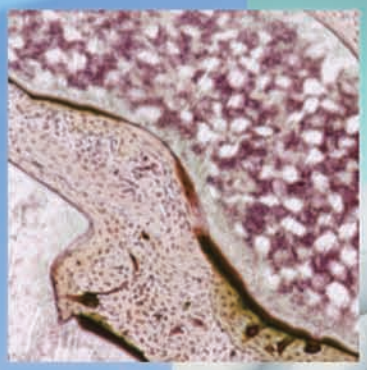
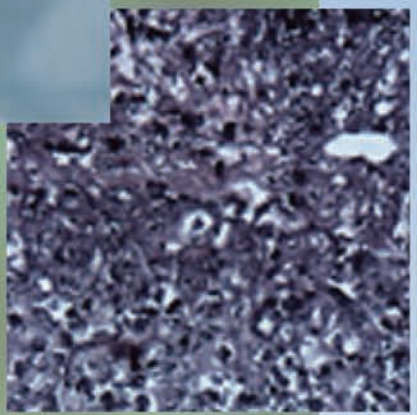
Bone and Joint Regeneration Technology





Bone and Joint Regeneration Technology

Collaboration between Medicine and Engineering Promotes the Process of Practical Application

Tetsuro Moriya
Collaboration coordinator

Medical technology for regeneration of bones and joints

Regenerative medicine has attracted increasing interest in past several years. The regenerative medicine in general includes diverse topics, ranging from clinical medicine and biotechnology for cell and tissue, cultivation to biomaterials and rehabilitation in the functional regeneration process. In this pamphlet, we focus on regeneration to bones and joints, "regeneration engineering," which comprises the interface between medicine and engineering.

In Japan, the juvenile population is rapidly decreasing while the labor shortage is becoming especially on prominent (Figure 1). To cope with the situation, we require an environment where seniors can overcome their declining physical and mental function and other disabilities in order to keep their activity in society (Figure 2). The most concrete among the technologies which address such demands is the bone and joint regeneration engineering. Regenerative medicine, in which no issue can be resolved by each elemental technology, is a typical comprehensive technology. Only cooperation of researchers of various fields can realize the medicine. The system commonly referred to as "medicine-engineering collaboration" needs to demonstrate its capacity in practical application including users, without ending in mere sloganeering by researchers.

Development of research at the National Institute of Advanced Industrial Science and Technology (AIST)

AIST sets forth "Realization of safe and effective health care through precise diagnosis and regenerative medicine" as a major strategic target in its "Research Strategy for the Second Period" published in April 2005. The bone and joint regeneration engineering the highest success through effective cooperation between medicine and engineering. Originally, engineering has dealt with rigid objects. When man started using tools, Engineering emerged from grinding rocks and shaving wood, and has been developped. Meanwhile, medicine deals humans, and thus centers on soft biological elements. Bones and joints, however, are distinctive in their position. As animals move and travel as individual beings, they are required to fully utilize mechanics. In the field of engineering, bones and joints can take advantage of the developments in conventional material and mechanical engineering, such as materials, mechanical properties and moving mechanisms. Meanwhile, In the fieled, the situation is also encouraging, with significant developments in bone tissue and cartilage regeneration, at cellular and tissue level in

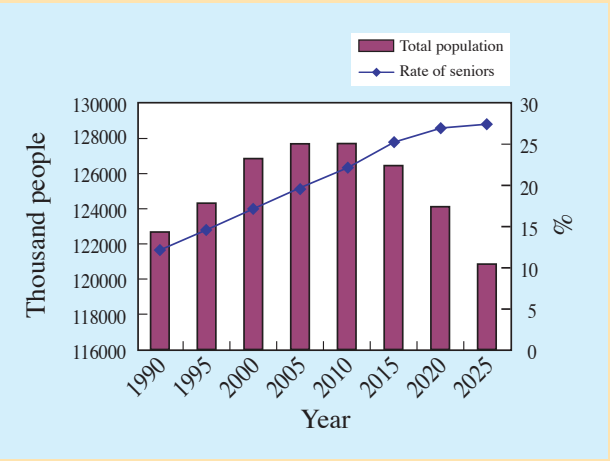


Figure 1: Future Estimate of Total Population and Rate of Seniors
Rate of seniors: Population over 65/ Total population

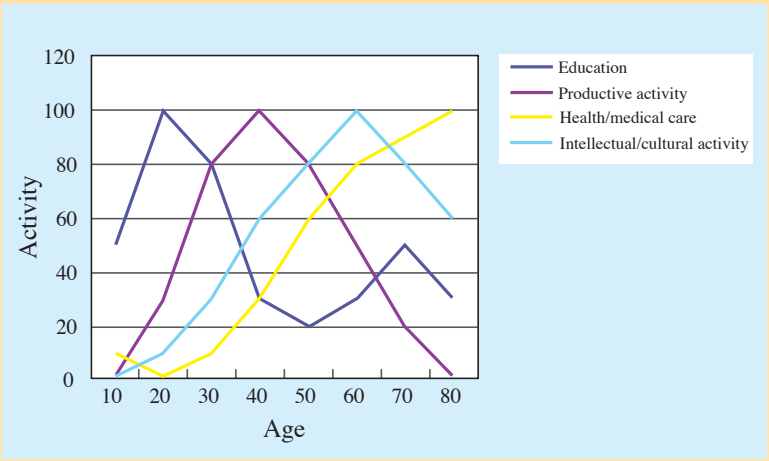


Figure 2: Dynamics of Fluctuating Factors in Lifetime Activity
In the future, regenerative medicine will become a key factor in the health/ medical care for securing activity of the aging society.

addition to new treatments in orthopedics.

Under such cir cwnstances, collaborative system amang diversed fields of AIST is very effective. Though we don't have a hospital, AIST has vast assets of researchers in diverse fields including life sciences, nanotechnology, materials, manufacturing, and standard fields, and fosters fusional research, where medical researchers also participate actively. AIST also boasts wide-rang of channels for collaboration with research institutes of the Ministry of Education, Culture, Sports, Science and Technology and the Ministry of Health, Labour and Welfare, as well as many hospitals, and is thus able to promote practical application of new technologies on a clinical level.

The topic featured here, effectively demonstrates the characteristics of cooperation and fusion, with the contributors of the articles coming from four different research units. It is my hope that these research as, will progress substantially to achieve clinical application.

Our Expectations towards AIST

Motoi Suwa,
Director, Research Institute, National Rehabilitation Center for Persons with Disabilities

As this future articles in regarding “Bone and Joint Regeneration Technology,” I will be published in AIST TODAY would like to express our great expectations towards AIST’s regeneration technology and all researchers involved in it.

National Rehabilitation Center for Persons with Disabilities is an institute under the Ministry of Health, Labour and Welfare. It was established to contribute to the welfare for the handicapped, through improving the QOL (Quality of Life) of persons with physical disabilities and encouraging their self-help and social participation. At the center, doctors, registered nurses, physiotherapists, occupational therapists, kinesipathists, speech-language-hearing therapists, orthoptists, medical social workers and prostheticians offer diagnosis and treatment as well as medical rehabilitation to persons with disabilities (those who have physical disability certificates) or those at risk of becoming physically disabled. The center also conduct training aiming at their successful rehabilitation into daily life and work. Research Institute conducts research and development on various rehabilitation-related issues with coordination of various areas including medicine, engineering, behavioral science, social science, and welfare engineering for . In order to enable social participation by disabled persons, it is also vital that we consider the human and physical environment surrounding the individual as well as social environment factors such as legal systems.

Recently, various technologies for treatment in regenerative medicine have been attracting interest. Of these, bone and joint regeneration technology is an area of great interest to those of us involved in rehabilitation. As decline in bone and joint functioning directly affects human behavior and thus has a large impact upon QOL, the technology is a crucial measure against the growing rate of disorders such as osteoporosis in the aging society. Although regeneration technology of human bodily function is, backed by sophisticated research, its success is also affected by how well the collaboration between medicine and engineering is achieved. In medical treatment using artificial bones and joints, different methods of rehabilitation and prognostic management are required according to the degree of functional recovery. Thus, pre- and post-treatment cooperation by doctors and engineering researchers is indispensable. Realization of an effective treatment and functional recovery requires backing by a comprehensive system of technology from various fields. As AIST covers very wide range of engineering fields, it serves as an elite research institution capable of catering to these demands. As we anticipate AIST’s great success through medicine-engineering collaboration, we also hope to further strengthen our cooperation with AIST in the future.

