Center for Experimental Medicine

Laboratory of DNA Biology and Embryo Engineering

Our major interest is to elucidate the physiological function of signal transduction molecules by analyses of mutant or transgenic mice generated from embryonic and genetic engineering. Our research concentrates on ras family genes and neurotransmitter receptor genes. We also created mouse models for human genetic diseases in which mouse genes are replaced by wild-type or mutant human homologues.

1. Functional analysis of *ras* family genes in the mouse using *ras* mutant mice and inducible expression system

Kenji Nakamura, Atsushi Yamada, Hirotake Ichise, Kazuki Nakao, Atsu Aiba and Motoya Katsuki

In mammals, Ras proteins are thought to be critical transducers of intracellular signaling in the regulation of cell proliferation and development. There are three *ras* genes located on different chromosomes, which encode highly homologous but distinct GTP-binding proteins: H-Ras, N-Ras, and K-Ras. To clarify the roles of each Ras protein and functional redundancy of three Ras proteins *in vivo*, we generated knockout mice lacking each *ras* gene by the gene targeting, and generated double and triple mutants for *ras* genes by interbreeding of *ras*-deficient strains. H-$ras^{-/-}$ mice and N-$ras^{-/-}$ mice as well as a substantial proportion of H-$ras^{-/-}$N-$ras^{-/-}$ mice that express only K-*ras* gene are viable, while K-$ras^{-/-}$ mice were previously shown to be embryonically lethal. Investigation of neonatal death of N-$ras^{-/-}$K-$ras^{+/-}$ and H-$ras^{-/-}$N-$ras^{-/-}$ mice showed that a part of these mice died because of chylous ascites and intestinal lymphedema. Most of *ras* mutant mice lacking 2 or more *ras* alleles exhibited similar phenotype. Incidence of chylous ascites increases as number of null alleles of any three *ras* genes are increased, suggesting the function of three *ras* genes is redundant. To prove this hypothesis, we introduce human H-*ras* transgene into a single and multiple *ras* mutant mice and found that H-*ras* transgene rescue all abnormality exhibited in the development of these *ras* mutant mice. Thus, we conclude the function of *ras* genes is redundant *in vivo*. We are now generating H-$ras^{-/-}$N-$ras^{-/-}$K-$ras^{-/-}$ mice carrying H-*ras* transgene, which have *loxP* sites and can be conditionally excised by Cre-mediated recombination.

2. Analysis of transgenic mice carrying human activated H-*ras* gene whose expression is induced by Cre

Atsushi Yamada, Kazuki Nakao, Atsu Aiba and Motoya Katsuki

To investigate the function of Ras, it is interesting to create the transgenic mice carrying activated *ras* genes which have their own promoter. However, we never obtained any transgenic pups carrying activated human H-*ras* genes which developed to term, because all transgenic embryos were malformed. Thus, we generated transgenic mice carrying activated human H-*ras* gene whose expression is stopped by insertion of a spacer sequence between promoter region and coding region in activated human H-*ras* gene which have their own promoter. We crossed this H-*ras* transgenic mice and CaMKIIα-cre transgenic mice which were reported to express *cre* gene only in CA1 pyramidal cells in the hippocampus. Unexpectedly, we found that many papillomas on the paws or faces of these mice and analysis of DNA and protein from these papillomas showed spacer sequences were excised and activated H-Ras proteins were expressed in the papillomas.

