Center for Experimental Medicine
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細胞機能研究分野

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Recent development of transgenic techniques has made it possible to directly analyze the functions of a particular gene in a living animal. These techniques have also made it possible to produce various animal disease models as well as tools to analyze them. Immune disorders and infectious diseases are our major concerns, and we are attempting to produce transgenic mouse models for these diseases.

1. Studies on rheumatoid arthritis models: human T cell leukemia virus type I transgenic mouse model and IL-1 receptor antagonist knockout mouse model

Shinobu Saijo, Akiko Nakajima, Noriyuki Fujikado, Katsuko Sudo, and Yoichiro Iwakura

Rheumatoid arthritis (RA) is one of the most serious medical problems world-wide with approximately 1% of the people in the world affected. The disease is autoimmune in nature and characterized by chronic inflammation of the synovial tissues in multiple joints that leads to joint destruction. High levels of autoantibodies in the serum and augmentation of proinflammatory cytokine expression in the joints are characteristics of the disease, although the pathogenesis has not been elucidated completely. We have been studying the pathogenesis of the disease using two arthritis models that we developed originally. One is the human T cell leukemia virus type I (HTLV-I) transgenic (Tg) mouse model (Iwakura et al., 1991) and the other is IL-1 receptor antagonist (Ra) knockout (KO) mouse model (Horai et al., 1998). Both of these models develop autoimmunity and chronic inflammatory arthropathy closely resembling RA in humans.

To elucidate roles of proinflammatory cytokines in the development of RA, effects of cytokine deficiencies were examined using these RA models. The development of arthritis was markedly suppressed in either IL-6 or IL-1 deficient mice in HTLV-I-Tg mouse model, while TNFα deficiency suppressed the disease in IL-1Ra-KO mouse model. We are now analyzing the role of these cytokines in the development of autoimmunity and arthritis. We are also analyzing genes that are activated in arthritic joints of these model mice using microarray techniques. The role of these disease-related genes will be analyzed by generating knockout mice or transgenic mice of these genes.

2. Generation of IL-17-deficient mice: Antigen-specific T cell sensitization is impaired in IL-17-deficient mice, resulting in the suppression of allergic cellular and humoral responses

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Interleukin-17 (IL-17) is a proinflammatory cytokine produced by T cells. The involvement of IL-17 in human diseases has been suspected because of its detection in sera from asthmatic patients and synovial fluids from arthritis patients. In this study, we generated IL-17-deficient mice, and investigated the role of IL-17 in various disease models. We found that contact, delayed-type, and airway hypersensitivity responses as well as T-dependent antibody production, were significantly reduced in the mutant mice, while IL-17-deficiency of donor T cells did not affect acute graft-versus-host reaction. The results suggest that impaired responses were caused by the defects of allergen-specific T cell activation. Our findings indicate that IL-17 plays an important role in activating T cells in allergen-specific T cell-mediated immune responses.

3. IL-1-induced TNFα elicits inflammatory cell infiltration in the skin by inducing interferon-γ-inducible protein-10 in the elicitation phase of contact hypersensitivity response

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Contact hypersensitivity (CHS) is a typical inflammatory response against contact allergens. Inflammatory cytokines, including interleukin-1 (IL-1) and tumor necrosis factor α (TNFα), are implicated in the reaction, although the precise roles of each cytokine have not been completely elucidated. In this report, we dissected functional roles of IL-1 and TNFa during CHS. CHS induced by 2, 4, 6-trinitorochlorobenzene (TNCB) as well as oxazolone was suppressed in both IL-1α/β$^{-/-}$ and TNFα$^{-/-}$ mice. Hapten-specific T cell activation, as examined by T cell proliferation, OX40 expression and IL-17 production, was reduced in IL-1α/β$^{-/-}$ mice but not in TNFα$^{-/-}$ mice, suggesting that IL-1 but not TNFα is required for hapten-specific T cell-priming in the sensitization phase. On the other hand, TNFα, induced by IL-1, was necessary for the induction of local inflammation during the elicitation phase. We also found that the expression of IFN-γ-inducible protein 10 (IP-10) was augmented at the inflammatory site. Although IP-10 mRNA expression was abrogated in TNFα$^{-/-}$ mice, both CHS development and TNFα mRNA expression occurred normally in IFN-γ$^{-/-}$ mice, indicating that the induction of IP-10 during CHS was primarily controlled by TNFα. Interestingly, CHS was suppressed by treatment with anti-IP-10 mAb, suggesting a critical role for IP-10 in CHS. Reduced CHS in TNFα$^{-/-}$ mice was reversed by IP-10 injection during the elicitation phase. Thus, it was shown that the roles for IL-1 and TNFα are different, although both cytokines are crucial for the development of CHS.

