Advanced Clinical Research Center

Division of Molecular Therapy
分子療法分野

Professor	Arinobu Tojo, M.D., D.M.Sc.	教　授	医学博士	東　條　有　伸
Associate Profesor	Satoshi Takahashi, M.D., D.M.Sc.	助教授	医学博士	高　橋　　聡
Research Associate	Jun Ooi, M.D.	助　手		大　井　　淳
Research Associate	Yasushi Soda. M.D., D.M.Sc.	助　手	医学博士	曽　田　　泰

The main theme of our research is toward the development of novel therapeutic options against intractable hematological disorders including leukemia and lymphoma. For this purpose, we are making every effort to master the mechanisms of normal and neoplastic hematopoiesis on the basis of molecular and cellular biology.

(1) Preclinical study of therapeutic gene transfer mediated by various viral vectors:
We have two main research projects in this field. One is a murine therapeutic model of tumor vaccine secreting GM-CSF (GVAX) in combination with nonmyeloablative allogeneic HSCT. The other is a human experimental model of ribozyme technology for inactivation of leukemogenic fusion mRNA such as BCR-ABL.

(2) Preclinical study of targeted drug delivery using various cell-targeting strategies and novel molecular target agents:
We are developing various cell-targeting strategies using cytokines, adhesion molecules as well as monoclonal antibodies. PEG-liposome has been applied for this purpose. In addition, we have made two types of cytokine derivatives by genetic engineering for preclinical study. We are also studying anti-leukemic effects of a novel signal transduction inhibitor and anti-GvHD effects of a novel cytokine synthesis inhibitor for the future clinical trial.

(3) Analysis of tumor stem cells and search for molecular targets for their elimination:
Cure of malignant tumors requires eradication of tumor stem cells. As a representative model for tumor stem cells, we are studying the identification and characterization of leukemia stem cells using cell tracking strategies and flow cytometry.

(4) Analysis of normal and neoplastic hematopoiesis based on their interaction with microenvironments:
Not only normal but also neoplastic hematopoiesis can be supported by the specific interaction between stem/progenitor cells and bone marrow microenvironments. To simulate this cell to cell contact in vitro, we are using a co-culture system in which stem/progenitor cells are overlaid on the layer of hematopoiesis-supporting stroma cells. This co-culture system is applied for determination of drug sensitivities and gene transfer effects.

1. Development of a unique *in vitro and in vivo* model system of Philadelphia chromosome-positive acute lymphoblastic leukemia (Ph-ALL) with emphasis on cell to cell interaction.

Izawa K, Sekine R, Kobayashi S, Tojo A

Philadelphia chromosome-positive acute lymphoblastic leukemia (Ph-ALL) is one of the most intractable hematological malignancies, readily acquires resistance to chemotherapeutic drugs including imatinib mesylate (IM), and shows a high relapse rate even after allogeneic stem cell transplantation. However, primary blast cells isolated from patients with Ph-ALL are generally susceptible to apoptotic cell death in sort-term suspension culture. Here, we established two Ph-ALL cell lines and characterized their growth properties supported by adhesive interaction with a murine bone marrow stromal cell line, HESS-5. IMS-PhL1 (L1) cells mainly expressed p210-type BCR-ABL mRNA with wild type sequences in the ABL kinase domain and were weakly positive for p190-type mRNA. IMS -PhL2 (L2) cells exclusively expressed p190-type transcripts with Y253H mutation and showed much lower sensitivity to IM, compared with L1 cells. The growth of L1 cells was slowly autonomous in suspension culture, but became more vigorous and their apoptosis was prevented by co-culture with HESS-5 cells. In contrast, the sustained growth and survival of L2 cells was absolutely dependent on direct contact with HESS-5 cells and did not respond to soluble cytokines including SCF, IL3and IL7. Both cell lines adhered to and migrated beneath the HESS -5 cell layer, resulting in the formation of cobblestone areas. This migration was significantly inhibited by the pretreatment of those with a neutralizing antibody against α4-integrin. While non-adherent L1 cells were eradicated by 1 μM IM, a portion of adherent L1 cells could survive even at 10 μM IM. Similarly, adherent L2 cells considerably resisted prolonged exposure to 10 μM IM. Intravenous injection of both cell lines caused leukemia in NOD-SCID mice after distinct latent periods. Leukemia cells appeared in peripheral blood, bone marrow as well as spleen. Interestingly, expression of α5-integrin was significantly down-regulated in both leukemia cells collected from those tissues, but was restored after co-culture with HESS-5. The study of L1 and L2 cells *in vitro* and *in vivo* will not only contribute to further insights into microenvironmental regulation of clonal maintenance and progression of Ph-ALL but also provide a unique model for experimental therapeutics against Ph-ALL.

2. shRNA targeting p190 successfully eliminates Ph-ALL cells with or without ABL kinase domain mutation and cooperates with imatinib.