3. NMDA receptor mediated signal transduction

Atsushi Yamada, Chieko Konishi, Yutaka Ohbu-

chi, Atsu Aiba and Motoya Katsuki

Glutamate receptors that are major excitatory neurotransmitter receptors in the central nervous system (CNS) lead to neuronal cell death when they are overactivated. Therefore, there may be some mechanisms that make a balance between excitotoxic and surviving signals when the receptors are normally activated. We found that NMDA stimulation, which conveys Ca^{2+} influx into the cell through the NMDA receptor channel, dramatically decreases tyrosine phosphorylation of NR2B subunit of the receptor in hippocampal slices as well as hippocampal cultured cells. In addition, NMDA stimulation activates extracellular-signal regulated kinases (ERKs) and p38 mitogen-activated protein kinase (MAPK), which mediate cell surviving and excitotoxic signals, respectively in the hippocampal neurons as well. This NMDA signaling is mediated by NR2B subunit because blockade of NR2B by a specific antagonist, ifenprodil, eliminates activation of the ERK and p38 MAPK activation and tyrosine dephosphorylation of NR2B and gene disruption of NR2B eliminates ERK activation by NMDA stimulation. Therefore, NR2B subunit of NMDA receptor regulates ERK pathway, p38 MAPK pathway and own phosphorylation upon NMDA stimulation to modulate a balance between excitotoxic and surviving signals.

4. Double knockout mice of *gp130* and K-*ras* genes exhibit erythrocytosis at the mid-gestation stage

Shoji Sawai and Motoya Katsuki

gp130 is the co-receptor of IL-6-family cytokines. Biochemical analyses have suggested that Stat3 and Ras signalings lie downstream of the gp130. *gp130* and K-*ras* knockout mice have some common phenotype. They exhibit, however, weaker defects than expected from their biochemical functions. Thus we assumed some genetic interaction of their mutations and have analyzed the phenotype of double knockout mice of *gp130* and K-*ras* genes. We found that the double knockout embryos (1) could not be recovered after 13.5 dpc, while the single knockout embryos could survive beyond 13.5 dpc (2) exhibited thin ventricular myocardium before 11.5 dpc, while the single knockout mice did not show the same phenotype before 13.5 dpc and (3) exhibited erythrocytosis around 12 dpc which the single knockout mice have never exhibited. These observations suggest that (1) gp130 pathway is involved in the erythropoiesis in the embryonic liver and (2) gp130 and K-Ras are functionally redundant. On the other hand, double knockout mice of *gp130* and N-*ras* or H-*ras* genes as well as triple knockout mice of *gp130*, N-*ras* and H-*ras* genes survived after 13.5 dpc and did not show such phenotypes as double knockout mice of *gp130* and K-*ras* genes, suggesting that K-Ras may be specifically functioning in the erythropoiesis in the embryonic liver.

5. Roles of dopamine receptors *in vivo*

Hiroo Wada, Seiji Hayashizaki, Masahiko Tsunoda, Chieko Konishi, Yumiko Ikeda, Shinobu Nakao, Kenji Nakamura, Kazuki Nakao, Atsu Aiba, and Motoya Katsuki

Dopamine is the principle neurotransmitter for several neural systems in the brain. The dopaminergic system is involved in the inhibitory regulation of the secretion of several peptide hormones in the pituitary as well as in the modulation of motor activity via the nigrostriatal dopaminergic pathway. Dopaminergic system is also implicated in motivated behaviors, emotional stability, and certain aspects of learning and memory. So far, five subtypes of dopamine receptor, designated as D1R to D5R, have been found by cloning. Based on gene structure, pharmacological properties, and signaling molecules, they are classified to two classes, D1-like receptors (D1R and D5R) and D2-like receptors (D2R, D3R and D4R). To investigate the physiological roles of these dopamine receptors in dopaminergic pathway and relationship between dopaminergic system and brain function, we planed to generate the mutant mice which lack each subtype of dopamine receptors. We have generated D1R, D2R, D3R, D4R, and D5R mutant mice. In addition, we generated the mice which lack D1-like receptors or D2-like receptors by crossing the single mutant mice. We have D1RD5R, D2RD4R, and D2RD3RD4R mutant mice. Pharmacological and behavioral analyses of these dopamine receptor mutant mice are under investigation.

6. Analysis of Dopamine D1R D2R double mutant mice

Tomoko Morita, Hiroo Wada, Atsu Aiba, and Motoya Katsuki

D1R and D2R are principal receptors of D1-like and D2-like dopamine receptors, respectively. D1R were shown to mediate stimulation of cAMP formation, whereas D2R were found to be negatively coupled to this cascade. The distribution of the D1R and D2R overlap extensively, especially in the main dopamine-containing systems arising in the midbrain. To elucidate the function of D1R and D2R, we generated the mice which lack both D1R and D2R (D1R(-/-)D2R(-/-) mice). D1R(-/-)D2R(-/-) mice were born at expected frequency but became hypoactive and stopped feeding during second week after birth and died during third week after birth.