4. Interleukin-1 is required for allergen-specific Th2 cell activation and the development of airway hypersensitivity response

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Interleukin-1 (IL-1) is a proinflammatory cytokine consisted of two molecular species, IL-1a and IL-1b, and the IL-1 receptor antagonist (IL-1Ra) is a natural inhibitor of both molecules. Although it is suggested that IL-1 potentiates immune responses mediated by Th2 cells, the role of IL-1 in asthma still remains unclear. In this study, we demonstrate that OVA-induced airway hypersensitivity response (AHR) in IL-1α/β-deficient (IL-1α/β$^{-/-}$) mice was significantly reduced from the levels seen in wild-type mice, whereas the responses seen in IL-1Ra$^{-/-}$ mice were profoundly exacerbated, suggesting that IL-1 is required for Th2 cell activation during AHR. OVA-specific T cell proliferation, IL-4 and IL-5 production by T cells, and IgG1 and IgE production by B cells in IL-1α/β$^{-/-}$ mice were markedly reduced compared with these responses in wild-type mice; such responses were enhanced in IL-1Ra$^{-/-}$ mice. Using IL-1α$^{-/-}$ and IL-1β$^{-/-}$ mice, we determined that both IL-1α and IL-1β are involved this reaction. Both IgG1 and IgE levels were reduced in IL-1β$^{-/-}$ mice while only IgE levels were affected in IL-1α$^{-/-}$ mice, indicating functional difference between IL-1α and IL-1β. These observations indicate that IL-1 plays important roles in the development of AHR.

5. Studies on the roles of cytokines in physiological and pathological bone remodeling

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We previously molecular cloned osteoclastogenesis-inhibitory factor (OCIF) (also called osteoprotegerin [OPG]), osteoclast differentiation factor (ODF; also called OPG ligand [OPGL], TNF-related activation-induced cytokine [TRANCE], and receptor activator of NF-κB ligand [RANKL]), and receptor activator of NF-κB (RANK), all of which are important for regulating osteoclast differentiation and activation. ODF/RANKL is a member of the membrane-associated tumor necrosis factor (TNF) ligand family and it induces osteoclast differentiation from progenitor cells co-treated with macrophage colony-stimulating factor (M-CSF) in the absence of osteoblasts/stromal cells and osteotropic factors. ODF/RANKL is a long-sought ligand expressed on osteoblasts/stromal cells in response to osteotropic factors, and it mediates an essential signal to osteoclast progenitors for their differentiation into

active osteoclasts. OCIF/OPG is a secreted member of the tumor necrosis factor receptor (TNFR) family, and it inhibits osteoclastogenesis *in vitro* and *in vivo*. RANK is the signaling receptor essential for ODF/RANKL-mediated osteoclastogenesis, and that OCIF/OPG acts as a decoy receptor for ODF/RANKL to compete against RANK. The discovery of ODF/RANKL, OCIF/OPG, and RANK opens a new era in the investigation of the regulation of osteoclast differentiation/function. Even though molecular mechanism of osteoclast differentiation and activation is almost clarified, factors (e.g. cytokines and hormones) regulating the expression of ODF/RANKL, OCIF/OPG, and RANK in vivo are not well studied.

Inflammatory cytokines, e.g. IL-1, play a major role in bone resorption in pathological conditions (e.g. rheumatoid arthritis and periodontal diseases). IL-1 also regulate the expression of ODF/RANKL and OCIF/OPG *in vitro*. However, the roles of these cytokines in bone development in physiological conditions are unknown. In addition, the relationship between inflammatory cytokines and ODF/RANKL or OCIF/OPG is not known in physiological conditions. Previous studies demonstrated that no obvious abnormality in bone in IL-1 receptor type I (IL-1R1) KO mice whose genetic background were C57BL/6 x 129/SV. We addressed the role of IL-1 in physiological bone remodeling using IL-1α KO mice, IL-1β KO mice, and IL-1α/β double KO mice, all of which were backcrossed to BALB/cA strain mice for 8 generations. Measurement of Bone Mineral Density (BMD) of femur with dual energy X-ray absorptiometry and peripheral quantitative computed tomography (pQCT) revealed significant increases in 8-week old mice with each genotype. Radiographs showed massive increase in bone density especially in the epiphysis and metaphysis of femur of these KO mice. Histological analysis also showed that marked increase of bone volume in trabecular bone of these KO mice. The thickness of cortical bone also was increased in these KO mice. The morphology of the growth plate and the columnar organization of chondrocytes are normal, but cartilaginous remnants were markedly observed in the cortical bone of these KO mice, which suggests a decrease in osteoclastic activity in resorption of bone and cartridge. Taken together these results indicate that IL-1 may have an important role in physiological bone development. The mechanism by which IL-1 KO mice have increased bone mass is under investigation.

