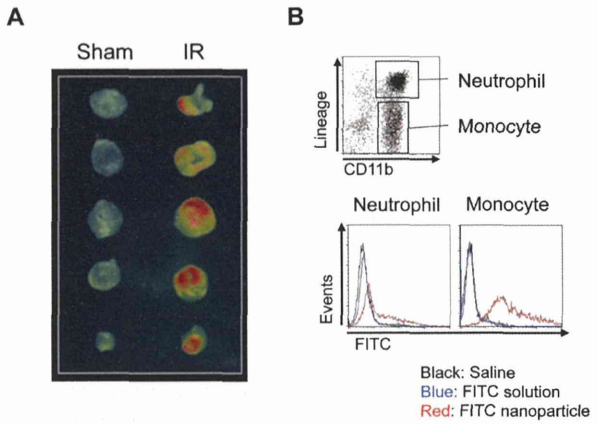

Figure 3. Nano-DDS for myocardial IR injury. **A:** Fluorescence reflectance imaging of the sections of heart in sham operated mouse or myocardial IR mouse. Accumulation of indocyanine green-loaded nanoparticle was noted in an IR heart after intravenous treatment at the time of reperfusion. **B:** Flow cytometric analysis in the leukocytes of an IR heart showed the incorporation of FITC-loaded nanoparticles was noted in macrophages and neutrophils.

mortality of MI patients has not improved despite significant reductions in door-to-balloon time in the last decade.[47] It is widely recognized that the reperfusion of coronary arteries paradoxically induces cardiomyocyte death, known as myocardial ischemia-reperfusion (IR) injury, for which several new therapeutic strategies are under investigation.[48,49] Myocardial IR induces the generation of reactive oxygen species (ROS), calcium overload, and rapid pH correction, all of which cause mitochondrial injury through the opening of the mitochondrial permeability transition pore (MPTP) and the activation of mitochondrial outer membrane permeabilization, leading to the necrosis and apoptosis of cardiomyocytes in the early phase (in several minutes) of IR injury. In the late phase of injury (over several hours), myocardial inflammation contributes to cardiomyocyte apoptosis and the healing of infarcted myocardium.[5,48,49]

The genetic ablation of cyclophilin D, a key regulatory molecule for MPTP opening, markedly reduces IR injury in mice.[50] In addition, intravenous administration of cyclosporine A (CsA), an inhibitor of cyclophilin D, at the time of reperfusion reduced myocardial IR injury in animals and in an early clinical trial in patients with acute MI.[51] The activation of pro-survival kinases, PI3K/Akt and Erk1/2 that are referred to as reperfusion injury salvage kinases (RISK), is another potential therapeutic target to attenuate reperfusion-induced necrosis and apoptosis and thus reduces MI size. Several pharmacological agents including statins and erythropoietin analogs have been shown to reduce MI size in animal studies. The recruitment of neutrophils and inflammatory monocytes is an established phenomenon after myocardial injury, and several animal studies have suggested a role of inflammation as a therapeutic target in IR injury.[31,49,52] However, several clinical trials on pharmacological cardioprotection for myocardial IR injury have failed to demonstrate a positive impact on clinical outcome in STEMI patients.[48,49] One possible explanation for the failure of current clinical trials is an insufficient drug delivery during a limited interventional time window, while administered at the time of reperfusion. Therefore, from a clinical perspective, it is feasible to apply an effective DDS that facilitates delivery to the sites of IR injury during reperfusion, a clinically feasible time point.

Nano-DDS may accumulate in injured tissues, including IR myocardium, where vascular permeability is enhanced.[5,24-26] Incorporation by circulating monocytes and other MPS is another mechanism targeting inflammation after myocardial injury.[31] Thus, nano-DDS may be feasible for myocardial IR injury targeting ischemic myocardium and inflammatory monocytes. Takahama, *et al* have tested PEGylated liposome-dependent delivery of adenosine during myocardial reperfusion, and found that liposomes attained a higher adenosine concentration in the ischemic myocardium and showed superior cardioprotection and less systemic hypotensive effect compared with free adenosine in a rat model.[26] Leuschner, *et al* have tested liposome-dependent delivery of siRNA against CCR2, and showed successful delivery to spleen, bone marrow, and liver, and the inhibition of monocyte/macrophage recruitment to the heart after IR. Treatment with siRNA-CCR2 successfully reduced MI size.[31]

We have examined the efficacy of PLGA nanoparticles as a DDS for myocardial IR injury. In a mouse model of myocardial 30-minute ischemia-reperfusion, PLGA containing indocyanine green-loaded PLGA nanoparticles was traced with fluorescence reflectance imaging. PLGA nanoparticles were found exclusively in the ischemic myocardium (Figure 3A). Flow cytometric analysis showed the incorporation of FITC-