7. Utilization of cryopreservation technique of embryos for the production of transgenic (Tg) and knockout (KO) mice.

Kazuki Nakao and Motoya Katsuki

This year, we have produced 8 Tg and 3 KO mouse lines using cryopreserved embryos.

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Center for Experimental Medicine
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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. Genetic diseases and infectious diseases are our major concerns, and we are attempting to produce transgenic mouse models for these diseases.

1. Studies on the roles of IL-1 in the development of autoimmune diseases and in maintaining homeostasis of the body

Reiko Horai, Susumu Nakae, Satoshi Ishii, Taizo Matsugi, Aya Nambu, Kyoko Kagiwada, Shinobu Saijo, and Yoichiro Iwakura

Interleukin (IL)-1 is a proinflammatory cytokine that plays important roles in inflammation, host defense, and the neuro-immuno-endocrine network. IL-1 receptor antagonist (IL-1ra) is an endogenous inhibitor of IL-1, and is supposed to regulate the IL-1 activity. However, its pathophysiological roles in a body remain largely unknown. To elucidate the roles of IL-1ra, IL-1ra-deficient mice were produced by gene targeting. We found that all the mice on a BALB/cA background spontaneously developed chronic inflammatory polyarthropathy resembling rheumatoid arthritis in humans. Elevated levels of serum immunoglobulins and autoantibodies were detected in these mice, suggesting development of autoimmunity. Bone marrow cell replacement experiments showed that bone marrow cells from IL-1ra deficient mice induced arthritis in wild-type mice, and those from wild-type mice suppressed development of arthritis in IL-1ra deficient mice. Moreover, peripheral T cells from IL-1ra deficient mice induced severe arthritis in BALB/c-*nu/nu* mice, while those from wild-type mice did not. These results indicated that bone marrow-derived cells, especially T cells, were impaired and responsible for the development of arthritis in IL-1ra deficient mice. Thus, we show that IL-1ra gene deficiency causes autoimmunity and joint specific inflammation, and suggest that IL-1ra is important in maintaining homeostasis of the immune system. We are now analysing IL-1ra gene in rheumatoid arthritis patients to examine possible involvement of IL-1ra gene mutations in the development of arthritis in humans.

We are also studying roles of IL-1 in the immune system using IL-1α, IL-1β, IL-1α/β and IL-ra-deficient mice. Preliminary experiments suggest that IL-1 plays important roles in T-cell dependent humoral immune response by augmenting expression of co-signaling molecules on T cells upon interaction with antigen presenting cells. Roles of various cytokines in contact hypersensitivity, delayed-type hypersensitivity, and asthma are also being investigated. Furthermore, we have studied the roles of IL-1 in the central nervous system and endocrine system. We found that IL-1 suppressed body weight by causing anorexia and inhibiting lipid storage in adipose tissues. To elucidate the functions of IL-1 in the central nervous system, we have cloned several genes down-stream of IL-1 in the brain, and now characterization of these genes is in progress.

2. Studies on the molecular mechanisms of bone metabolism in normal physiology and in diseases

Hisataka Yasuda, Youngmi Lee, and Yoichiro Iwakura

Bone remodeling is regulated by bone-forming osteoblasts and bone-resorbing osteoclasts, both of which are modulated by a variety of hormones and local factors. An imbalance between bone formation and bone resorption causes various diseases affect-

ing bone metabolism. Osteoclasts are multinucleated cells that derive from hematopoietic cells of the monocyte/macrophage lineage. A co-culture system of spleen cells with osteoblasts or bone marrow stromal cells has been established to produce osteoclasts. In the co-cultures, osteoclast-like cells (OCLs) are formed from spleen cells in the presence of stimulators of bone resorption. The cell-to-cell interaction between osteoblasts/stromal cells and osteoclast progenitors in the co-cultures has been found to be essential for the OCL formation. It was hypothesized that a membrane-bound factor, designated as "osteoclast differentiation factor (ODF)", is expressed on osteoblasts/stromal cells in response to osteotropic factors, and that it transduces a signal essential for osteoclastogenesis to osteoclast progenitors through cell-to-cell interaction.

