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Special Lecture I

Therapeutic utilization of human embryonic stem cells

Hirofumi Suemori

Laboratory of Embryonic Stem Cell Research, Stem Cell Research Center
Institute for Frontier Medical Sciences, Kyoto University, Japan

Embryonic stem (ES) cell lines are derived from the inner cell mass (ICM) of blastocysts, and they proliferate indefinitely in vitro while retaining the ability to differentiate into tissues derived from the three embryonic germ layers. The establishment of human ES (hES) cell lines has indicated possibilities of the application of ES cells in medical research and applications such as cell therapy and drug discovery. Advance in protocols for directed induction of differentiation of ES cells into specific cell types would realize the therapeutic potential of ES cells. We have established three hES cell lines and distributed them to researchers who have obtained permission to use hES cells. hES cells are also expected as a source of human functional cells used for pharmaceutical researches such as drug screening.

Special Lecture II

Regenerative medicine in oral and maxillofacial surgery using bone morphogenetic proteins

Kazuhisa Bessho

Department of Oral and Maxillofacial Surgery, Graduate School of Medicine, Kyoto University, Japan

There is a long history of tissue restoration in the field of dentistry with autograft and biomaterials used frequently in clinic situations. However, serious problems that neither can easily solve remain. A new field of tissue engineering has recently developed with treatment using various cytokines. Further, in the field of oral and maxillofacial surgery, induction of bone, cartilage and teeth etc. by bone morphogenetic protein (BMP) as a regenerative medicine has been considered. However, though the problems with BMP have been diminished, BMP is still not widely used, although clinical use has been worldwide for about ten-year. We have performed basic research and preclinical study in respect to the various problems that needed resolution. In this lecture, I would like to introduce the outline of BMP briefly, then talk about the bone regeneration, then to prospect of application to clinical practice.

2005 Award special Lecture

Gene expression of matrix proteins in Cbfa1-knockout mice

Hitoshi Nagatsuka

Department of Oral Pathology and Medicine, Graduate School of Medicine,Dentistry and Pharmaceutical Sciences,
Okayama University, Japan

Cbfa1 is well-recognized as the master regulator of osteoblastic differentiation, little is known of its precise role during odontogenesis. We sought to clarify this by examining morphogenesis of teeth and matrix protein gene expression in cbfa1-knock-out mice. Results demonstrated morphological and functional differentiation differences between these two processes: incisor tooth germ showed morphological and functional differentiation of odontoblasts with expression of osteopontin. Stage-specific and cytodifferentiation differences were also observed between incisor and molar tooth germs. Present findings suggest that 1. The transcription factor Cbfa-1 is associated with morphogenesis of teeth and matrix protein gene expression. 2. Compared to the incisor tooth germ, the molar tooth germ is more strongly subjected to control by Cbfa1, and 3. In Cbfa1-knockout mice, the odontoblast-like cells in the incisor showed different patterns of gene expression of matrix proteins common to teeth and bones.

Session 1

5th Asian Science Seminar

Preferential expression of heparanase protein correlates with early invasion and progression of oral mucosal melanoma

Rosario Santos Rivera[1, 2], Hitoshi Nagatsuka[2], Ryo Tamamura[2], Jing Xiao[2, 3] and Noriyuki Nagai[2]

[1]College of Dentistry, University of the East, Manila, Philippines
[2]Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama University, Japan
[3]Department of Oral Pathology, College of Stomatology, Dalian Medical University, China

Oral mucosal melanoma (OMM) is an aggressive tumor with frequent metastasis. Most OMMs begin with an in situ phase and then progressed to invasive phase. Heparanase is an endo-beta-D-glucuronidase, which cleaves heparan sulfate (HS) chains found in basement membrane (BM) and extra-cellular matrix (ECM). Heparanase released by invading neoplastic cells removes HS chains from the BM resulting to increased permeability. The expression of heparanase has been studied in various neoplasms and its expression has been related to invasion, progression, angiogenesis and metastasis. The objective of the study was to determine the immunohistochemical expression of heparanase in normal oral mucosa, oral melanosis, primary OMM and those that metastasized to the lymph nodes. Results revealed that the melanocytes in normal mucosa and in oral melanosis were negative to heparanase. However, melanoma cells in in situ, invasive and metastatic OMM were positive. A gradual increase in the expression was observed during the transformation from in situ to invasive phase and in the progression to early invasive phase. However, in deeper tumor areas, heparanase was limited to melanoma cells individually invading the ECM, near the vessels and at the invasive fronts. Several tumor cells that metastasized to the lymph nodes also expressed heparanase. In conclusion, heparanase was only expressed by melanoma cells and not by normal melanocytes. Heparanase expression was most intense during the transformation from in situ to invasive phase and in early invasive phase suggesting its correlation with the early invasion and progression of OMM.