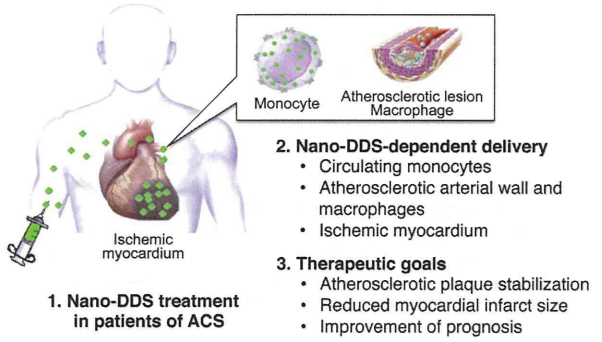

Figure 4. Perspective of nano-DDS-mediated treatment for acute coronary syndrome. Nano-DDS-mediated treatment for cardiovascular disease comprises 1) timely intravenous injection of nano-DDS, 2) nano-DDS-dependent drug delivery to circulating monocytes, atherosclerotic arterial wall and macrophages, and ischemic myocardium, and 3) therapeutic goals including atherosclerotic plaque stabilization, reduced myocardial infarct size, and improvement of patient prognosis.

loaded PLGA nanoparticles in macrophages and neutrophils in the IR heart (Figure 3B). Nano-DDS-dependent delivery to inflammatory cells and ischemic myocardium is a desirable property for the treatment of myocardial IR injury. PLGA nanoparticles and other nano-DDS including liposomes will be tested in clinical trials in the future to test whether they will enhance therapeutic efficacy through targeting specific mechanisms of myocardial IR injury.

Summary and Clinical Perspective

In this review, we summarized the properties of selected nano-DDSs and their preclinical studies in animal models of cardiovascular diseases, namely atherosclerosis and myocardial infarction. Current applications of nano-DDS for cardiovascular disease utilize mainly 2 major mechanisms of drug delivery, enhanced vascular permeability and incorporation by MPS, which underpins enhanced drug delivery for circulating monocytes, atherosclerotic arterial wall and macrophages, and ischemic myocardium. These properties of nano-DDS may be applicable for the treatment of acute coronary syndrome (coronary plaque destabilization and acute myocardial infarction) (Figure 4).

Although preclinical studies have reported nano-DDS have therapeutic effects on atherosclerotic plaque stabilization and myocardial IR injury, there is a wide variety of opportunities to combine nano-DDSs and various therapeutic agents, including chemicals, nucleotides, peptides, and others, which may expand the potential of current pharmacotherapy for several cardiovascular diseases. Possible application of nano-DDS in other cardiovascular diseases may include pulmonary hypertension,[12,17] vein graft disease,[53] and therapeutic neovascularization for clinical limb ischemia[15,18,54] and coronary stents.[14,19] We have started a phase I/IIa investigator initiated clinical trial in Kyushu University Hospital to test the efficacy of PLGA nanoparticle-mediated delivery of pitavastatin in patients with critical limb ischemia (UMIN000008011). Future clinical trials conducted in the next decade may prove the safety and efficacy of nano-DDS for cardiovascular diseases.

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重症肺高血圧症用ナノ粒子製剤の実用化と臨床試験

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はじめに

重症肺高血圧症は生活の質（QOL）の悪化をもたらす生命予後不良の希少難治性疾患であり、かつ、効果的治療法が無い疾患です。ホスホジエステラーゼⅤ阻害薬、エンドセリン受容体拮抗薬などの血管拡張薬が新しい治療法として導入されていますが、その効果は限定的（5年生存率<50%）です。従って、肺小動脈病変の進行を阻止し、さらには、治癒に導く事の出来る、効果的かつ安心安全の医薬品の実用化が期待されています。

スタチン封入PLGAナノ粒子製剤の開発

重症肺高血圧症に対する新規治療法開発のために、生体吸収性高分子ポリマー（PLGA）製ナノ粒子を用いたドラッグデリバリーシステム（DDS）を開発しました。このナノDDSによって、治療薬を肺動脈病変（肺動静脈平滑筋細胞、炎症性細胞など）へ安定送達がさせる事が出来ます。私たちは、LDL-コレステロール低下薬として世界で広く用いられているスタチン（HMG-CoA還元酵素阻害薬）の血管保護作用に注目しました。スタチンには、LDL-コレステロール低下作用とは独立した多面的作用として血管内皮細胞機能改善作用、血管平滑筋細胞増殖抑制作用、抗炎症作用を有しています。