We previously purified and molecularly cloned osteoclastogenesis-inhibitory factor (OCIF) (also called osteoprotegerin [OPG]). OCIF/OPG is a secreted member of the tumor necrosis factor receptor (TNFR) family, and it inhibits osteoclastogenesis *in vitro* and *in vivo*. Subsequently, we succeeded in molecular cloning of ODF as a ligand for OCIF/OPG. ODF (also called OPG ligand [OPGL], TNF-related activation-induced cytokine [TRANCE], and receptor activator of NF-κB ligand [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 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. Furthermore, we demonstrated that the receptor activator of NF-κB (RANK) is the signaling receptor essential for ODF-mediated osteoclastogenesis, and that OCIF/OPG acts as a decoy receptor for ODF to compete against RANK. It is believed that ODF, RANK, and OCIF/OPG play essential roles in osteoclastogenesis. Recently we demonstrated the importance of osteoblasts in osteoclast differentiation and function as a source of ODF and OCIF. ODF and OCIF produced by osteoblasts up- and down-regulate osteoclast differentiation/function, respectively. The discovery of ODF, OCIF/OPG, and RANK opens a new era in the investigation of the regulation of osteoclast differentiation/function. Identification of novel factors involved in the regulation of osteoclasts is under investigation. It will let us make transgenic or knock-out mice of these genes to understand their functions and such mice would be good models to investigate human diseases.

3. Mechanisms of HIV-1 gene activation in transgenic mice and roles of chemokine receptor in the pathogenesis of AIDS

Jun Tanaka, Hidenori Ozaki, Byung-il Choi, Jiro Yasuda, Reiko Horai, Yoichi Tagawa, Masahide Asano, Shinobu Saijo, Mitsunobu Imai[1], Kenji Sekikawa[2], Manfred Kopf [3], and Yoichiro Iwakura: [1]Department of Virology, Kanagawa Prefectural Health Laboratory, [2]Department of Immunology, National Institute of Animal Health and [3]Basel Institute for Immunology, Switzerland

Since serum HIV-1 load correlated well with the prognosis of the disease, it was suggested that the viral load is one of the major determinants of the disease progression of AIDS. Accordingly, HIV-1 activation mechanisms were extensively studied *in vitro*, and involvement of cytokines including TNF-α, IL-1, IL-6 and IFN-γ was suggested in this process. However, so far roles of these cytokines in the HIV-1 expression *in vivo* have not been well elucidated because of the lack of appropriate animal disease models. In this study, we attempted to elucidate the roles of cytokines in HIV-1 activation *in vivo*.

Transgenic mice carrying a defective HIV-1 genome, which we previously produced, were used as a model for HIV-1 carriers. To examine possible involvement of cytokines in HIV-1 expression, we produced TNF-α-, IL-1-, IL-6- and IFN-γ-deficient HIV-1 transgenic mice, and HIV-1 expression was analyzed after activation with bacterial lipopolysaccharides (LPS). HIV-1 expression in the transgenic mouse spleen was activated 10- to 20-fold by LPS, and the serum p24 Gag protein levels reached 400 pg/ml, nearly equal levels to that seen in AIDS patients. However, this augmentation was suppressed by 60% in TNF-α-deficient mice and by 40% in IL-1α/β-deficient mice. In contrast, no suppression was observed in either IL-6-, IFN-γ-, IL-1α, or IL-1β-deficient mice. These results suggest that TNF-α and IL-1 play important roles in HIV-1 gene activation and selective suppression of these cytokines could improve clinical prognosis and potentially slow progression of the disease. Furthermore, we have found that this activation of HIV-1 by LPS requires DNA replication of the host cells. We showed that this DNA replication is required for the demethylation of the HIV-1 gene.

We have also investigated roles of a chemokine receptor, CXCR4, which is known as a receptor for HIV-1 in the immune system. We have produced CXCR4 conditional targeted mice using a Cre-loxP system, and found that this receptor is important for the survival of T lymphocytes. We are now examining the possibility that CXCR4 is involved in the pathogenesis of AIDS.