6. Genomic structure of the mouse 2',5'-oligoadenylate synthetase gene family

Shigeru Kakuta, Shinwa Shibata and Yoichiro Iwakura

The 2′,5′-oligoadenylate synthetase (2-5OAS) is one of the IFN-induced proteins and mediates the antiviral action of interferon (IFN). In human, three classes of 2-5OAS genes (OAS1, OAS2, and OAS3) and one OAS-like gene (OASL) are reported. However, in mice, OAS genes corresponding to human OAS2 and OAS3 have not been identified. In this report, we have identified six novel OAS family genes in mice by screening mouse genomic library and EST database. These genes include three homologues of the human OAS1, and each homologous gene of the human OAS2, OAS3, and OASL, respectively. Each gene displays 52-65% amino acid identity to the corresponding human homologues. Nine 2-5OAS genes, except for two OASL genes, locate within 210 kb genomic region and form a cluster. Each novel 2-5OAS gene showed a characteristic expression pattern among different tissues, while all of them were induced by polyinosinic-polycytidylic acid. Biochemical analyses using recombinant proteins produced in *E. coli* showed that all the novel mouse 2-5OAS molecules have dsRNA binding ability but they have not 2-5OAS activity except the OAS2 and OAS3 mouse homologues. These results show that there are at least 11 OAS genes, which are classified into 4 groups, in the mouse.

7. The role of inflammatory cytokines in stress response

Dai Chida, Taizo Matsuki, Kyoko Kagiwada and Yoichiro Iwakura

IL-1 and TNFα are major mediators of inflammation and exert pleiotropic effects on the neuro-immuno-endocrine system. To evaluate the role for IL-1 and TNFα in stress response, we made use of IL-1α/β and TNFα triply deficient mice (IL-1/TNF KO) along with IL-1α/β KO mice, and TNFα KO mice. Peripheral administration of recombinant murine IL-1α (rmIL-1α) but not recombinant mTNFα triggered a febrile response and anorexia in IL-1/TNFα KO mice, while central administration of rmTNFα did trigger a febrile response and anorexia. We further analyzed the molecular mechanism for the difference of peripheral IL-1 and TNFα induced stress response, and found that the induction of IL-6 in the brain is differently regulated in response to IL-1 and TNFα, while both cytokines induced cox-2. Induction of IL-1 and TNFα in the brain is required for the induction of IL-6 in the brain in response to peripheral administration of TNFα, while they are not required in response to IL-1.

We are also analyzing the role of IL-1 in the control of appetite and lipid metabolism.

8. Generation of AIDS disease models and analysis of the pathogenesis using animal models

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In an attempt to generate AIDS disease models, we have produced a line of transgenic mice that carried the HIV-1 genome deficient in the *pol* gene. In this model, HIV-1 genome can be reactivated *in vivo* via lipopolysaccharide (LPS) administration through induction of IL-1α/β and TNF-α, although the HIV-1 genome in the lymphocytes was dormant under normal physiological conditions. We have analyzed the reactivation mechanism of the latent HIV-1 using this transgenic mouse model. Possible involvement of CpG methylation in the HIV-1 latency was examined by treating transgenic lymphocytes with a demethylating agent, 5′-Azacytidine (5′-AzaC). CpG methylation in the HIV-1 LTR was analyzed using the bisulfite genomic sequencing method. Since previous studies suggested that CpG demethylation depended on the cell cycle progression, we analyzed the relation between cell cycle progression and LPS-induced reactivation of HIV-1 by labeling lymphocytes with an intracellular fluorescein, CFSE. We found that 5′-AzaC enhanced HIV-1 expression 9-fold compared to treatment with LPS alone. Furthermore, HIV-1 p24 induction by LPS was observed only in cells that had undergone cell division, while induction was prevented in cells in which cell cycle progression was blocked either with mimosine, aphidicolin, or nocodazole. LPS-induced HIV-1 reactivation was associated with demethylation of two CpG sites located in the CREB/ATF binding sites in the HIV-1 LTR in a cell cycle dependent manner. These observations indicate that cell cycle progression-dependent demethylation of the CREB/ATF sites in the LTR is crucial for the reactivation of latent HIV-1 genome in transgenic mice.

We are also trying to generate HIV-1 sensitive mice by replacing all the host factor genes which form host range barrier into human genes. To this end, we have already produced mice that carry humanized receptor genes and a transcription factor gene. Since these mice are still resistant against HIV-1 infection, we are now trying to identify genes that are involved in host range barrier in the mouse.

9. Pathological changes of renal epithelial cells in mice transgenic for the TT virus ORF1 gene

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TT virus (TTV) is a newly identified human DNA virus of *circoviridae*, and its genome consists of six putative ORF genes. TTV was originally isolated from a patient with cryptogenic hepatitis and the association with hepatitis has been studied extensively, while leaving the significance in other diseases totally unknown. In this study, in order to examine its pathogenicity, we produced transgenic mice for the ORF genes in various combinations. We obtained 11 independent founder mice, and found that two that carry the ORF1 gene showed pathological changes in the kidney without affecting other tissues. In these mice, the transgene was expressed most strongly in the kidney, and the transcript was shown to be spliced to encode a protamine-like highly basic protein. Mice from a line with high transgene expression developed renal failure with severe renal epithelial cell abnormalities resembling nephrotic syndrome. The mice died before 5 weeks of age with severe ascites. Another founder mouse with low expression levels also showed similar, but milder, renal epithelial cell changes, indicating that this effects is not caused by the insertion of the trasgene but is rather caused by the ORF1 product. These observations suggest the possibility that TTV affects renal epithelial cells as a naturally occurring infection.