基礎研究の結果、（1）ピタバスタチンがもっとも強力な血管保護作用を有すること、（2）培養ヒト肺動脈平滑筋細胞においてピタバスタチン封入ナノ粒子製剤はピタバスタチン単独と比較して、より優れた細胞増殖抑制作用を示すこと、（3）本製剤の静脈内投与によって肺高血圧症モデルの病態が著明に改善すること、を明らかにしました（図1）。

臨床への橋渡し研究

ピタバスタチン封入PLGAナノ粒子製剤を臨床応用するために、GLP（Good Laboratory Pracitce）準拠での各種安全性試験、安定性試験、治験薬のGMP（Good Manufacturing Pracitce）準拠での製造等を行ってきました。その結果、治験薬としての安全性に大きな問題はないことを明らかにしました。規制当局である独立行政法人医薬品医療機器総合機構（PMDA）との2回にわたる対面助言を行い、現状の非臨床試験の成果をもとにして、医師主導治験（健常成人男性を対象とした第Ⅰ相試験）を実施する事に科学的、倫理的に問題はないことを合意いたしました。本治験は、健康成人男性日本人志願者を対象として、本製剤の安全性および薬物動態を検討することを目的としています。九州大学病院ARO次世代医療センターの支援を受けて治験の準備を進めています。平成26年度中には医師主導治験が終了し、その安全性を明らかにする予定です。医薬品として承認申請を得て、実用化に結びつくまでの道のりは未だ長く、少なくとも5年以上を要すると思われます。

本剤が実用化されれば、重症肺高血圧症に対する革新的低侵襲治療法となり、患者のQOL・生命予後の改善や早期社会復帰を可能とする高効果・低副作用の低侵襲医療が達成できるでしょう。日

本発の革新的低侵襲ナノ治療が創出される点で臨床的意義は大きいと期待されます。

また、本剤は肺高血圧症以外の難治性肺疾患（特発性間質性肺炎・肺線維症、びまん性汎細気管支炎）や閉塞性肺疾患、肺ガンの治療にも応用できることから、重要性が高いとされています。

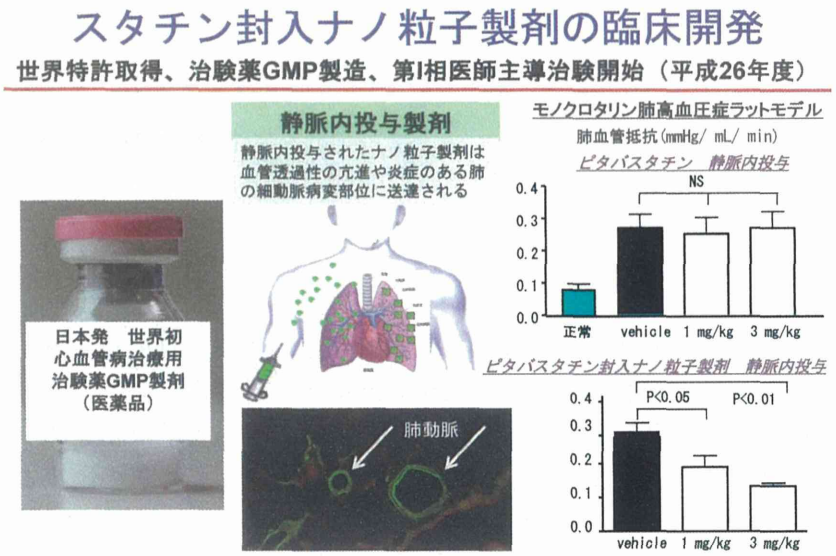

図1

本研究は革新的技術の開発を阻害している要因を克服するため、研究資金の特例や規制を担当する部局との並行協議などを試行的に行う「先端医療開発特区」、いわゆる「スーパー特区」の研究課題の一環として行われていることから、政府からもその進捗状況、研究開発内容について注目されています。

平成25年 5月19日、安倍晋三内閣総理大臣が九州大学病院と医学研究院を視察のため訪れました。有川総長、久保病院長、片野医学研究院長等の挨拶を受けた後、本研究概要説明をいたしました。安倍総理自ら実験装置の前に立ち、日本発世界で最初のナノ粒子製剤を手に取り、ご確認されました。また、日本の国策として難病の先端医療研究の重要性について、活発な意見交換を行いました。

さいごに重症肺高血圧症という難病で希少疾病に対する新規医薬品を臨床開発し、実用化するまでにはいくつもの大きなハードルをクリアしていく必要がありますが、一刻も早く本疾患で苦しまれている患者様やそのご家族様に本製剤を還元し、生命予後改善、QOL向上をめざして、一心に取り組みたいと思っております。

江頭健輔教授による説明