4. Development of nephrotic syndrome with severe ascites in mice transgenic for the TT virus ORF1 gene

Hiroshi Yokoyama, Jiro Yasuda, and Yoichiro Iwakura

TT virus (TTV) was discovered from patients with post transfusion hepatitis of unknown etiology in 1997. However, an association between TTV infection and hepatitis is still unclear. To investigate the pathogenesity of TTV *in vivo*, we produced transgenic mice that expressed TTV open reading frame (ORF) 1 under the control of the CMV-β-actin promoter. F1 mice of a founder transgenic mouse (Fst09) expressed high levels of the transgene in the kidney, and all of them developed severe ascites and died before 5 weeks of age. The protein concentration of the serum was low and high levels of protein were detected in the urine. Similar, but milder, phenotype was observed in another founder mouse (Snd15) with a lower level of transgene expression. Histological examination showed abnormal Bowman's capsule cells and vacuolar degeneration of distal tubule cells in both Fst09 F1 mice and Snd15. As compared with wild-type mice, PCNA positive cells were increased in the renal epithelia of both lines of transgenic mice, suggesting their aberrant proliferation. In Fst09 F1 mice, electron microscopic study revealed that the podocytes were immature with complete absence of their foot process. Thus, it was shown that the expression of TTV ORF1 inhibits the normal development of renal epithelial cells and results in the development of nephrotic syndrome in mice. These observations suggest possible involvement of TTV in renal failure in humans.

5. Cloning of a novel 2', 5'-oligoadenylate synthetase-like molecule, Oasl5, in mice

Shinwa Shibata, Shigeru Kakuta, and Yoichiro Iwakura

The 2′, 5′-oligoadenylate synthetase (2-5OAS) represents a family of proteins that are inducible by interferon (IFN) and show activity to polymerize ATP into 2′, 5′-linked oligomers, $pppA(2'p5'A)_n$ (1<<30), upon activation with dsRNA. The 2-5A produced by 2-5OAS is known to activate RNase L, which inhibits viral replication by degrading the mRNA. Thus, the 2-5A system is considered to be an important mechanism for the antiviral action of IFN. In humans, 2-5OAS consists of three isoforms and one related protein, these are p40/46 (short form; OAS1), p69/71 (middle form; OAS2), p100 (long form; OAS3) and p59 (OAS-related protein; OAS-RP). On the other hand, four short form genes of 2-5OAS and one OAS-RP gene have been identified in mice, but so far no middle form or long form genes have been reported. Here, we report cloning of a novel 2-5OAS-like molecule, termed Oasl5 in mice.

The size of *Oasl5* cDNA is about 2 kb and that encodes a protein consisting of 362 amino acids. The amino acid sequence shows 76% similarity to the major mouse 2-5OAS molecule, although several motifs being important for the enzyme activity are not conserved. *Oasl5* mRNA is most significantly expressed in the brain, and relatively weak expression can be found in other organs such as the spleen, kidney, ovary and testis. It is also expressed in embryonic stem (ES) cells. The expression level of *Oasl5* mRNA in ES cells is elevated up to 5-fold in the presence of IFN and about 2-hold in the brain when stimulated with IFN inducer poly(I:C). *In situ* hybridization analysis revealed that *Oasl5* is expressed at high levels in the central nervous system in adult mice. When expressed in *E. coli*, *Oasl5* yields 42 kDa protein that binds to double-stranded RNA, but it does not have enzyme activity as oligoadenylate synthetase. These findings suggest that *Oasl5* plays some roles other than oligoadenylate synthetase in the brain and developing embryos, and imply its function independent of 2-5A systems.