Sequential expression of Notch1, HES5, Jagged2, and Math1 in molar tooth germ of mouse

Silvia Susana Borkosky[1, 2], Naoki Katase[2], Phuu Pwint Han[2, 3], Mehmet Gunduz[2] and Hitoshi Nagatsuka[2]

[1]Department of Oral Pathology, Tucuman National University, School of Dentistry, Tucuman, Argentina
[2]Department of Oral Pathology and Medicine, Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama University, Okayama, Japan
[3]Institute of Dental Medicine, Yangon, Myanmar

The Notch signaling is an evolutionary conserved mechanism that plays an important role in cell-cell communication and cell fate in a wide range of tissues. Notch regulates cell fate decisions through two different mechanisms: lateral specification and inductive signaling.

Expression of Notch1, HES5, Jagged2 and Math1 were analyzed at the molar tooth germ during embryonic stage (E) 13 and E15 and during postnatal stage (PN) 1, PN3, PN5, PN10 and PN14 by using in situ hybridization. Positive Notch1 expression was found at the tooth bud during embryonic stages, but its expression was absent from the basal cells in contact with the dental mesenchyme. Later, during postnatal stages Notch1 appeared briefly expressed at the differentiated ameloblasts and odontoblasts. HES5 showed weak or absence of expression through all stages. Jagged2 and Math1 were strongly expressed at the differentiated ameloblasts and odontoblasts and Math1 strong expression was even maintained until PN14 stage. Math1 showed the strongest expression in contrast to the weakest expression of HES5. Our results suggest that the Nocth1 signaling pathway through Jagged2 has an important effect on Math1, regulating odontogenesis through both mechanisms lateral specification and inductive signaling.

Mutation analysis of the EGFR pathway in head and neck squamous cell cancer

Mahmoud AL Sheikh Ali[1, 2], Mehmet Gunduz[2], Hitoshi Nagatsuka[2], Beyhan Cengiz[2, 3], Esra Gunduz[2], Ryo Tamamura[2] and Noriyuki Nagai[2]

[1]College of Dentistry, Jordan University, Jordan
[2]Department of Oral Pathology and Medicine, Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama University, Japan
[3]Gaziantep Medical School, Turkey

The EGFR-RAS-RAF-MAPK signaling cascade is an important pathway in cancer development and recent reports show that the EGFR and its downstream signaling molecules are mutated in a number of cancers. ErbB2 is another member of the EGFR family with a strong kinase activity and it's the preferred heterodimerization partner for EGFR. Ras activates raf which is also an important oncogene in this pathway. In order to clarify the mutation status of this pathway and in order to find potential targets for molecular therapy, we used PCR and direct sequencing to detect the mutation status of the hot spot regions of EGFR (exon 19,20,21) and Kras (codons 12,13,61).

Out of the 79 head and neck squamous cell carcinoma samples used in this study we couldn't find any mutation in the EGFR nor in the Kras gene. Our data shows that mutations of the TK domain of EGFR and the Kras gene are not common in head and neck squamous cell carcinoma. Currently, we are investigating the

mutation status of the TK domain of erbB2 (exons 18, 19, 20, 21, 22, 23) and exons 11 and 15 of Braf gene using single strand conformation polymorphism (SSCP) and direct sequencing.