10. Attempts to reprogram adult tissue-specific stem cells into pluripotent stem cells

Chie Soeta, Kenjiro Adachi, and Yoichiro Iwakura

Successful production of cloned animals by nuclear transplantation has demonstrated that maternal cytoplasmic factors are capable of reinitialize differentiated somatic cells into undifferentiated state. Moreover, it was shown recently that adult somatic stem cells (haematopoetic stem cells, neural stem cells, etc.) had still high developmental potential. These stem cells could differentiate into all the three embryonic germ layers when they formed chimeras with normal blastocysts, suggesting that microenvironment is important for the cells to differentiate. We are now trying to find conditions that induce reprogramming of the lineage-specific somatic stem cells.

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Center for Experimental Medicine
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遺伝子機能研究分野

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Gene targeting technology has revealed many aspects of gene functions in vivo. Knock out mice offer the opportunities of not only analyzing the complex gene functions in vivo, but also presenting various human disease models, where new therapeutic approaches can be explored. To allow more detailed dissection of gene function, we introduce a point mutation or disrupt genes in certain lineages (or stages) using Cre-loxP system, a method of conditional gene targeting. In the process of analyzing knock out mice, we have isolated spontaneous mutant mice which develop chylous ascites and edematous limbs. In order to understand the mechanism of lymphatic development and functions in more detail, we are also generating various knock-out/knock-in mouse lines including a conditional knock out mouse. ES cells, which are used for gene targeting, are the only stem cells being cultured in vitro. To elucidate the molecular mechanism regulating self-renewal of pluripotent ES cells, we have tried to identify a factor(s) cooperating with Oct-3/4, the critical transcription factor for maintaining undifferentiated state of ES cells. We also intend to elucidate the etiopathogenesis of systemic autoimmune disease by analyzing nucleobindin (Nuc), an autoimmunity-augmenting factor.

1. Mice Lacking the EDB Segment of Fibronectin Develop Normally but Exhibit Reduced Cell Growth and Fibronectin Matrix Assembly *in Vitro*

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Fibronectins (FNs) are major cell-adhesive proteins in the extracellular matrix and are essential for embryonic development. FNs are encoded by a single gene, but heterogeneity is introduced by alternative pre-mRNA splicing. One of the alternatively spliced segments, extra domain B (EDB), is prominently expressed during embryonic development and in tumor tissues, although it is mostly eliminated from FN in normal adult tissues. To examine the function of the EDB segment *in vivo* , we generated mice lacking the EDB exon using the Cre-loxP system. Although EDB-containing FNs are highly expressed throughout early embryogenesis, EDB deficient mice developed normally and were fertile. Despite the absence of any significant phenotypes observed *in vivo* , however, fibroblasts obtained from EDB-deficient mice grew slowly *in vivo* and deposited less FN in the pericellular matrix than fibroblasts from wild-type mice. These results indicate that ex-

pression of EDB-containing isoforms is dispensable during embryonic development, yet may play a modulating role in the growth of connective tissue cells via the FN matrix.

2. SH2-B Is Required for Both Male and Female Reproduction

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Many growth factors and hormones modulate the reproductive status in mammals. Among these, insulin and insulin-like growth factor I (IGF-I) regulate the development of gonadal tissues. SH2-B has been shown to interact with insulin and IGF-I receptors, although the role of SH2-B in these signals has not been clarified. To investigate the role of SH2-B, we generated mice with a targeted disruption of the SH2-B gene. Both male and female SH2-B$^{-/-}$ mice showed slight retardation in growth and impaired fertility. Female knockout mice possess small, anovulatory ovaries with reduced number of follicles and male SH2-B$^{-/-}$ mice have small testes with a reduced number of sperm. SH2-B$^{-/-}$ cumulus cells do not respond to either follicle-stimulating hormone or IGF-I. These data suggest that SH2-B plays a critical role in the IGF-I-mediated reproductive pathway in mice.