Bone and Cartilage Regeneration Technology Using a Patient's Own Mesenchymal Stem Cells

Hajime Ohgushi and Noriko Kotobuki,
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Bodily tissue vital to healthy living: bone and cartilage

As Japan is faced with an aging society and a declining birthrate at a level without parallel in the world, issues are arising regarding disorders particular to senior citizens. Osteoarthritis - a disease in which joint cartilages gradually deform - is one of these diseases, and it is a major factor impeding everyday activities of seniors.

Bone and cartilage are the tissues in our bodies which are vital to movement. Now, do bones and cartilages, when impaired, heal naturally as does a cold? The answer is "No." Although the bone has a natural self-repair capability (the ability to heal itself), as seen where a fractured bone heals when fixed in a cast, self-repair is difficult in the case of compound fractures, and in some regions of the body, may result in non-union (the bone does not connect normally). Cartilages have an extremely poor self-repair capability thus do not heal naturally. Figure 1 compares conventional bone transplantations with regenerative therapy[1]. As the Figure indicates, conventional treatment methods of bone and cartilage damage involve using biomaterials such as artificial bones and joints as replacements or transplanting bone/cartilage from other regions of the body. However, these methods require considerations for the useful life of artificial components, and also subject healthy parts of the body to damage.

Stem cells exist in adults as well

Our research team is working to develop technologies which hopefully will contribute to society in the area of "regenerative medicine", in an effort to come closer to resolving the issues above. For example, we implement comprehensive studies covering the entire process from *in vitro* regeneration of bone and cartilage to their transplantation into the body of the patient. Figuratively comparing regenerative medicine to growing a plant, we are planting the seed (stem cell) in the flowerbed (patient) to make various flowers (tissue, organs) bloom (regenerate). Further, in some cases, we plant the seed (stem cell) with fertilizer (differentiation-inducing factor) in the seedbed (test tube) and grow it to a seedling (for example, tissue-engineered bone created from cells), then transplant the seedling to the flowerbed (patient). This article mainly illustrates the process of creating tissue-engineered bone.

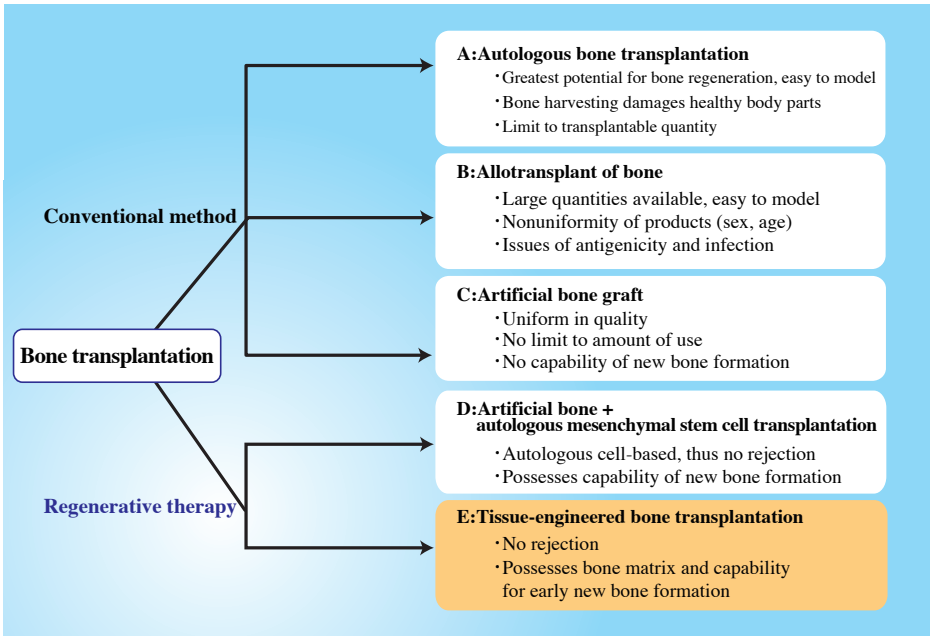

Figure 1: Comparison of conventional methods and regenerative therapy in bone transplantation

The stem cell, as the term "stem" - meaning trunk - in its name implies, refers to a cell in a state (an undifferentiated state) prior to specialization (differentiation) into various cells which constitute different tissues. Well-known stem cells include the embryo stem cell (ES cell) obtained from the fertilized ovum, and the mesenchymal stem cell (MSC) found in bone marrow. These cells, when cultured with the appropriate differentiation-inducing factors, have the potential of differentiating not only into bone and cartilage, but into every type of tissue or organ, including nerves and liver (Figure 2)[2].

The undifferentiated stem cell is commonly believed to exist only in babies and growing children, however, our studies have found it to exist in the bone marrow taken from a patient in his/her 80s[3]. This revelation means that a patient is able to receive regenerative therapy using his/her own bone marrow at any age. Viewing potential clinical application of this technique to patients, the possibility of using the patient's own bone marrow becomes extremely significant, especially compared to ES cells which pose ethical hesitations, and also in terms of safety.

Regenerating bones and cartilages *in vitro*

Although MSC exists for certain in bone marrow, its percentage is a mere 0.01-0.1% of

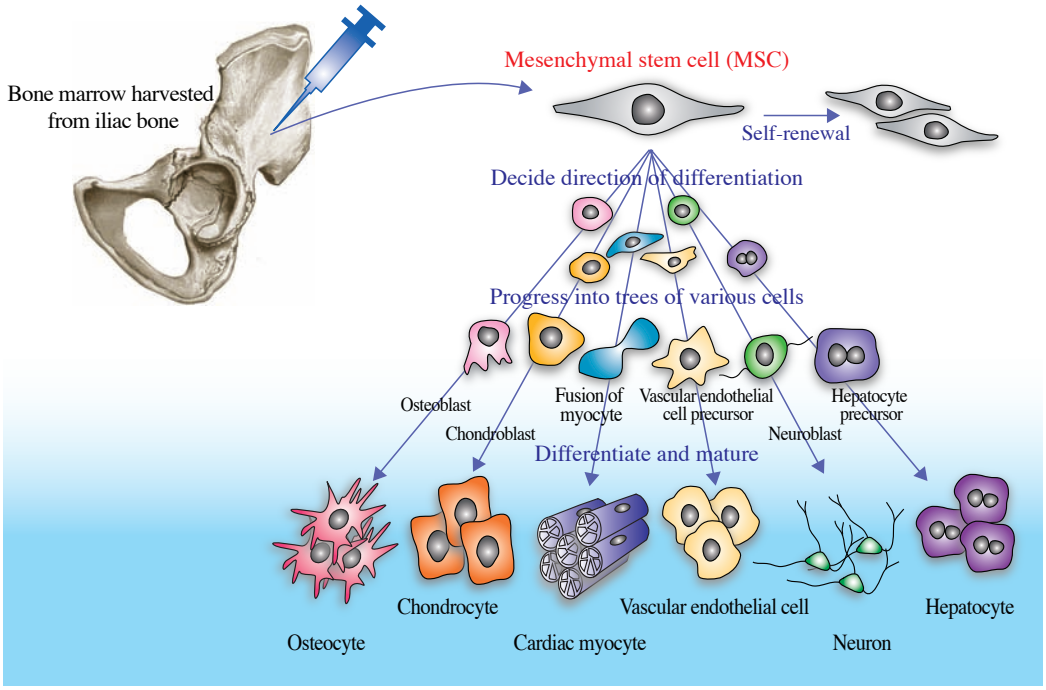

Figure 2: Differentiation tree of MSC
MSC found within bone marrow differentiates into various cells including bone and cartilage when cultured *in vitro* in the presence of the appropriate differentiation-inducing factors.

all marrow cells. Thus regenerative therapy which uses large quantities of MSC would consequently require enormous volumes of bone marrow, the harvesting of which would be all too unrealistic. Given this fact, we had been working to multiply MSC from the bone marrow *in vitro* while keeping its differentiation ability intact and have succeeded, through culturing, in securing the number of cells required for regenerative therapy[4]. As shown in Figure 3a, the MSC is spindle-shaped in the culture dish[5], but when cultured in the presence of a differentiation-inducing factor, differentiates into the constituent cells of bone and cartilage (called osteoblasts and chondrocytes, respectively) (Figure 3b)[6].