Biological analysis of bone marrow stromal cells *in vivo* cell culture method

Mathieu Bernard Lefevre[1, 2], Andrea Paola Rodriguez[2, 3], Hidetsugu Tsujigiwa[2], Hitoshi Nagatsuka[2] and Noriyuki Nagai[2]

[1]Paris V University, France
[2]Department of Oral Pathology and Medicine, Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama University, Okayama, Japan
[3]Department of Oral Pathology, Tucuman National University, School of Dentistry, Tucuman, Argentina

KUSA/A1 cells are immature mesenchymal stem cells isolated from bone marrow. These cells can differentiate to at least three types of mature cells like osteoblast, adipocyte and myoblast after treatment with 5-Azacytidine. Study showed that subcutaneous implantation of mature KUSA/A1 osteoblasts in diffusion chambers resulted in bone formation. It is well know that immature mesenchymal stem cells have better proliferation capabilities than mature cells. So, we hypothesized that the use of immature stem cells rather than mature cells could yield better results in tissue engineering due to the possibility of obtaining more cells to promote tissue repair.

For this purpose, we have evaluated an appropriate medium to maintain KUSA/A1 cells (kindly provided by Dr. Umezawa, Keio University) in their immature stage. In order to determine the ability of these immature KUSA/A1 cells to differentiate under the influence of the host microenvironment, immature KUSA/A1 cells in diffusion chamber have been implanted in intraperitoneal site in SCID mice. The results indicated that immature KUSA/A1 cells in vivo cell culture differentiated to osteoblasts and produced bone-like tissue. This study supports that immature KUSA/A1 cells show osteogenic potential activity in *in vivo* culture method.

Mechanism of new bone formation by using immature stem cell KUSA/A1 for bone tissue engineering in vivo study

Sathi Gulsan Ara[1, 2], Andrea Paola Rodriguez[2, 3], Hidetsugu Tsujigiwa[2], Hitoshi Nagatsuka[2] and Noriyuki Nagai[2]

[1]Bangladesh Dental College, Dhaka, Bangladesh
[2]Department of Oral Pathology and Medicine, Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama University, Okayama, Japan
[3]Department of Oral Pathology, Tucuman National University, School of Dentistry, Tucuman, Argentina

The basic principle of bone tissue engineering is to seed stem cells in porous scaffold. Stem cells can proliferate and differentiate into various types of mature cells. On the contrary, mature cells have low proliferation potential thereby not being able to obtain sufficient amount of cells to promote tissue repair. In previous study, we established an appropriate medium to maintain KUSA/A1 cells in their immature stage. These immature cells placed in a diffusion chamber and implanted intra-peritoneally, differentiated into osteoblast-like cells and produced bone-like tissue. In order to induce new bone formation, immature KUSA/A1 cells were seeded into atelocollagen honeycomb carrier. We evaluated the behavior of immature KUSA/A1 cells alone or with honeycomb carrier implanted in subcutaneous tissues of SCID mice. Transplants were subjected to radiographic, histological and immunohistochemical (CD34, Osteopontin, PCNA and BMP-2) examinations after 1, 2 and 4 weeks of implantation. KUSA/A1 cells alone showed few, small islands of new bone formation surrounded by scanty cells. On the other hand, KUSA/A1 with atelocollagen revealed abundant new bone formation as well as cellular proliferation. To clarify the cells implicated in new bone formation, same implantation was done in GFP (green fluorescent protein) mice. The results showed evidence that GFP positive host cells and GFP negative immature KUSA/A1 cells were both responsible for this new bone formation. From this study we concluded that there is a possibility of new bone formation induced by immature KUSA/A1 and host stem cell within atelocollagen honeycomb carrier *in vivo*.

Development of $CaTiO_3$-C coating material for dental implant

Mika Okauchi[1], Ryo Tamamura[1], Ko Kai Ryuu[1, 2], Kan Rui[1, 2], Jing Xiao[1, 3], Keisuke Nakano[1], Noriyuki Nagaoka[1], Katsunori Kanata[1], Toru Takagi[1], Masaru Akao[1] and Noriyuki Nagai[1]

[1]Department of Oral Pathology, Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama University, Japan
[2]China Medical University, China
[3]Department of Oral Pathology, College of Stomatology, Dalian Medical University, China

One of the most relevant advances in restorative dentistry is the replacement of lost teeth by osseointegrated implants. Titanium implants coated with hydroxyapatite (HA) is commonly used but bonding between these two materials is weak. In this regard, a binding material called calcium titanate with amorphous carbon ($CaTiO_3$-C) has been developed to enhance the bond strength and stability without increasing the interface between HA and titanium. Modified thermal decomposition method was used to increase Ca/P and Ca/Ti ratios and to decrease sintering temperature. Results revealed that a thin and homogenous coating was created between HA and titanium enhancing the bond strength as well as the stability of the implant.