3. Paranodal junction formation and spermatogenesis require sulfoglycolipids

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Mammalian sulfoglycolipids comprise two major members, sulfatide (HSO_3–3-galactosylceramide) and seminolipid (HSO_3–3-monogalactosylceramid). Sulfatide is a major lipid component of the myelin sheath and serves as the epitope for the well known oligodendorocyte-marker antibody O4. Seminolipid is synthesized in spermatocytes and maintained in the subsequent germ cell stages. Both sulfoglycolipids can be synthesized *in vitro* by using the isolated cerebroside sulfotransferase. To investigate the physiological role of sulfoglycolipids and to determine whether sulfatide and seminolipid are biosynthesized *in vivo* by a single sulfotransferase, *Cst*-null mice were generated by gene targeting. *Cst*$^{-/-}$ mice lacked sulfatide in brain and seminolipid in testis, proving that a single gene copy is responsible for their biosynthesis. *Cst*$^{-/-}$ mice were born healthy, but began to display hindlimb weakness by 6 weeks of age and subsequently showed a pronounced tremor and progressive ataxia. Although compact myelin was preserved, *Cst*$^{-/-}$ mice displayed abnormalities in paranodal junctions. On the other hand, *Cst*$^{-/-}$ males were sterile because of a block in spermatogenesis before the first meiotic division, whereas females were able to breed. These data show a critical role for sulfoglycolipids in myelin function and spermatogenesis.

4. Sera with high anti-human nucleosome but with low anti-human nucleosome DNA antibody levels well reflect the presence of lupus nephropathy

Yoshiyuki Kanai, Tetsuo Kubota[14], Yuji Yamada, Yoshiaki Tokano[15] and Hiroshi Hashimoto[15]: [14]Tokyo Medical and Dental University, [15]Juntendo University

Yesteryear's studies indicate that anti-nucleosome(NS) antibody is an excellent marker for lupus nephropathy. For safety estimation of anti-NS antibodies, however, crossreaction between nucleosome(ns)DNA and NS, in which nsDNA is packaged, should be carefully precluded. For this purpose we evaluated the significance of anti-human(h)NS antibodies in predicting the presence of lupus nephropathy using carefully-prepared hNS as an autoantigen. We first purified mono-nucleosome from nuclei of human HL-60 cells by HPLC with a Superdex column after extraction of crude NS in Tris-buffered 0.25M NaCl solution from micrococcal endonuclease-digested chromatin. Human nucleosome(hns) DNA with 150-bp DNA was isolated from the purified hNS. ELISA was developed with Immulon 2HB flat plates directly coated with antigens prepared as above, respectively. Thus prepared hNS was of 99% purity and di-NS was totally excluded. Under these conditions, 90 sera from 90 patients with SLE fulfilled with diagnostic criteria by

ACR were assayed by ELISA. Overall positivity of anti-hNS and anti-hnsDNA antibodies were 47.7 % and 16.6%, respectively. In the mean time, 70% were positive for anti-hNS antibodies in SLE patients who had at least nephropathy above class II defined by WHO. However, positive cases for anti-hnsDNA were as low as 25%. Few cases(3/17) showed high levels of anti-hnsDNA antibodies. Taken together, it is cocluded that autoantibodies to NS purified from human HL-60 cells is useful for predicting the presence of lupus nephropathy. Prallel assay for hnsDNA antibodies would further help the consolidation.

5. Induction of IgG subclass restricted anti-mouse nucleosome autoantibodies in mice immunized with human nucleosome

Yoshiyuki Kanai and Tetsuo Kubota[14]

Since the report by Theophilopoulos et al in 1993 that the immune response to subnucleosome in lupus-prone mice is of special importance as a marker of early appearance of lupus syndrome, mounting evidences in human lupus suggest that levels of anti-nucleosome(NS) antibodies in serum are closely related to the presence of nephropathy. Concern on anti-NS antibodies, however, is that there really exist human NS specific autoantibodies that are able to distinguish from nonhuman nucleosome. To address this naive question, it is essentially required to prepare the pure human NS and a counterpart such as mouse NS. Thus, we have isolated purified mononucleosome (hereafter, it is referred to as NS) from human HL-60 cells and mouse lymphoid cells with less than 1% association of di- or oligo-NS as far as judged by SDS-PAGE, agarose gel electrophoresis, Superdex HPLC and by electronmicroscope. We immunized BALB/c mice with 5 μg human NS as protein in Freind's incomplete adjuvant three times. Four months after initial immunization, blood was collected through a tail vein and serum was subjected to ELISA in which 2μg human or mouse NS were coated to Immulon 2HB plates directly in Tris-buffer containing 0.25M NaCl. Of 6 mice, two elicited significant amount of immune responses to both human and mouse NS ; one was high, while another was moderate. As was expected, immune responses to human NS were higher than those to mouse NS. However, interestingly enough, the response to mouse NS of both mice was confined to IgG1, while that to human NS of both mice was mainly IgG2b, and IgG1 was followed. In conclusion, NS said to be highly conserved did induce hetro- and auto immune responses, suggesting epitope spreading even in conglomerated antigen complexes. Moreover, immunologically disclosed interspecies structure difference of NS could give a new clue not only in autoimmunity but also in chromatin study.

6. Genetic analysis of lymphatic development and functions in mice

Hirotake Ichise, Taeko Ichise, Akiko Hori, Seiji Shiozawa, Takeshi Kuraishi, and Nobuaki Yoshida

The lymphatics are thought to be responsible for edematous condition in patients, especially in those suffering from lymphedema. Recent studies show that lymphangiogenesis, as well as angiogenesis, also plays some roles on tumor metastasis. However, the lymphatic development in mammals has been unknown from lack of useful mutant animal that has obvious lymphatic abnormality.