6. Impaired learning with enhanced hippocampal long-term potentiation in PTPδ-deficient mice

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Protein tyrosine phosphatase δ (PTPδ) is a receptor-type PTP that is expressed in the specialized regions of the brain including the hippocampal CA2 and CA3, B lymphocytes and thymic medulla. To elucidate physiological roles of PTPδ, mutant mice were produced by gene targeting. PTPδ-deficient mice were semi-lethal due to insufficient food intake. They exhibited learning impairment in Morris water maze, reinforced T-maze, and radial arm maze tasks. Interestingly, although the histology of the hippocampus appeared normal, the magnitude of long-term potentiation (LTP) both in the hippocampal CA1 and CA3 synapses was significantly enhanced with augmented paired-pulse facilitation (PPF) in the CA1 region. Thus, these results suggest that PTPδ plays a decisive role in hippocampal LTP and learning processes, and that hippocampal LTP is inversely correlated with the spatial learning ability in PTPδ-deficient mice. To our knowledge, this is the

first report that a specific PTP is involved in the regulation of synaptic plasticity, and learning and memory processes.

7. Involvement of EphA2 in the formation of the tail notochord via interaction with ephrinA1

Chie Naruse-Nakajima, Masahide Asano, and Yoichiro Iwakura

Eph receptors have been implicated in the cell-to-cell interaction during embryogenesis. We generated *EphA2* mutant mice using a gene trap method. Homozygous mutant mice developed short and kinky tails, and they had ectopic vertebrae in the tail. In situ hybridization using a *Brachyury (T)* probe found the notochord to be abnormally bifurcated at the caudal end between 11.5 days post coitum (dpc) and 12.5 dpc. Sonic hedgehog (Shh) is known as the inducer of the scrotome from somite, and it is secreted from the notochord. We found that the expression of *Shh* became abnormally bifurcated in the mutant embryos, in consistent with the finding that the notochord formation was abnormal. Furthermore, we found that ectopic scrotomes were formed when scrotome formation was examined using *Pax1* probe. *EphA2* was expressed at the tip of the tail notochord, while one of its ligands, *ephrinA1*, was at the tail bud in normal embryos. In contrast, *EphA2* deficient notochordal cells were spread broadly into the tail bud. These observations suggest that *EphA2* and its ligand are involved in the positioning of the tail notochord cells through repulsive signals between cells expressing these molecules on the cell surface.

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Center for Experimental Medicine

Laboratory of Gene Expression and Regulation

Gene targeting technology has revealed many aspects of gene functions in vivo. Knock out mice offer the opportunity not only to analyze complex gene function in vivo, but also to present various human disease model where new therapeutic approach can be explored. To allow a more detailed dissection of gene function, we try to introduce a point mutation or to disrupt gene in certain lineages (or stages) by conditional gene targeting using Cre-loxP system. In the process of analyzing knock out mice, we have isolated spontaneous mutant mice which develop chylous ascites and edematous limbs. ES cells, which are used for gene targeting, are the only stem cells being cultured in vitro. To elucidate the moecular mechanism that regulates self-renewal and differentiation of pluripotent ES cells, we try to identify a factor(s) cooperating with Oct-3/4 which is a critical transcription factor for maintaining of 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. Prostaglandin D_2 as a Mediator of Allergic Asthma

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Allergic asthma is caused by the aberrant expansion in the lung of T helper cells that produce type 2 (T_H2) cytokines and is characterized by infiltration of eosinophils and bronchial hyperreactivity. This disease is often triggered by mast cells activated by immunoglobulin E (IgE)-mediated allergic challenge. Activated mast cells release various chemical mediators, including prostaglandin D_2 (PGD_2), whose role in allergic asthma has now been investigated by the generation of mice deficient in the PGD receptor (DP). Sensitization and aerosol challenge of the homozygous mutant ($DP^{-/-}$) mice with ovalbumin (OVA) induced increases in the serum concentration of IgE similar to those in wild-type mice subjected to this model of asthma. However, the concentrations of T_H2 cytokines and the extent of lymphocyte accumulation in the lung of OVA challenged $DP^{-/-}$ mice were greatly reduced compared with those in wild-type animals. Moreover, $DP^{-/-}$ mice showed only marginal infiltration of eosinophils and failed to develop airway hyperreactivity. Thus, PGD_2 functions as a mast cell-derived media-

tor to trigger asthmatic responses.