Session 2

15th Annual Meeting of the Society for Hard Tissue Regenerative Biology

Induction of bone and cartilage by functionally graded-HAp / BMP-2 on rat skull periosteum

Jun Hino[1], Masaru Murata[1], Toshiyuki Akazawa[2], Mie Naraoka[2], Junichi Tazaki[1], and Makoto Arisue[1]
[1]Second Department of Oral and Maxillofacial Surgery, School of Dentistry, Health Sciences University of Hokkaido
[2]Department of Materials Technology, Hokkaido Industrial Research Institute

The objective of this study is to estimate the induction of bone and cartilage by absorbable hydroxyapatite block (fuctionally graded-hydroxyapatite:fg-HAp)/ rhBMP-2 (5μg) on rat skull periosteum, the absorption of fg-HAp and the observation of the periosteum histologically. As a result, body fluid permeated the inside of fg-HAp scaffold which were fragmented by degradation and absorption. The formation of hard tissue wasn't found in the fg-HAp alone, while in the fg-HAp/BMP-2, the formation of bone and cartilage started in the outer layer of the implanted materials and the soft tissues including periosteum existed between the induced tissues and the skull bone. Therefore, it seems that periosteum has homeostasis as a boundary membrane of the hard tissue formation.

Properties of osteoinduction and BMP-2 release in functionally graded HAp

Junichi Tazaki[1], Masaya Yamamoto[2], Yasuhiko Tabata[2], Toshiyuki Akazawa[3], Jun Hino[1], Masaru Murata[1], and Makoto Arisue[1]

[1]Oral and Maxillofacial Surgery, School of Dentistry, Health Sciences University of Hokkaido
[2]Department of Biomaterials, Institute for Frontier Medical Sciences, Kyoto University
[3]Department of Materials Technology, Hokkaido Industrial Research Institute

The aim of this study is to investigate *in vivo* local BMP-2 PK and bone induction in two bioceramics blocks(3×3×3mm), based on different surface structures. Spongy and cortical bones in bovine femur were calcined at 800℃ by the step-wise calcinations to obtain bulk HAp (b-HAp). The functionally graded HAp (fg-HAp) was designed by the partial dissolution-precipitation method. We estimated the *in vivo* release profile of ^{125}I–labeled BMP-2 and induction of hard tissues histologically. Specific nano-micro structures and biodegradation of fg-HAp is more effective for both BMP-2 retention and bone induction, compared to b-HAp, in the ectopic model.

Geometry of artificial extracellular matrices: BMP-induced ectopic osteogenesis with three geometrically different titanium-webs

Youichi Uramoto[1], Hiroko Takita[2], Yoshinori Kuboki[3] and Masato Tamura[1]

[1]Department of Biochemistry and Molecular Biology, Graduate School of Dental Medicine, Hokkaido University
[2]Central Research Institute, [3]Koken Bioscience Institute

We examined whether geometry of titanium-web (TW) as the cell-substratum controls BMP-induced osteogenesis. Three geometrically different TW which were created by altering the diameter of the titanium fiber (8, 50 or 80 mm; TW-8, TW-50 or TW-80) were mixed with BMP-2 and implanted subcutaneously into the back of rats. The highest calcium and osteocalcin content of implants was found in TW-8 at 2 or 4 weeks after implantation. Histological observations indicated that the bone-like tissue volume in TW-8 was much higher than in TW-50 or TW-80. This study clearly demonstrated that a certain geometrical size (8 mm fibers) is most favorable for BMP-induced bone formation.