When osteoblasts differentiate, they produce collagen type I and an enzyme called alkaline phosphatase, known as an osteogenic differentiation marker. Then, a mineral consisting mainly of hydroxyapatite - a component made of calcium phosphate and which constitutes bones - precipitates around the osteoblasts. The clusters such as indicated by the arrow in Figure 3b are this mineral. In addition, as seen in Figure 4, staining confirms that chondrocytes produce large quantities of collagen type II and a glycoprotein called proteoglycan. By producing these proteins, cartilages are able to fulfill their key function of retaining large amounts of water to serve as cushions in the joints.

In this way, stem cells differentiate into specialized cells and produce extracellular substances such as different type of collagen to form (regenerate) tissue within a test tube. In other words, bone and cartilage tissue can be formed from undifferentiated cells through *ex vivo* (active) culture procedures.

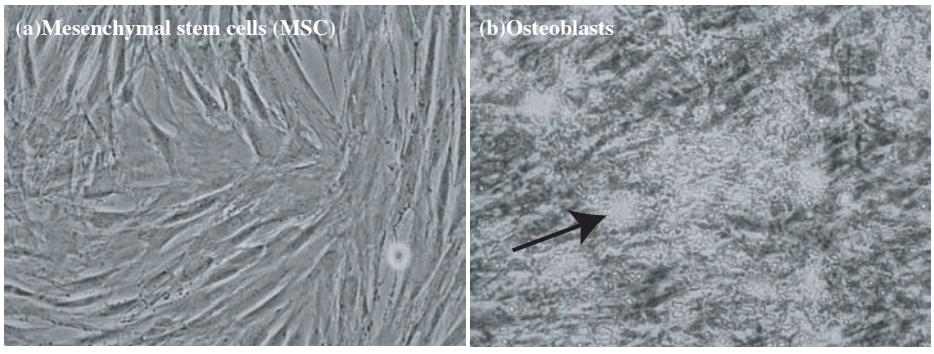

Figure 3a: Shape of MSC: MSC is spindle-shaped as shown
Figure 3b: Shape of osteoblasts differentiated from MSC: Arrow indicates precipitated mineral.

Preparing the regenerated bone/cartilage to a state suitable for transplantation

As described above, bone and cartilage can be regenerated *in vitro*. However, they must be further combined with biomaterials in order to become suitable for transplantation into the body, the reason being that whereas the body is three-dimensional in construction, the bone and cartilage tissue created *in vitro* from cells can

only form in a two-dimensional structure. One may think it easier to simply create a three-dimensional structure *in vitro* using cells alone in the first place. However, using the current technology, it is as yet too difficult for us to create three-dimensionally structured tissue, of a size and function suitable for transplantation, solely from cells. Thus, in order to compensate for any insufficiencies in bodily functions and strength, biomaterials which are easily adaptable to cells and the body are used.

The term biomaterial refers generally to materials used within the body or in direct contact with biological components such as cells or proteins. Biomaterials used for bone regeneration include ceramics such as hydroxyapatite, calcium phosphate and alumina, while those for cartilage regeneration include collagen sponge and polylactic acid, a biodegradable polymer. Past studies have revealed that porous substances, such as hydroxyapatite, calcium phosphate and collagen sponge, serve as effective scaffolds for cells to proliferate and differentiate. Especially as these biomaterials possess multitudes of tiny internal hollows for cells to penetrate, they allow cells to regenerate tissue not only on the surface but internally as well and can be progressively replaced by the new tissue!

As mentioned earlier, biomaterials are sufficiently effective in treatment when used alone and are thus used in clinical practice. To them we have added the tissue-regeneration capability of cells, and have developed a material for transplantation which combines the benefits of both. Since 2001 up to present, we have already applied the technique in about 70 cases in collaboration with university hospitals.

Bone and joint regeneration using the patient's own MSC

Our research group possesses a facility (Cell Processing Center) for culturing cells of a quality suitable for transplantation to patients. In the facility, we have a biological clean room of high air cleanliness where operations can be carried out under sterile conditions (Figure 5).

Figure 6 shows the flow of clinical application research conducted at this facility. Once the patient's bone marrow and blood are transferred to the facility from the university hospital, the serum which is to be used as the nutrient in the cell culture is separated from the blood to make a cell culture medium. The bone marrow is then cultured in a flask using the cell culture medium, and the MSC multiplies. In bone regeneration, this MSC is seeded onto a biomaterial such as alumina and cultured for about 2 weeks in a culture medium in the presence of the osteogenic differentiation-inducing factor (Figure 7a).

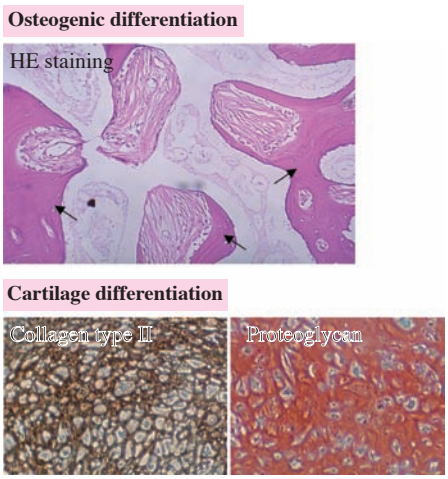

Figure 4: Image of stained bone and cartilage tissue produced by induced differentiation on biomaterial
Bone tissue is stained pink by HE staining as the arrow indicates. In cartilage tissue, collagen type II is stained brown by antibody staining and proteoglycan is stained red by safranin O staining.

Figure 5: Clean room
Bone marrow harvested from the patient is handled in an extremely clean work environment.

The result is a hybrid, of the cell which produced large amounts of extracellular substances and the biomaterial, or in other words, the tissue-engineered bone(Figure 7b). This tissue-engineered bone is known to further induce new *in vitro* bone formation. Once the absence of any contamination during culture is confirmed, the tissue-engineered bone is transplanted into the patient. In the case of cartilage creation, the multiplied MSC is mixed into a gel substance consisting of collagen and then seeded onto collagen sponge (Figure 7c). Whereas in the case of bone regeneration, MSC must be induced *in vitro* to differentiate into bone tissue before transplanting into the patient[6], in cartilage regeneration, MSC is known to be sufficiently effective when transplanted as is. *In vitro* induced differentiation is not required, thus the MSC is used for transplantation in a relatively short time following harvesting of the bone marrow.

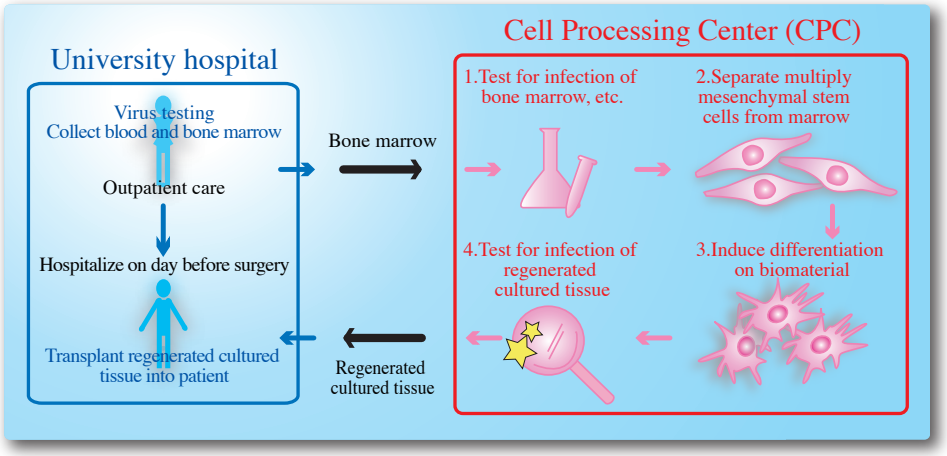

Figure 6: Flow of clinical application research conducted in collaboration with a university hospital

Future developments

Roughly four years have passed since we pioneered transplantation of tissue-engineered bone in the world. Although follow-up is as yet short-term, we are maintaining extremely good results, with no occurrence of adverse effects such as inflammatory reactions or infection. As described above, we are now able, through regenerative medical technology, to create bone and cartilage tissue which possesses the same structure and function as living bones from bone marrow collected through minimum invasion and without sacrificing the patient's healthy own tissues. The era for this technology to be applied to treatment of various bone diseases has arrived. Further, cartilages, which had poor prospects for natural healing within the body now hold new potential for healing through regenerative medicine.