In order to understand the mechanism of lymphatic development and functions, we are generating genetically-engineered mice for some genes that are thought to regulate lymphangiogenesis; VEGF-C conditional transgenic mice, *prox-1* conditional knockout mice, and tamoxifen-inducible cre transgenic mice under transcriptional regulation of either lymphatic endothelial-specific gene (VEGFR-3 promoter) or ubiquitous promoter. We have already established some tamoxifen-inducible cre transgenic mouse lines, so we are investigating their recombination efficiencies of floxed ROSA26 (ROSA26R) loci and seeking for efficient methods of tamoxifen administration.

We are also under investigation of an original spontaneous mutant mouse line developing chylous ascites and lymphedema that are thought to be due to lymphatic abnormality. In the homozygous mutant mice, the blood flow is found not only in blood vessels but also in lymphatic vessels of intestine and a part of skin. The peripheral capillary-lacteal shunt at the intestinal villi is observed in homozygous mutant mice. It is thought to be one of the cause for blood flow observed in lymphatics of the homozygous mutant mice. In our immunohistochemical study, VEGFR-3, one of the receptor tyrosine kinase regulating lymphangiogenesis, is expressed intensely in the intestinal lymphatic endothelial cells of the wild-type mice, but not in those of the homozygous mutant mice. The intestinal lymphatic vascular structure of the homozygous mutant mice is immature and dilated. In addition to the lymphatic defect, the intestinal vascular sturucture of blood vessels is also impaired in them. The candidate gene for this mutation is responsible for both angiogenesis and lymphangiogenesis on late stage of embryogenesis, and is thought to regulate them in tissue-specific manner. We are trying forward genetic approaches to find the candidate for this mutation. We have already generated our mutant strain in 129/SvEv genetic background (N8). As they have an autosomal recessive mutation, we are planning to perform F2 intercrosses between wild-derived inbred strain. 129/SvEv-+/mutant F1 hybrids in order to map the mutant locus.

7. Protein purification and cloning of ROX that binds to Rex-1 gene promoter.

Mitsuharu Sato, Yuhki Nakatake and Nobuaki Yoshida

The mechanism that establishes undifferentiated state in mouse embryonic stem (ES) cells is not fully understood. Although many ES specific genes have been identified and analyzed extensively, we do not have a whole picture of self-renewal mechanism of ES cells.

One of the POU-family transcription factors, Oct-3/4 is essential for mouse ES cells to be maintained in undifferentiated state. However, it was shown that Oct-3/4 functions as a transcriptional activator only in undifferentiated cells suggesting some factor cooperates with Oct-3/4 in undifferentiated cells. To identify the factor that cooperates with Oct-3/4, we focused on Rex-1 gene promoter because new DNA binding activity, termed Rox, was identified on the adjacent site to Oct-3/4 binding motif in this region.

We performed denature-renature cycle to recover Rox peptide from SDS-PAGE and isoelectoric point gel electrophoresis. Rox activity of each fraction was traced by EMSA and we identified the peptide showing Rox property. With a great help of Dr. Isobe and Dr. Izumi (Division of proteomics research) amino acid sequence of Rox was determined by LC-MAS spectrometry. We are analyzing mRNA level of Rox along with ES differentiation and physical interaction of Rox with Oct-3/4.

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Center for Experimental Medicine
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幹細胞治療動物モデル分野

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Stem cells are generally defined as clonogenic cells capable of both self-renewal and multilineage differentiation. Because of these unique properties, stem cells offer the novel and exciting possibility of organ reconstitution in place of transplanted or artificial organs in the treatment of organ failure. In addition, stem cells are considered as ideal target cells for gene therapy. The goal of this laboratory is to provide new insights into stem cell biology as well as approaches to therapeutic intervention for various diseases.

1. The mechanism of hematopoietic stem cell differentiation

a. Role of Gfi-1B zinc finger protein in hematopoiesis

In the search for genes expressed in hematopoietic stem cells, we identified that the expression of *Gfi-1B (growth factor independence-1B)* is highly restricted to hematopoietic stem cells, erythroblasts, and megakaryocytes. Gfi-1 and Gfi-1B are zinc finger proteins that share highly conserved SNAG and six zinc finger domains. *Gfi-1* has been characterized as an oncogene involved in lymphoid malignancies in mice. In contrast, role of Gfi-1B in hematopoiesis has not been well characterized. We therefore analyzed its function in human hematopoiesis. Enforced expression of *Gfi-1B* in human $CD34^+$ hematopoietic progenitors induced a drastic expansion of erythroblasts in an erythropoietin-independent manner. Expression of *Gfi-1B* did not promote erythroid commitment, but enhanced proliferation of immature erythroblasts. Erythroblasts expanded by exogenous *Gfi-1B*, however, failed to differentiate beyond proerythroblast stage and showed massive apoptosis. These biological effects of Gfi-1B were mediated through its zinc finger domain, but not by the SNAG or non-zinc finger domain. Proliferation of erythroblasts was associated with sustained expression of GATA-2 but not of GATA-1, indicating a potential link between Gfi-1B and GATA family regulators. Importantly, the function of Gfi-1B to modulate transcription was dependent on promoter context. In addition, activation of transcription of an artificial promoter was mediated through its zinc finger domain. These findings establish Gfi-1B as a novel erythroid regulator and reveal its specific involvement in the regulation of erythroid cell growth through modulating erythroid-specific gene expression.