Rationale for hydroxyapatite coating on 3D-titanium web enhancing bone formation

Yoshinori Kuboki [1, 2], John A. Jansen[3], Johan W. M. Vehof [3], Ryota Yoshimoto[4], Tohru Kaku[5], Hiroyuki Shiota[6], Yasuo Seki[6] and Hiroko Takita[7]

[1]Professor Emeritus, Hokkaido University, [2]Koken Bioscience Institute
[3]School of Dentistry, University of Nijmegen, Nijmegen, The Netherlands University
[4]Second Department of Oral Maxillofacial Surgery and
[5]Department of Oral Pathology, Hokkaido Health Sciences University of Hokkaido,
[6]Medical Device Department, Hi-Lex Corporation
[7]Central Research Institute, Graduate School of Dental Medicine, Hokkaido University

Hydroxyapatite coating on titanium implants is generally believed to increases isotopic bone formation, but there is no direct quantitative evidence. In this paper, we demonstrated that, for the first time, a new method of sputtering of calcium phosphate (2 micron thickness) on a new device of 3-dimensional titanium web (TW, composed of titanium fibers with 50 micron diameter) clearly induced 4 - 5 times higher amount of bone formation in the Ca-P coated Ti mesh than the non-coated one, judging from Ca contents and alkaline phosphatase activity at 6 weeks. A new insight on the mechanism of enhancement bone formation is to be presented.

RANKL induces the expression of carbonic anhydrase II, cathepsin K, and matrix metalloproteinase-9 in RAW264.7 cells

Kyosuke Fujisaki[1], Natsuko Tanabe[2,3], Naoto Suzuki[3,4], and Masao Maeno[2,3]

[1] Department of Endodontics, Nihon University School of Dentistry
[2] Department of Oral Health Sciences, Nihon University School of Dentistry

[3] Division of Functional Morphology, Dental Research Center, Nihon University School of Dentistry
[4] Department of Biochemistry, Nihon University School of Dentistry

We examined the effects of RANKL and/or M-CSF in the presence of IL-1á or IL-1á alone, on the expression of carbonic anhydrase II (CAII), cathepsin K, matrix metalloproteinases-9 (MMP-9), M-CSF receptor (cfms), RANK, and c-Fos using RAW264.7 cells as osteoclast precursor. In the presence of IL-1á, CAII, cathepsin K, and MMP-9 expressions increased markedly in cells cultured with RANKL, or M-CSF plus RANKL, whereas their expressions were difficult to detect in cell culture with IL-1á alone and M-CSF. RANK and c-Fos expressions also increased in cells cultured with RANKL, or M-CSF plus RANKL in the presence of IL-1á, whereas c-fms expression was not change. These results indicated that the expression of CAII, cathepsin K, and MMP-9 in RAW264.7 cells is induced by not IL-1á and M-CSF but RANKL.

Runx2 expression in mandibular condylar cartilage development

Takako Shimizu[1], Hitoshi Nagatsuka[2], Hidetsugu Tsujigiwa[2], Takehiro Watanabe[1], Norimasa Okafuji[1], Saburo Kurihara[1], Noriyuki Nagai[2], and Toshiyuki Kawakami[1]

[1]Deparment of Hard Tissue Research, Matsumoto Dental University, Nagano, Japan
[2]Department of Oral Pathology and Medicine, Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama University, Okayama, Japan

The purpose of this study was to investigate the expression pattern of Runx2 in mandibular condylar cartilage, a type of secondary cartilage. Mandibular condyles of ddY mice were fixed from embryonic days E14 to E18, and just after birth (equivalent to E19). Immunohistochemistry(IHC) and in situ hybridization(ISH) results for E14 showed Runx2 expression in coagulating mesenchymal cells. In both IHC and ISH results after E17, Runx2 factors appeared in the cells of the condylar cartilage sheath. These results suggest that Runx2 plays an essential role for mandibular condylar cartilage development.

Effects of phosphate on mineralization related gene expression of dental follicle cell

Sunao Sato[1], Masae Kitagawa[2], Shinji Iizuka[1], Kiyako Sakamoto[1], Hiroko Oka[1], Mutsumi Miyauchi[1], Brian Foster[3], Martha Somerman[3], and Takashi Takata[1]

[1]Department of Oral Maxillofacial Pathobiology, Graduate School of Biomedical Sciences, Hiroshima University
[2]Center of Oral Clinical Examination, Hiroshima University Hospital
[3]Department of Periodontics, School of Dentistry, University of Washington

The mouse disordered in phosphate and pyrophosphate metabolism exhibits abnormalities of bone formation and marked increase in cementum formation, however precise mechanism is still unclear. To examine the role of phosphate in cementogenesis, we cultured follicle cells with phosphate and assayed expression of mineralization related genes. After 48H culture with 0-5mM phosphate, 3mM phosphate showed most increased BMP-2 and OPN expression. Then we did time course experiment (1-120H) with 3mM phosphate. Most increased BMP-2 and OPN expression was seen in 72H group. These results suggest that phosphate regulates the mineralization related gene expression of follicle cells.