In the future, by utilizing MSC's ability to differentiate into various tissue-constituting cells, we can anticipate clinical application of this technology to regenerate diverse tissues and organs, in addition to treating bone and cartilage disorders.

Unfortunately, our current regenerative therapy has not matured to the level which may render it acceptable for use in every medical institution. Our team believes that in order to propagate this therapy, we must make further progress in basic research as a matter of course, but also make it our important role to implement standardization of regenerative therapy and promote development of the equipment to be used for culture and evaluation (systems) in partnership with universities and corporations.

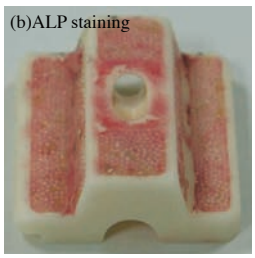

Figure 7a: Seeding MSC onto alumina artificial joint
Figure 7b: Osteogenic differentiation confirmed by ALP staining of tissue-engineered bone
Alumina was seeded with MSC, and osteogenic differentiation was induced to produce this tissue-engineered bone. The red staining represents the parts which are active as bone.
Figure 7c: Collagen sponge seeded with MSC for cartilage regeneration

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Mosaic Artificial Bones
Free Design and Manufacture of Artificial Bones by Aggregating Microscopic Units

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Introduction

As regenerative medicine becomes prominent, artificial bones are regarded as interfaces for actively appreciating life, rather than as mere prosthetic appliances. Porous ceramic is attracting the most interest[1] as an artificial bone material, which will fulfill the demands of regenerative medicine.

In an ideal mechanism of porous artificial bones in the body, the artificial bone takes biogenic factors such as cells and growth factors within its fine pores, and the cells are transformed into tissue. The artificial bone eventually disappears, and is replaced by the tissue. It is known that geometric factors, such as shape, size, and distribution of pores in the artificial bone, have a large effect on the rate of bone regeneration. However, the factors which actually contribute to bone regeneration have not been elucidated, because the geometry of pores in a typical porous ceramics is in wide diversity. Other issues include the absence of definite assurances regarding interconnectivity (linkage) of the pores, as well as large variations among production lots. Limited moldability and poor workability of ceramics also cause difficulty in practical application.

In order to resolve these issues, we have developed a new manufacturing process called the "mosaic artificial bone" method. Features of this process are the following:

1) by aggregating microscopic units, artificial bones can be designed in any shape, and

2) a network of interconnected gaps between the artificial bone units can be created in the aggregate. The shape of the interconnected spaces can be controlled by selecting artificial bone units. If a structure which promotes bone formation, *i.e.* "a bone conductive structure", is introduced in the artificial bone unit, each unit wo rks as the minimum unit with full function. For example, if one bone conductive structure is implemented per unit, "one per unit volume" distribution of the structure is assured over the mosaic artificial bone. In this way, the mosaic artificial bone method provides a platform for designing the shape and "bone conductive structure" of artificial bones.

Fabricating the mosaic artificial bone using spherical apatite beads

The mosaic artificial bone is made by filling hydroxyapatite beads (HA beads, approx. 1 mmϕ) into a cylindrical cell (5 mmϕ × height 5 mm) as shown in Figure 1[2]. Each HA bead has cylindrical through-holes (approx. 300 μmϕ) as the "bone conductive structure".

As aggregated spheres do not have closed space, the inter-bead gaps and the through-holes form a "completely interconnected pore network". The overall volume fraction of the pore network was estimated to be 47.7 ±1.9% by using microfocus X-ray CT. As the biogenic factors can easily penetrate into the network, satisfactory bone formation is expected. It is also possible to form the mosaic artificial bone through injection therapy, as the HA beads can be easily ejected through an injection needle.

The inter-bead gaps, in addition to through-holes, are the key to designing, interpreting, and understanding the macrospace structure of the artificial bone. Figure 2 shows the inter-bead gaps of the HA beads close packing. The gap structure which becomes tighter nearing

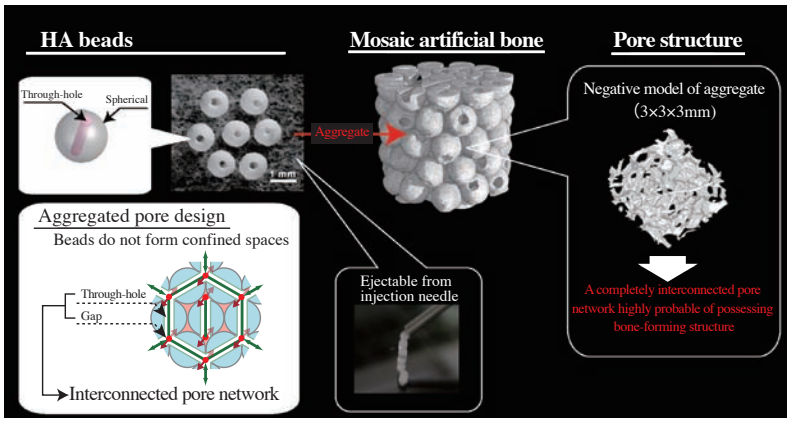

Figure 1: Mosaic artificial bone made using spherical artificial bone units (HA beads)
HA beads can be aggregated into any arbitrary shape, upon which they form a completely interconnected pore network. The 1 mm ϕ HA beads can be ejected from a 16G injection needle. The image of the HA bead aggregate is a 3D-CG model based upon microfocus X-ray CT data.

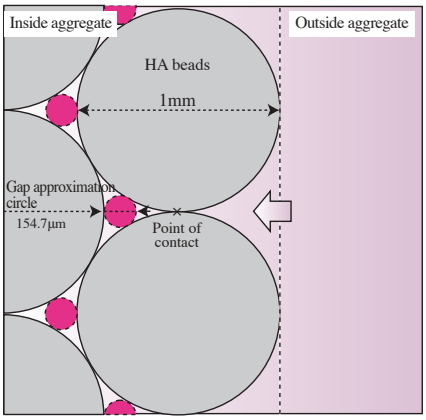

Figure 2: Pattern diagram of HA beads of 1 mm diameter at the closest packing structure

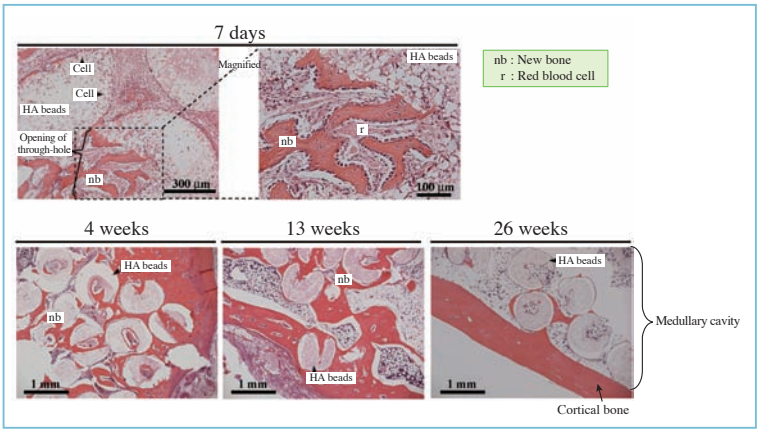

Figure 3: Tissue specimen demonstrating bone formation ability of through-holes and HA beads gaps
By filling a bone-deficient region, of diameter 5 mmϕ × height 5 mm made in the proximal tibia of a healthy male SPF rabbit (12 weeks old), with HA beads, a completely interconnected pore network was formed by the bead gaps and through-holes. It is also possible to fix the HA beads in the aggregate.