b. Essential and instructive roles of GATA factors in eosinphil development

GATA transcription factors are major regulators of hematopoietic and immune system. Among GATA factors, GATA-1, GATA-2 and GATA-3 play crucial roles in the development of erythroid cells, hematopoietic stem and progenitor cells, and Th2 cells, respectively. A high level of GATA-1 and GATA-2 expression has been observed in eosinophils, but their roles in eosinophil development remain uncertain both *in vitro* and *in vivo*. We showed that enforced expression of *GATA-1* in human primary myeloid progenitor cells completely switches myeloid cell fate into eosinophils. Expres-

sion of *GATA-1* exclusively promotes development and terminal maturation of eosinophils. Functional domain analyses revealed that the C-terminal finger is essential for this capacity while the other domains are dispensable. Importantly, GATA-1-deficient mice failed to develop eosinophil progenitors in the fetal liver. On the other hand, GATA-2 also showed instructive capacity comparable to GATA-1 *in vitro* and efficiently compensated for GATA-1-deficiency in terms of eosinophil development *in vivo*, indicating that proper accumulation of GATA factors is critical for eosinophil development. Taken together, our findings establish essential and instructive roles of GATA factors in eosinophil development. GATA-1 and GATA-2 could be novel molecular targets for therapeutic approaches to allergic inflammation.

c. Reciprocal roles for C/EBP and PU.1 transcriptional factors in Langerhans cell commitment

Myeloid progenitor cells give rise to a variety of progenies including dendritic cells, but the mechanism controlling the diversification of myeloid progenitors into each progeny is largely unknown. PU.1 and C/EBP family transcription factors have been characterized as key regulators for development and function of myeloid system. However, roles of C/EBP transcription factors have not been fully identified because of functional redundancy among family members. Using high titer retroviral infection, we demonstrate that a dominant negative C/EBP completely blocked the granulocyte/macrophage commitment of human myeloid progenitors. Alternatively, Langerhans cell (LC) commitment was markedly facilitated in the absence of TNFα, a strong inducer of Langerhans cell development. Conversely, expression of wild-type C/EBP in myeloid progenitors promoted granulocytic differentiation and completely inhibited TNFα-dependent LC development. On the other hand, expression of wild-type PU.1 in myeloid progenitors triggered LC development in the absence of TNFα, and its instructive effect was canceled by co-expressed C/EBP. Taken together, our findings establish reciprocal roles for C/EBP and PU.1 in LC development, and provide a new insight into the molecular mechanism of LC development that has not yet been well characterized.

d. Lentiviral vector-mediated transduction of murine CD34-negative hematopoietic stem cells

Efficient gene transfer into murine hematopoietic stem cells (HSCs) provides a powerful tool for exploring hematopoietic stem cell biology. Lentiviral vector has been to be useful to introduce genes into human hematopoietic progenitor cells. However, it was not know whether lentiviral vector would also be useful for murine HSCs. We evaluated the efficiency of lentiviral vector–mediated gene transfer into murine $CD34^{-/low}$c-$Kit^{+}Sca\text{-}1^{+}Lin^{-}$ ($CD34^{-}$ KSL) cells that are highly enriched for HSCs. FACS-sorted $CD34^{-}$ KSL cells were transduced with the vesicular stomatitis virus G glycoprotein-pseudotyped HIV-1–based lentiviral vector containing the green fluorescent protein (GFP) gene under the control of the cytomegalovirus promoter, and then 50 transduced cells were transplanted into lethally irradiated mice. Transduction efficiency was assessed by FACS analysis for GFP expression in peripheral blood (PB) cells. FACS-sorted GFP^{+} KSL bone marrow (BM) cells from primary recipients were used for secondary transplantation, and GFP expression in PB cells of reconstituted mice was analyzed by FACS. GFP expression was detected in PB cells of all primary recipients (n = 10) at an average of 40% (range 26–58%) when the lentiviral vector containing the woodchuck hepatitis virus posttranscriptional regulatory element was used. GFP^{+} cells were found in multilineage cells in PB, BM, spleen, and thymus for at least 8 months posttransplantation. In secondary recipients, donor-derived GFP^{+} KSL BM cells could reconstitute and GFP expression was detected in both myeloid and lymphoid cells in PB. Our results indicate that lentiviral vectors can efficiently transduce highly enriched murine HSCs and sustain long-term expression of the transgene in the multilineage differentiated progeny in reconstitutedmice.