2. Forced expression of terminal deoxynucleotidyl transferase in fetal thymus resulted in a decrease in γδ cells and random dissemination of Vγ3Vδ1 T cells in skin of newborn but not adult mice

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The repertoire of lymphocyte receptor genes encoded in a germline is further diversified by a number of processes, including the template-independent addition of nucleotides (N regions) by means of terminal deoxynucleotidyl transferase (TdT). Normally, mouse γδ T cells in the early fetal thymus, whose T cell receptor (TCR) genes lack N regions and are encoded by Vγ3-Jγl and Vδl-Dδ2-Jδ2 with canonical junctions (invariant Vγ3Vδl), are thought to be the precursors of dendritic epidermal T cells (DETC). We generated mutant mice whose endogenous TdT promotor was replaced with the *lck* promoter through homologous recombination. These mutant mice expressed TdT in fetal thymus, had abundant N regions and infrequent canonical junctions in γ and δ rearrangements, and showed a decreased number of γδ T cells. Various Vγ3Vδl T cells, most of which had N regions in their TCR genes, were found to disseminate in the skin of newborn mutant mice, whereas normal numbers of DETCs with the invariant Vγ3Vδl rearrangement were observed in adult mutants. These data demonstrate that the regulation of TdT expression during fetal development is important for the generation of γδ T cells, and that Vγ3Vδ1 T cells, which have various junctional sequences in their TCR genes, randomly disseminate in skin, but invariant Vγ3Vδ1 T cells have a great advantage for proliferation in skin.

3. Abnormalities of Synapses and Neurons in the Hippocampus of Neuropsin-Deficient Mice

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In the present study, we produced null-mutant mice of neuropsin, an extracellular matrix serine protease, to examine the neural functions of this protein particularly in the hippocampus. Golgi-Cox impregnation and Nissl staining revealed morphological change of cell soma in the mutant mice compared to wild-type mice. However, Golgi-Cox impregnation revealed no apparent change in the dendritic arborization and spine density. Quantitative electronmicroscopic analysis revealed that number of asymmetrical synapses were significantly decreased in the stratum radiatum, the major terminal field of Schaffer-collaterals, whereas free boutons still holding synaptic vesicles but with no synaptic specialization were increased in number in the same microscopic fields. An increased number of parvalbumin-immunoreactive cells (known as fast spiking cells) in mutant was also observed. These results strongly suggest that neuropsin is involved in connectivity of a group of CA1 synapses and consequently in the hippocampal networking.

4. Not only anti-dsDNA monoclonal antibodies but also polyclonal serum antibodies of lupus-prone mice react with histone H3 dimer--- Can anti-histone H3 dimer antibodies replace anti-nucleosome antibodies ?

Yoshiyuki Kanai

For the study of nucleosome(NS) immunology, we tried to purify core histones from swollen lymph node cells of MRL/lpr mice to make pure NSs in vitro. First, whole histones were purified by the previous method. Core histones were clearly separated from histone H1 by a Hitrap SP column with acid-NaCl linear gradient elution. Core histones thus obtained were dialyzed against Tris-saline buffer (25mM Tris, 250mM NaCl,pH7.4). Upon checking their purity on SDS-PAGE , we found one clear band at 32kD over core histones. But H3-depleted core histones could not yield a 32kDa band and the 32kDa band disappeared by heating in the presence of 2-mercaptoethanol, indicating that the 32kD band was a H3 dimer. Historically, H3 dimer formation in the context of NS has been reported in 1977, 3 years after the establishment of the concept/existence of NS in 1974, and recently, it is supposed to be localized outside relative to NS core. Moreover, H3 tail (N-terminal) plays important roles in NS positioning as well as signal transductions through acetylation and phosphorylation. However, it has not been focused in the field of autoimmunity because of its difficulty for preparation. Unexpected advent of H3 dimer facilitated us to study of the presence of antibodies to H3 dimer. Given the recent reports on the preference of so-called anti-dsDNA monoclonal antibodies to NS over dsDNA, we first tested for the reactivity murine monoclonal anti-dsDNA antibodies 2C10 and H241 to H3 dimer by Western blot and

have found that both antibodies did react strongly with H3 dimer; H4 did not at all, but H3 monomer, H2b and H2a reacted weakly. Moreover, reactivity with H3 dimer of polyclonal serum antibodies of lupus prone mice was detected as well. Of note was the finding that the reactivity was increased just before the aggravation of glomerulonephritis/vasculitis. Taken together, it will be worth studying whether anti-H3 dimer antibodies are the same as anti-NS autoantibodies. Timely, we succeeded in the purification of NS in large amounts not only from normal but also from apoptotic cells. So we currently study the fine differences.