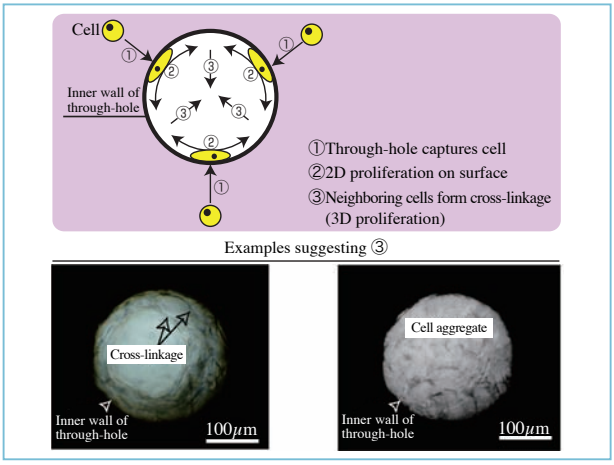

Figure 4: Conceivable cell proliferation mechanism within the through-hole, and image of cells within through-hole suggesting this mechanism

the contact point of the beads is a characteristic not found in conventional artificial bones. The diameter of circle inscribing the gap between 1mm spheres is 154.7 μm. Size of inscribed circle can be controlled by diameter of beads.

Figure 3 shows bone formation ability of the HA bead aggregate in an animal. Seven days after implantation to the proximal tibia in a rabbit, new bone (nb) tissue (membranous ossification), a characteristic of the natural healing process of bones, and red blood cells (r), which suggest angiogenesis, were observed inside the through-holes. Cells were also found inside the beads (7 days). Extensive new bone formation was observed, 4 to 13 weeks after the implantation. After 13 weeks, a boundary was beginning to be formed between the cortical bone and the medullary cavity due to intrusion of bone marrow.

Evaluation of HA bead through-hole diameter

Through-holes of 200-300 μm diameter are thought to be suitable, but is it right? In order to determine the optimum through-hole size for bone regeneration, a evaluating method for bone formation in the holes is studied using HA beads. The HA beads of various through-hole sizes are immersed in cell suspensions, and the numbers of cells in the through-holes and the subsequent proliferation and differentiation are observed. The relation between hole size and bone penetration was investigated.

Most significant cell proliferation was observed with the HA beads of 226 μmϕ through-hole, so far. The beads also showed the highest tissue density (number of cells/surface area of beads) 5 days after the immersion. Cell clusters block the through-holes after 5 days or longer cultivation (Figure 4). These results suggest that cell proliferation in the 226 μmϕ through-holes occurs not only along the inner wall. We can propose a proliferation mechanism where neighboring cells form cross-linkage, which in turn offers a new scaffold for further cell proliferation.

Summary

In this article, our studies on the mosaic artificial bones with a core competence of "unitilization of artificial bone" and related technologies have been discussed. Using this manufacturing process, artificial bones with completely interconnected pores can be produced in any shape. The method can also be potentially applied to artificial bone formation by injection therapy. Artificial bone units are proposed as a touchstone to investigate correlation between pores and bone regeneration.

Regenerative medicine requires cooperation across many fields. The view seems plausible that lack of scaffold materials causes the delay in clinical application. In future, we would like to respond to such frustrations through research of artificial bones, and contribute to regenerative medicine through material development.

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Construction of Large-Scale Three-Dimensional Cartilage Tissue Using a Microgravity Bioreactor

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A new technique for cartilage regeneration

There is a demand for the rapid establishment of cartilage regeneration technology to treat joint disorders such as osteoarthritis (Figure 1). However, owing to obstacles during the *ex vivo* cell culture that cause the engineered tissue to become necrotic, the technique for regenerating a large-scale cartilage tissue has not yet been achieved. In an effort to resolve the setbacks of conventional cartilage regeneration technology and render it applicable to large-scale three-dimensional cartilage tissue regeneration, we have developed a new technique using a bioreactor which simulates a microgravity environment with few obstacles for the cell.

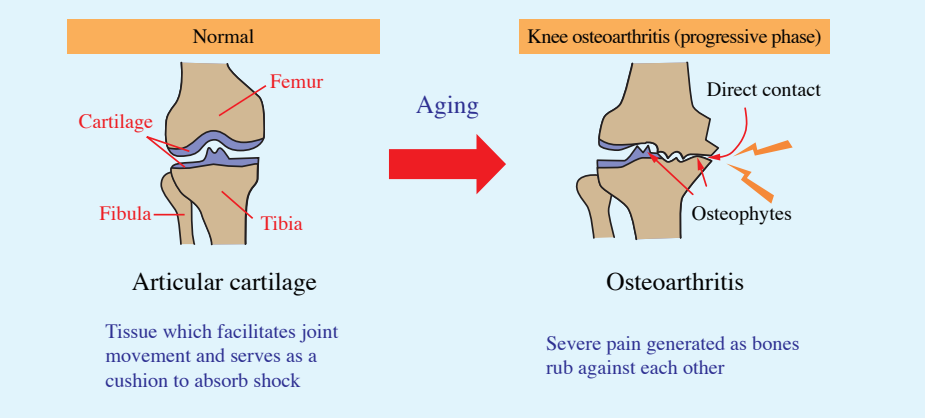

Figure 1: Articular cartilage and osteoarthritis

The bioreactor escapes the effect of earth's gravity

A technology for regenerating lost cartilage tissue using mesenchymal stem cells derived from the patient's own bone marrow is one of the anticipated new methods for treating cartilage disorders. The mesenchymal stem cell possesses high abilities to proliferate and differentiate into various tissue including bone, cartilage, adipocytes and ligament. If cultured three-dimensionally in the presence of an appropriate differentiation-inducing factor, it can differentiate into cartilage tissue (Figure 2). However, when the culture is conducted *ex vivo*, the earth's gravity causes the cells to sink to the bottom of the dish so that the engineered tissue can only form in a two-dimensional sheet structure. Cartilage cells, also dedifferentiate into fibroblast-like cells when cultured two-dimensionally. Due to these difficulties, the methods actually performed include three-dimensional culture using artificial biomaterials, or spinner cultures, but even so, other problems remain such as impaired penetration of the culture solution into the tissue when the cells aggregate at high density, or cell damage due to the stress of agitation causing the engineered tissue to become necrotic. As a method to resolve the problems, a three-dimensional culture in an environment close to zero gravity, namely a microgravity environment, was conceived. The rotating bioreactor, RWV (Rotating Wall Vessel) (Figure 3), was developed to enable cell culture under microgravity. The RWV is a circular vessel (container) equipped with a gas exchange membrane on the backside. When the vessel rotates, the direction of gravity on the cells constantly changes, thereby successfully simulating a microgravity environment of a hundredth of the earth's gravity on a time-average basis. As the vessel

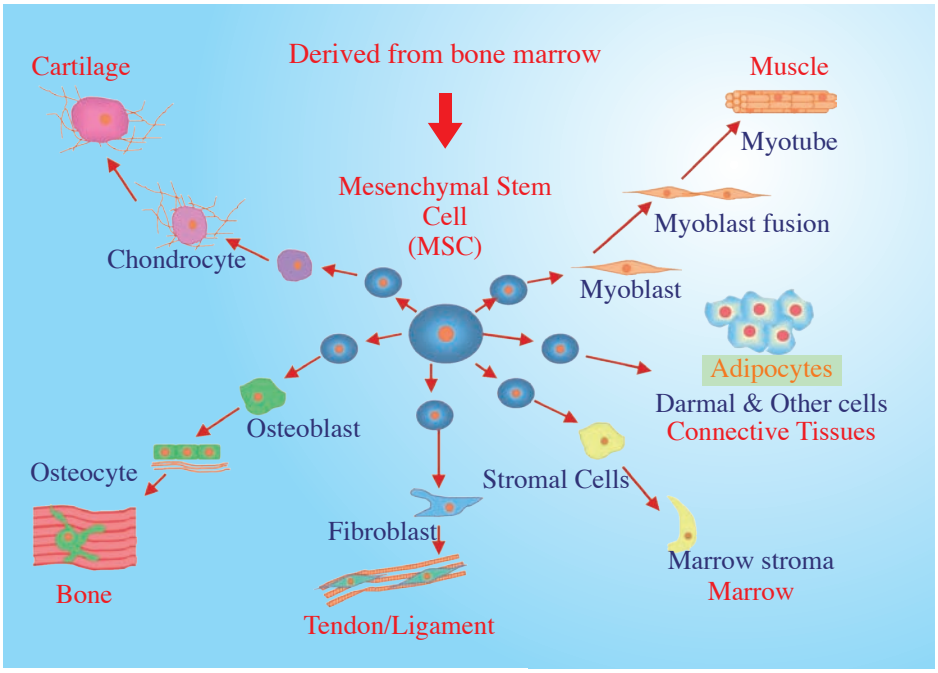

Figure 2: Differentiation process of mesenchymal stem cells into various tissues

rotates, the cells do not sink to the bottom, but gradually form a three-dimensional cluster in a state freely suspended in the culture solution.