Forming mechanism of extracellular matrix containing periodontal ligament-like collagen fiber and its bone inducing activity

Kikuji Yamashita[1], Akemichi Ueno[2], Jun Ishibashi[1], Naohiro Ohshita[1], and Seiichiro Kitamura[1]

[1]Department of Oral and Maxillofacial Anatomy, Institute of Health Bioscience, University of Tokushima Graduate School
[2]Department of Hygiene Chemistry, School of Pharmaceutical Sciences, Ohu University

In order to clarify the forming mechanism of extracellular matrix containing calcified collagen fibers and its bone inducing activity, we analyzed the structure formed by cultured MC3T3-E1 osteoblasts on the calcified pure titanium, after removal of the cells. The one tip of beads-like collagen fiber with spherical granules bound to the calcified bioactive layer of titanium. The collagen fibers embedded in the calcium salt on titanium as Sharpey's fibers embedded in the calcium salt of alveolar bone or cement. Then, it was clarify that the extracellular matrix secreting into culture medium was collected by centrifugation and showed bone inducing activity.

Mechanisms of HERS formation and development of tooth root formation technology

Hidemitsu Harada[1], Tadayoshi Kagiya[1], Naoki Fujiwara[1], Yasuhiko Tabata[2], and Kiyoto Ishizeki[1]

[1]Department of Oral Anatomy II, Iwate Medical University, School of Dentistry
[2]Department of Biomaterials, Institute for Frontier Medical Science, Kyoto University

HERS is formed at the initiation of tooth root development. BrdU proliferation assay showed that although inner enamel epithelial cells had nearly finished proliferating, the outer enamel epithelial proliferated more actively. Immnostaining analysis showed that HERS expressed Notch2, which is a marker of outer enamel epithelium. These results suggested that HERS was derived from outer enamel epithelium in tooth development. Based on

the results, we tried to develop the technology inducing root formation by gelatin sheets including human recombinant Fgf10.

Application of micro-CT to observe the tissue surrounding dental implants at rabbit's bone

Yasuko Numata [1] , Toshiro Sakae [2] , Hiroshi Nakada [3] , Taketoshi Suwa [1] , Takao Kato[4], Takahiro Yazaki [3] , Katsuhisa Kuwahara [3] , and Kihei Kobayashi [3]

[1]Gnatho-Oral Prosthetic Rehabilitation, Nihon University School of Dentistry at Matsudo
[2]Department of Histology and Cytology, Nihon University School of Dentistry at Matsudo
[3] Department of Gnatho-Oral Prosthetic Rehabilitation, Nihon University School of Dentistry at Matsudo
[4] Department of Oral and Maxillofacial Implantology, Nihon University School of Dentistry at Matsudo

The aim of this study is application of micro-CT to observe the tissue surrounding dental implants at rabbit's bone. The rabbit's bone with the implants were removed 6 weeks after implantation and preserved in formalin. Micro-CT analysis was performed using three types of machine, and data obtained were compared. The best conditions were taken to obtain the best images for each equipment. Many artifacts appeared with TOSCANER-31300 μhd®, but disappeared with TDM1000®. SkyScan-2011® provided the most detailed images. Micro-CT made possible quantitative analysis of newly formed bone surrounding the dental implants.

Phase contrast imaging of dental tissues

Toshiro Sakae[1], Taketoshi Suwa[1], Yasuko Numata[1], Hiroshi Nakada[1], Yukie Sato[1], Hiroyuki Okada[1], Hirotsugu Yamamoto[1], Tohru Hayakawa[1], Kaoru Suzuki[2], Takao Kuwada[3], Yasushi Hayakawa[3], Toshinari Tanaka[3], Ken Hayakawa[3], and Isamu Sato[3]

[1]School of Dentistry, Nihon University at Matsudo,
[2]Faculty of Science and Technology, Nihon University
[3]LEBRA, Nihon University

When X-rays penetrate the material, the X-ray absorption image is obtained according to the composition of the material. The composition can be measured by using monochromatized X-ray. Using coherent X-ray, the phase contrast image is obtained based on the change in the phase of X rays penetrated through the materials. X-ray phase contrast image was possible only by super research facilities because of the X-ray source needed to be highly controlled. Parametric X-ray was generated from a 80MeV LINAC at LEBRA, Nihon University, and it succeeded in taking a picture of the phase contrast image of the ground specimens of teeth. [Nihon University Science Research Subsidy (Sogo 05-029) and the LEBRA Science Frontier Promotion].