2. Identification and characterization of the endodermal stem/progenitor cells

The enormous regenerative capacity of the liver following partial hepatectomy or chemical injury is well known. However, it remains unclear how the liver is regenerated and what cells are involved in such regeneration. While by analogy with hematopoiesis liver regeneration can be regarded as mediated by proliferation and differentiation of hepatic stem cells, it has become increasingly apparent that fully differentiated hepatocytes themselves possess great growth potential and that stem cells may not be required for liver regeneration.

By contrast, it is believed that in the developing liver both hepatocytes and cholangiocytes differentiate from a common cell component, the hepatoblast . It was recently reported that isolated fetal rat liver cells, transplanted into retrorsine-treated liver, reconstituted bile duct and hepatocyte structures . This strongly suggested that the donor cell population included at least bi-potent hepatic stem / progenitor cells. However, it was not possible to determine if the regenerated structures had differentiated from stem cells or from lineage-committed cells. Thus, while the hepatoblast is a possible candidate for the hepatic stem cell, in the absence of clonal analysis, severe controversy persists regarding its potential for differentiation and self-renewal.

Using flow cytometry and *in vitro* single-cell-based assays, we prospectively identified hepatic

stem cells with multilineage differentiation potential and self-renewing capability. These cells could be clonally propagated in culture, where they continuously produced hepatocytes and cholangiocytes as descendants while maintaining primitive stem cells. When cells that expanded *in vitro* were transplanted into recipient animals, they morphologically and functionally differentiated into hepatocytes and cholangiocytes, with reconstitution of hepatocyte and bile duct structures. Furthermore, these cells differentiated into pancreatic ductal and acinar cells or intestinal epithelial cells when transplanted into pancreas or duodenal wall. These data indicate that self-renewing multipotent stem cells persist in the developing mouse liver and that such cells can be induced to become cells of other organs of endodermal origin under appropriate microenvironment. Manipulation of hepatic stem cells may provide new insight into therapies for diseases of the digestive system.

3. An experimental model of stem cell therapy for prevention of atherosclerosis

Apolipoprotein E (apoE), a key regulator in cholesterol-rich lipoprotein metabolism, is a 34-kD glycoprotein that is major structural component of cholesterol-rich plasma lipoproteins. It serves as a ligand for receptor-mediated clearance of lipoproteins, including very low density lipoproteins (VLDL) and chylomicron remnants, through LDL receptors and LDL receptor-related proteins (LRP). Deficiency of apoE results in severe hypercholesterolemia and early development of atherosclerosis in humans (type III hyperlipoproteinemia; HLP) and in gene targeted mice. Although most apoE is synthesized in the liver, apoE is synthesized in a wide variety of tissues and cell types, including macrophages/monocytes.

Because macrophages/monocytes are derived from HSCs, the effects of bone marrow transplantation (BMT) were tested in apoE-deficient (apoE(-/-)) mice with phenotypes similar to those in type III HLP. BMT of normal bone marrow cells into apoE (-/-) mice normalized serum cholesterol and triglyceride levels, and prevented atherosclerosis in arterial walls. This indicates that apoE synthesized not by hepatocytes, but by macrophages/monocytes derived from HSCs play a central role in protecting against arterial atherosclerosis.

These studies suggest that stem cell gene therapy may provide a new clinical approach for patients without suitable bone marrow donors. Efficient transduction of HSCs has been difficult with current gene transfer technologies, however, since most HSCs are in G_O phase of cell cycle. It therefore is crucial to determine the minimal levels of chimerism required prevent atherosclerosis. To address this issue, we transplanted normal bone marrow cells, mixed at various ratios with those of apoE(-/-) mice, into lethally irradiated apoE(-/-) mice and analyzed changes of serum cholesterol levels and the extent of atherosclerosis in arterial walls every 3 weeks for up to 42 weeks post transplant. Areas of atherosclerotic lesions in aortas were quantified 6 months post-transplant. The serum cholesterol levels in mice with >60% chimerism were normalized by 6 weeks post-transplant. Mice with >40% chimerism showed significant reductions, but not normalization, in serum cholesterol levels even at 42 weeks post-transplant. However, atherosclerotic areas observed in 10%-chimeric mice were significantly smaller than those in control mice ($P<0.01$). Immunohistochemical studies in 10%-chimeric mice revealed foam cells derived from donor marrow (apoE (+/+)) and expressing immunoreactive apoE in atherosclerotic lesions.

These data indicated that chimerism of 10%, the minimum level analyzed, was sufficient to reduce the severity of atherosclerosis, even though serum cholesterol levels were not completely normalized. The results suggest that stem cell gene therapy and mini-transplant are possible new therapeutic approaches in patients with abnormal lipid metabolism.

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