Success in formation of large-scale, homogenous three-dimensional cartilage tissue

As an initial step, we have attempted to construct cartilage tissue from rabbit marrow cells using the RWV bioreactor. Marrow cells were harvested from the long bone of a Japanese white (JW) rabbit (10 days old) and multiplied by culture for 3 weeks under normal conditions, after which they were cultured rotationally in the RWV bioreactor in a culture solution containing cartilage-inducing factor TGF-β. After 4 weeks, we succeeded in the formation of a large-scale, homogenous three-dimensional cartilage tissue of major axis 1.5 cm by minor axis 0.8 cm, having a strength of one half to a quarter of natural cartilage in the body (Figure 4). By comparison, in the pellet culture where masses were merely agglutinated through centrifugation, the strength was poor and the only tissues obtained were necrotic inside. We prepared tissue sections and performed safranin O staining to stain the cartilage matrix. These results, as well as the fact that mRNA expressions of aggrecan (a representative large-scale keratan sulfate/chondroitin sulfate proteoglycan found in cartilage tissue) specific to cartilage and collagen type II were observed, enabled us to confirm the tissue to be cartilage. We transplanted the cartilage-like tissue developed in the RWV bioreactor into a model of a rabbit knee joint with full-thickness defect and monitored subsequent developments. Through observations by the naked eye, safranin O staining of the tissue section and amounts of expression of aggrecan and collagen type II, we confirmed excellent cartilage formation and strong binding with the host cartilage and bone. Thus in the above transplantation model using rabbit, the results were extremely favorable.

Future plans

In order to cater to various forms of cartilage disorders, we are currently investigating various types of scaffold materials to which cells may attach. We are also using the RWV bioreactor to reconstruct cartilage tissue from marrow cells harvested from patients, obtained thorough informed consent, and investigating culture methods suitable for each individual patient.

At present, in a method being attempted, chondrocytes are harvested from autologous cartilage tissue, cultured, and then transplanted. However, there is a limit to the number of cells which can be harvested when using autologous cartilage, and damage to healthy cartilage can not be avoided. Meanwhile, using marrow cells offers a large advantage as they include mesenchymal stem cells which can be cultured and multiplied to obtain large numbers of chondrocytes. In our quest to enable restoration of large-scale cartilage loss,

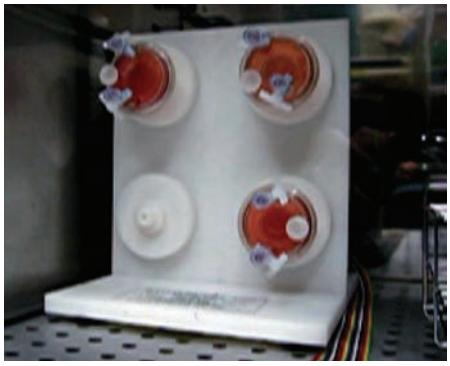

Figure 3: RWV (Rotating Wall Vessel) bioreactor

we have organized joint research schemes with the Biomaterials Center of the National Institute for Material Science, Department of Orthopaedic Surgery, the University of Tsukuba, and Suzuka University of Medical Science, where we promote diverse research aimed at achieving clinical application.

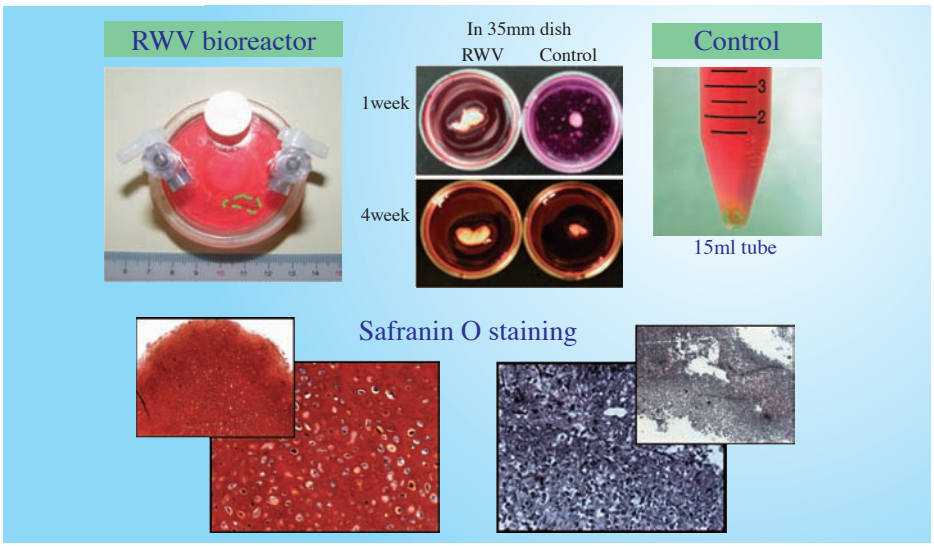

Figure 4: Top left: Rabbit marrow cells are cultured in the RWV bioreactor. Cartilage tissue is being formed (inside green wavy line).
Top right: *In vitro* culture for the control
Top center: Photo observation in 35-mm dish (unstained)
Bottom left: Cartilage tissue is stained red by safranin O
Bottom right: The *in vitro* culture does not stain

Biomaterials for Osteoporosis Therapy

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The number of osteoporosis patients in Europe, the United States and Japan is said to total about 75 million, of which Japan alone is responsible for roughly 10.75 million (estimate of year 2000). According to WHO estimates, the number of cases of fractured neck of femur (fracture of the narrow hourglass-shaped region of the femur near the hip) caused mainly by osteoporosis is 1.3-1.6 million worldwide, and is expected to increase to 6 million by 2025. Considering the rise in average life expectancy, 4 of these 6 million cases in particular are expected to occur in the Asian region including Japan.

Drug products such as calcium, bisphosphonate, estrogen, steroids, calcitonin and vitamin K_2 are currently being used clinically in osteoporosis therapy worldwide, while sodium fluoride and sodium monofluorophosphate are presently under examination by the United States Food and Drug Administration (FDA). In the case of humans, other factors such as magnesium, zinc, and copper are effective in preventing bone resorption and in promoting bone formation. Recent knowledge suggests that, vitamin D, calcium, zinc and copper deficiencies are implicated in bone loss of elderly males and females exceeding 10 years post menopause while the bone loss of females up to 10 years post menopause. A clinical study on osteoporosis patients who were administered zinc (15.0 mg/day), manganese (5.0 mg/day) and copper (2.5 mg/day) in addition to calcium (1000 mg/day) over 2 years revealed these trace minerals in addition to calcium to be effective in preventing deterioration in bone density (J. Nutr. 124: 1060-1064, 1994).

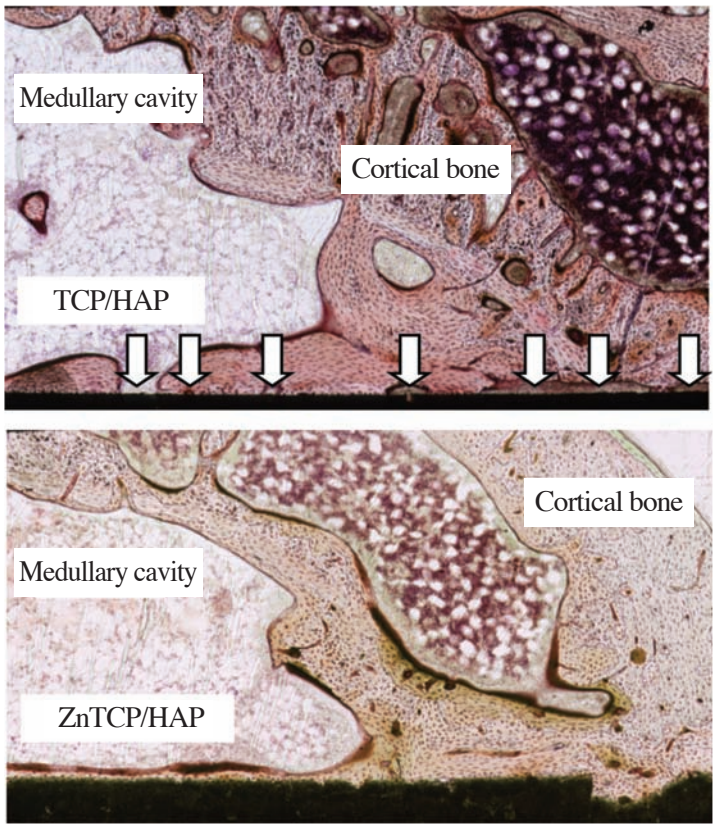

Figure 1: Bone tissue 24-weeks after implantation of TCP/HAP (Ca/P=1.60, zinc content 0 wt%) (top) and ZnTCP/HAP ((Ca+Zn)/P=1.60, zinc content 0.316 wt%) (bottom) into femur of rabbit.
The interface of TCP/HAP and bone shows gaps (indicated by arrows) due to bone resorption, but the cortical bone remains attached to ZnTCP/HAP.
(Reprinted from H. Kawamura, A. Ito *et al.*, J. Biomed. Mater. Res., 65A, 468-474, 2003, Weily Periodocals, Inc.)