Functional scaffold for tissue engineering

Yoshiaki Hirano

Department of Applied Chemistry, Faculty of Engineering, Osaka Institute of Technology.

Tissue engineering aims to create new tissues and organs to replace those lost to disease, trauma, or congenital defects. However, considerable instruction must be given to the cells forming these new tissues if one is to create a tissue structurally and functionally similar to the native tissue. Materials (Scaffold) play a key role in virtually all tissue engineering approaches. Scaffold create and maintain a space for tissue formation, provide mechanical support to the forming tissue, deliver inductive molecules or cells to the site of interest, and provide cues controlling the structure and function of the newly created. The cell-binding domain of fibronectin and many other adhesive proteins in ECM contains the peptide Arg-Gly-Asp-Ser (RGDS), and this sequence is recognized by integrin receptors. An overview of techniques to present RGDS peptides from scaffold, and the utility of these systems in engineering tissues *in vitro* and *in vivo* is presented.

Hepatocyte growth factor in tissue regeneration and anti - fibrosis

Kunio Matsumoto

Division of Molecular Regenerative Medicine,
Osaka University Graduate School of Medicine

Hepatocyte growth factor (HGF) was originally identified as a mitogenic protein for mature hepatocytes. HGF exerts multiple biological actions involved in cell proliferation, migration, morphogenesis, and apoptosis, through the Met receptor tyrosine kinase. Physiologically, HGF plays roles in regeneration and protection of organs such as the liver and kidney. Administration of HGF or HGF gene therapy has potent therapeutic effects on various acute and chronic diseases in distinct organs. Particularly HGF induces recovery from sclerotic disorder, including liver cirrhosis and chronic renal disease. In case of liver cirrhosis, HGF suppresses expression of TGF-b, the most potent fibrogenic growth factor, enhances proteolysis of extracellular matrix, stimulates proliferation of hepatocytes, and these are associated with remarkable improvement of liver cirrhosis. Because HGF orchestrates different biological activities, depending on target cell types, the recovery from fibrotic diseases associated with irreversible pathology seems to be achieved. Clinical trials of HGF are ongoing.

Regeneration induction therapy based on DDS technology of growth factors

Masaya Yamamoto and Yasuhiko Tabata

Department of Biomaterials, Institute for Frontier Medical Sciences, Kyoto University

Tissue engineering is a newly emerging biomedical form to create

a local environment which enables cells to promote the proliferation and differentiation for induction of tissue regeneration. The regeneration and repairing of tissues and organs have been achieved by making use of tissue engineering technology or methodology in a surgical or internally medical manner. For successful growth factor-induced tissue regeneration, it is important to develop drug delivery system (DDS) which can efficiently deliver the factor to the target site at the right concentration and the right timing over an appropriate time period. This paper overviews the recent development of growth factor delivery technologies necessary to realize the induction therapy of tissue regeneration.

Evaluation technique for regenerative bone tissue

Takayoshi Nakano

Course of Materials Science & Engineering, Division of Materials & Manufacturing Science, Graduate School of Engineering, Osaka University

Recent remarkable progress in medical technique for regenerating bone defects requests to develop a new method for evaluating the functional integrity of regenerative tissue. We applied the Materials Scientific technique such as microbeam X-ray diffractometer system and nano-indentation test to the regenerative bones in order to analyze the preferential alignment of the biological apatite (BAp) c-axis and the related mechanical function as a parameter of bone quality, focusing on the *in vivo* stress distribution. The BAp nano-crystals basically precipitate as an anisotropic hcp lattice on the extended collagen fibers, and the preferential alignment controls the mechanical function in bones.

We finally conclude that the BAp orientation distribution in bones is a new measure to evaluate *in vivo* stress distribution, nano-scale microstructure and the related mechanical function, healing process of the regenerated bone and progress of the bone diseases.