5. Novel purification method of nucleosomes by an immunoaffinity-HPLC combination system from cells undergoing apoptosis

Yoshiyuki Kanai

This method is based upon the finding that anti-DNA antibody producing hybridoma 2C10 undergoes apoptosis when hybridoma cells are cultured in serum-free medium at around $5x10^5$ cell/ml in place of serum-containing medium. After 48-h culture, serum-free medium was concentrated ten times with amicon 10 membrane and applied to a Hitrap protein A column. After washing the column with Tris-buffered saline (TBS) until effluent absorbance at 260nm becomes approximately zero, histone-DNA complexes or nucleosomes were detached with a linear gradient of NaCl in TB. Each eluate was concentrated with Ultrafree devices or with Centricon 10 and was filtrated to a Superdex 200 HPLC column which had been equilibrated with 0.25 M NaCl in TB. The HPLC pattern in parallel with agarose gel ectrophoresis and SDS-PAGE has revealed that mono- or oligonucleosomes could be detached at around 0.5-0.6 M, longer oligomers being detached with higher M NaCl ; high yield of oligonucleosomes was achieved with high M NaCl. In order to obtain momers or at least up to dimers, pooled fraction from 0.8-1.2 M NaCl eluates was digested with micrococcal nuclease. Superdex 200 HPLC filtration of the digests clearly separated the mono- or dinucleosomes, and fragmented nucleosome linker DNA.

6. Genetic analysis of lymphatic development and functions in mammals

Hirotake Ichise and Nobuaki Yoshida

The lymphatics are thought to be resonsible for edematous condition in patients, especially in patients suffering from primary lymphedema. However, the lymphatic development in mammals has been unknown for this century 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 knock-out/knock-in mice for some genes, each of which codes potential growth factor or receptor in lymphangiogenesis. We also found a new spontaneous mutant mouse line developing chylous ascites and lymphedema that are thought to be due to lymphatic abnormality. The mutant mouse line has been found and maintained in the animal center of IMSUT. The homozygous mutant mice are viable, but they develop chylous ascites and intestinal lymphedema after suckling and have edematous hindlimbs. Surprisingly, the blood flow is found not only in blood vessels but also in lymphatic vessels of their tissues. This mutant mouse line is useful for the histological and genetic studies of lymphatic development.

7. Identification of a factor which cooperates with Oct-3/4 in undifferentiated ES cells

Mitsuharu Sato and Nobuaki Yoshida

Many studies have shown that the Oct-3/4 is a critical transcription factor for the maintenance of undifferentiated state of ES cells. Actually, in the study of Oct-3/4 deficient ES cells, it was demonstrated that the expression of Oct-3/4 must be controlled at a certain level to hold the ES cells in undifferentiated state. In addition, it was shown that the expression of Oct-3/4 alone can not maintain the state of ES cells unless LIF was added to the culture medium. These results suggest that some factor which is regulated by LIF signaling may cooperates with Oct-3/4. However, how the LIF stimulation and Oct-3/4 contributes to the maintenance of undifferentiated state of ES cells is unclear. *Rex-1* gene, one of the target gene of Oct-3/4 and encoding zinc finger protein, is expressed in undifferentiated cells (e.g. teratocarcinoma and ES cells) and downregulated as the cells to be differentiated. *Rex-1* has a typical octamer binding motif in its promoter region and a novel DNA binding activity, termed Rox-1, was identified on the adjacent site to the octamer motif. The factor(s) which consists of Rox-1 DNA binding activity has not been nailed down yet. To identify the factor consisting Rox-1, we perform DNA affinity chromatography using Oct-3/4-Rox-1 binding site from *Rex-1* gene promoter and analyze how Rox-1 contributes to the maintenance of undifferentiated state of ES cells. We further examine whether LIF signaling affects the activity of Rox-1 function.

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