Zinc-containing calcium phosphate promotes bone regeneration

We have been developing a controlled zinc-releasing tricalcium phosphate ceramic material (ZnTCP), fabricated by sintering bioabsorbable tricalcium phosphate (TCP:$Ca_3(PO_4)_2$) containing an adequate amount of zinc[1,2]. In terms of bone metabolism, as zinc activates osteoblasts and inhibits activity of osteoclasts, ZnTCP itself can be considered a biomaterial with tissue regeneration-stimulating capability (*in situ* tissue regeneration). In the process of development, we discovered the following phenomenon which suggested ZnTCP to be preventing development of osteoporosis. When apatite ceramic containing ZnTCP (ZnTCP/HAP) was implanted long-term in the femoral shaft of a rabbit, bone resorption became prominent after the 12th week due to immobility and aging of the rabbit, and the cortical bone region displayed a pseudo-osteoporosis state (pores opening in the bone). With the TCP/HAP containing no zinc, similar bone resorption occurred at the interface where the bone contacted the TCP/HAP, and at 24-60 weeks, the TCP/HAP was found to be partially detaching from the bone (Figure 1, top). Meanwhile, in the case of ZnTCP/HAP of zinc content 0.316 wt%, bone resorption

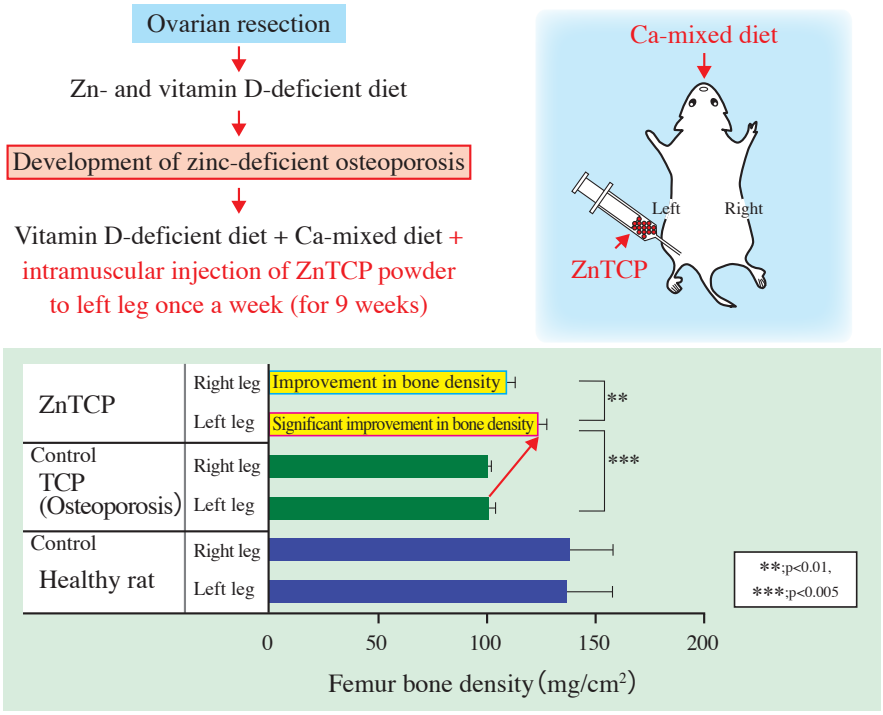

Figure 2: Improving effect of ZnTCP powder intramuscular injections on zinc-deficient osteoporosis
ZnTCP has a zinc content of 12.05 wt%, and is a mixture with $CaZn_2(PO_4)_2$ (Reprinted from Ito *et al.*, "Controlled zinc-releasing ceramics to control bone formation," Biomaterial. 21(2003)383-388, Japanese Society for Biomaterials).

occurred in regions far from the ZnTCP/HAP, but was minimal at the interface (Figure 1, bottom). These results suggest that ZnTCP/HAP prevents bone resorption, a symptom of osteoporosis.

Prospects for osteoporosis therapy

In light of the results above, we performed an experiment to confirm the effectiveness of ZnTCP in treating osteoporosis[3]. After the ovaries were resected from a 5-week-old female Wistar rat, it was kept on a diet low in zinc and vitamin D to induce a state of zinc-deficient osteoporosis. Subsequently, the rat was given Ca-mixed feed and was injected with 10 mg ZnTCP powder (Zn content 12.05 wt%) once a week in the muscle near the femur of the left leg. ZnTCP was not implanted within the bone, but merely injected into the muscle as a powder. Nonetheless, the bone density of the femur of the left side which received the injections improved considerably after 9 weeks (Figure 2) (a joint research effort with former Assistant Professor Makoto Otsuka of Kobe Pharmaceutical University). The bone density of the right femur which did not receive ZnTCP injection was lower than that of the left. There were no abnormal findings such as redness or fur loss in the skin where ZnTCP was injected; the injected ZnTCP disappeared within 1 week; and no inflammation, fluid exudation, or granulation tissue was observed. Thus, it is confirmed that by combining ZnTCP intramuscular injections with oral administration of calcium, the bone density of a rat in a state of zinc-deficient osteoporosis can be recovered on a local basis. These results suggest prospects for implementing localized therapy to prevent bone fracture, by promoting bone formation in target regions of low bone density at high risk of fracture.

Magnesium and fluorine have been included besides zinc in order to make a composite material of even higher effect, and the study is in progress as a National Institutes of Health (NIH) grant-supported project (Project Title: Biomaterials (Mg/Zn/F-BCPs) for osteoporosis therapy; Grant Number: 1R01EB003070).

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Standardization Research Aiming at Stimulating the Implant Industry

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Performance evaluation of medical equipments

Orthopedic implant technology is expected to merge with regenerative medicine technologies. As mechanical (structural) safety of orthopedic implants affects the long-term clinical performance, metallic materials with high strength such as titanium materials are frequently used. Problems with the implant such as breakage tend to increase with increasing number of implants, with deteriorated condition due to increased body weight, or with prolonged using period. We are thus investigating methods for evaluating the performance of orthopedic implants, mainly focused on design-based problems including material manufacturing processes.

"The Revised Pharmaceutical Affairs Law" was enforced in April 2005, and a JIS (Japanese Industrial Standards)-based third-party certification system is introduced for manufacturing approval of Class II medical instruments such as diagnostic equipment. For Class III devices including orthopedic implants and Class IV medical equipment directly linked to life safety, specifications/standards-based examination is required. As a result, the process for manufacturing approval become more transparent and speedy, however, the evaluation criteria including JIS must be established as rapidly as possible.

This article introduces recent activity in standardization of metallic materials which are indispensable to orthopedic implants, and our studies on the evaluation of mechanical properties of osteosynthesis devices.

Evaluation of mechanical properties of osteosynthesis devices

Evaluation of mechanical properties is crucial in osteosynthesis devices, as the major problem of the devices is breakage. The key properties are strength and rigidity to withstand the weight in the early stage before the bone unites. Commercially pure titanium and titanium alloys are used most, and stainless steel is not used much. The evaluation method of mechanical properties of a bone plate is shown in Figure 1. As almost all breakages of bone plates occur from a screw hole near the fracture line, a 4-point bend test is recommended. The bone plate is placed with the side which contacts bone facing up, and a compressive load is applied. Once a load-displacement curve is determined, the bending strength and bending rigidity are calculated as indicated in Figure 1. Long distance (about 2 holes for an 8-hole plate) should be secured between support and load rollers. The results of a 4-point bend test on titanium straight plates of identical shape with different material are shown in Figure 2. Roughly equivalent fatigue strength was obtained using the new titanium material compared to the conventional material, demonstrating that the 4-point bend test is also effective in evaluating durability. CHS (compression hip screw), screws, nails and intramedullary nails may be evaluated in the same way.

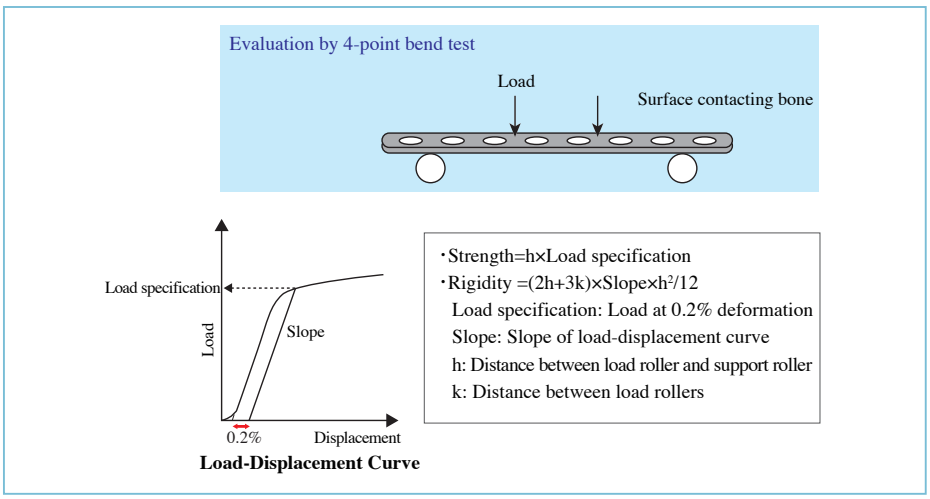

Figure 1: Method for mechanical properties evaluation of bone plate
Types of bone plates include straight, angled, end, and others which are used bended, while the materials used are commercially pure titanium, titanium alloys, and stainless steel.

Standardized metallic materials

The International Organization for Standardization (ISO) is implementing standardization focused on surgical implants. As metallic materials are used largely in orthopedic surgeries, standards regarding the composition and strength of titanium materials, stainless steel, and cobalt-chromium alloys are being revised. Titanium materials, which have the highest biocompatibility, are most commonly used, and Ti-6Al-4V alloy is most popular among them. Though elution of Ni and allergy-related problems are negligible with orthopedic implants, use of Ni-containing stainless steel is decreasing. Co-Cr-Mo alloys are used mainly in artificial joint stems, heads of bones, and sliding parts of artificial knee joints. Co-Cr-Ni-Mo-Fe alloy doped with Ni and Fe has superior workability and is showing growing use in wire products.

Titanium materials differ in corrosion resistance, mechanical property, and fatigue characteristics depending upon differences in

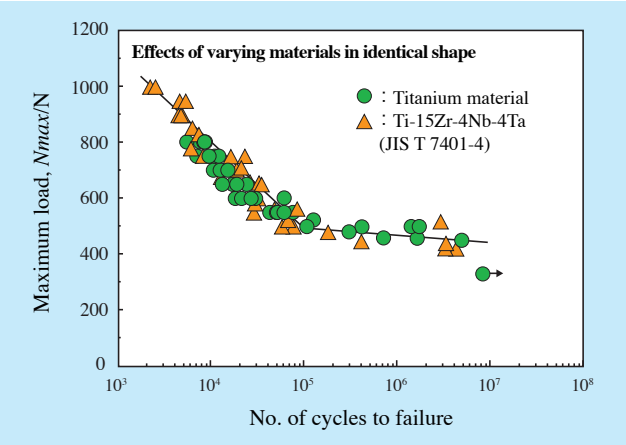

Figure 2: Results of 4-point bend test on straight plates

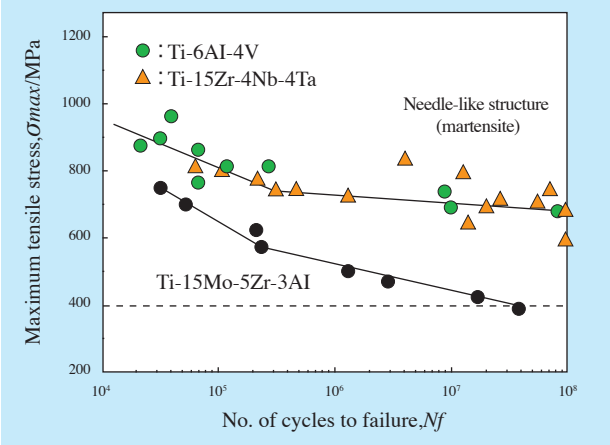

Figure 3: Comparison of fatigue characteristics of titanium alloys

composition and grain structure (microstructure). Commercially pure titanium demonstrates increased tensile strength with increased amount of Fe and O. Therefore, Grade 4 commercially pure titanium which has been cold-worked achieves a tensile strength and elongation after fracture almost equal to titanium alloys. JIS for titanium alloys stipulate a tensile strength of 800 MPa or higher and elongation of 12% or higher. As shown in Figure 3, Ti-6Al-4V and Ti-15Zr-4Nb-4Ta alloys[1,2] yield microscopic needle-like (martensite) structures with annealing (700°C, 2h), and demonstrate high fatigue strength. The relationship between maximum stress and number of cycles to failure, S-N curves, are shown in Figure 3. The fatigue strength at 10^8 cycles (100 million cycles) is over 700 MPa, which is higher than that of the solution-annealed Ti-15Mo-5Zr-3Al alloy (approx. 400 MPa).

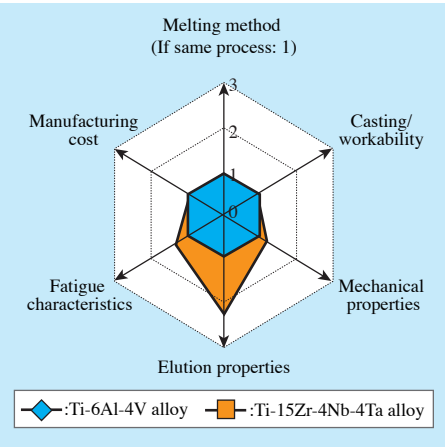

Figure 4: Comparison of titanium alloys

A Ti-15Zr-4Nb-4Ta alloy coatining Zr, Nb and Ta which are biocompatible and enhance corrosion resistance was developed in Japan as a titanium alloy for implants, and is standardized in JIS. As shown in Figure 4, the alloy has almost the same melting method (assigned 1 when identical), manufacturing process, manufacturing cost, heat treatment and processing conditions, etc. as the Ti-6Al-4V alloy, while it shows low levels of metal ion elution[3,4]. The alloy shows high corrosion resistance under friction with apatite ceramics simulating bone tissue[5]. Use of this alloy is anticipated as a measure against the crevice corrosion which has been reported in the artificial joint stems of cement type Ti-6Al-4V alloy. The grounds for such high corrosion resistance are that when Zr, Nb and Ta are doped, ZrO_2, Nb_2O_5 and Ta_2O_5 are formed in the passive film, and the ZrO_2 reinforces the film.

The increasing role of standardization

In the sections above, the effectiveness of mechanical properties evaluation of products has been demonstrated. It would have deepened the reader's understanding that implementation of standardization enables clear definition which are required for application of manufacturing approval, and also facilitates design changes and use of superior materials. I hope that Japan's implant industry will be stimulated, and our fine technology will be exported mainly in Asia.

At AIST, Industrial Standards Division leads efforts to promote research integrally with standardization, aiming to reinforce international competitiveness of industries. AIST sets industrial standardization policies, as well as implement and support steady standardization. "Center for JIS in Daily Living" of AIST is also promoting and publicizing our standardization research. In addition, AIST contributes to efforts in preparing the ground for streamlining pharmaceutical examinations and in establishing the guidelines for defining judgment criteria, including utilization of JIS. The role to be played by standardization efforts, such as JIS establishment, in stimulating the Japanese implant industry will become more prominent.

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Bone and Joint Regeneration Technology

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