Futami M, Soda Y, Kobayashi S, Tojo A

In the majority of Philadelphia chromosome (Ph)-positive acute lymphoblastic leukemia (Ph-ALL) cases, the resulting BCR-ABL fusion gene generates 190 kD active tyrosine kinase (p190) which is responsible for leukemogenesis and can be a molecular target for therapy. Although a number of ABL kinase inhibitors including imatinib, nilotinib and dasatinib reveal potent activities against Ph-ALL, acquired resistance caused by point mutations in the kinase domain such as T315I still remains to be overcome. That is why novel strategies are desired in the treatment of Ph-ALL. We previously reported that lentiviral delivery of maxizyme targeting p190 specifically induced apoptosis of Ph-ALL cells. Since RNA interference proved to be a more powerful tool in selective gene silencing, we applied this technology to test whether specific and efficient killing of Ph-ALL cells could be achieved by down-regulation of p190. We designed a series of 21-mer and 27-mer small hairpin RNA (shRNA) targeting p190 and constructed U6 promoter-based plasmid vectors expressing those shRNA, which were screened by transfection of 293T/p190 cells to determine optimal target sites. As a result, three candidate sequences were identified; junctional 27-mer, junctional 21-mer and ABL 21-mer. Then, we inserted each of the shRNA expression cassettes into the lentiviral vector (HIV-U6/shRNA) and prepared high titer virus stock for infection of leukemia cells. shBCR-ABL/21, but not shBCR-ABL/27, induced significant and specific cell death of p190+ Ph-ALL cells in a time-dependent manner. shABL was more potent than shBCR-ABL/21 and also active against p 210+ CML cells as well as 293 cells, but did not substantially affect Ph-negative leukemia cells. Both shABL and shBCR-ABL/21 completely inhibited growth of Ba/F3 cells harboring either wild-type or mutant p190, which renders Ba/F3 resistant to imatinib. Furthermore, both shRNA at low multiplicity of infection cooperated additively with imatinib in growth inhibition of Ba/F3-p190 cells. These data suggest that shRNA targeting p190 may become a therapeutic option in Ph-ALL by improvement of its delivery system such as liposomal encapuslation.

3. Identification and comparative analysis of Pax5 C-terminal isoforms expressed in human cord blood-derived B cell progenitors.

Sekine R, Tojo A

We identified three Pax5 isoforms due to alternative splicing of the C-terminal exons of its gene in cord blood (CB)-derived B cell progenitors cultivated on the murine bone marrow stromal (HESS-5) cells. Apart from wild type (wt), one isoform skips exon 9 without subsequent frameshift (del9), while the other has a frameshift insert between exons 8 and 9, resulting in novel C-terminal sequences (ins8′). Quantitative reverse transcription-polymerase chain reaction analysis revealed that wt mRNA could be detected in CB $CD34^+$ cells, but that del9 and ins8′ isoforms only appeared after one or three weeks of co-culture, respectively. Expression of each isoform mRNA was markedly upregulated during B cell differentiation *in vitro*, and wild type continued to be the most abundant isoform. In a luciferase reporter assay using a synthetic CD19 enhancer, del9 isoform revealed slightly lower activity and ins8′ isoform showed much lower activity, compared with Pax5-wt. Furthermore, retroviral expression of each Pax5 isoform in CB $CD34^+$ cells induced aberrant CD 19 expression in a fraction of immature myeloid cells after one week of culture, although del9 and ins8′ isoforms showed much less potent activity than Pax5-wt. These results suggest that Pax5-wt is quantitively and qualitatively dominant over other C-terminal isoforms during human B cell differentiation program.

4. Efficient retroviral transduction of primary human B cell progenitors: their growth inhibition by Pax5 transgene

Sekine R, Tojo A

We developed a convenient *in vitro* system adapted for genetic manipulation of primary human B cell progenitors using RD 114-pseudotyped retroviral vectors and the murine bone marrow stromal cell support. Cytokine-stimulated human cord blood $CD34^+$ cells could be infected at high efficiency (over 80%) with RD114-pseudotyped retroviral vectors, which were produced by transient transfection of a packaging cell line Plat-F with vector plasmids such as pMX-Ig, pMY-Ig and pMCs-Ig encoding enhanced green fluorescent protein (EGFP) downstream of the internal ribosomal entry site. Transduced cells were seeded on HESS-5 stromal cells in the presence of stem cell factor and granulocyte colony-stimulating factor, allowing extensive production of $EGFP^+$ progenitor B cells beneath the HESS-5 cell layer during several weeks of culture. Using this system, we investigated the consequences of forced Pax5 expression in early B cell progenitors. Eventually, the growth of Pax5/EGFP-transduced B cell progenitors were substantially impaired throughout the culture periods, and after 6 weeks of culture, the number of Pax5/EGFP-transduced $CD19^+$ cells was 40-fold lower than that of EGFP-transduced $CD19^+$ cells. However, surface expression of CD20 and light chain on Pax5/EGFP -transduced $CD19^+$ cells suggests that Pax5 transgene may not interfere with their differentiation. This system is sole and useful method for genetic manipulation of human B cell progenitors.

5. JTE-607, a multiple cytokine production inhibitor, ameliorates disease in a SCID mouse xenograft acute myeloid leukemia model.

Uesato N, Fukui K, Tojo A, Tajima N

Accumulating findings suggest that in acute myeloid leukemia (AML) patients, proinflammatory cytokines and growth factors play important roles in the proliferation and survival of AML cells in an autocrine and paracrine manner, leading to deterioration of AML. JTE-607 is a multiple cytokine inhibitor that potently suppresses production of proinflammatory cytokines. In the present study, we investigated the potency of JTE-607 as an antileukemic agent by exploiting a SCID mouse acute leukemia model. SCID mice injected with anti-asialo-GM1 antibody were exposed to sublethal total-body irradiation at a dose of 3 Gy and then inoculated intravenously with AML cells. JTE-607 was administered using osmotic minipumps. The effects of JTE-607 on mouse survival time, human interleukin (IL)-8 levels in mouse plasma, and proportion of human CD45(+) cells in the bone marrow were studied. The survival time of the mice was strictly dependent on the number of U -937 cells proliferating in vivo. Administration of JTE-607 during the initial 7 days significantly prolonged survival of the mice, suggesting killing activity of JTE-607 against AML cells in vivo. Delayed administration of JTE-607 also prolonged the survival of mice bearing established leukemia with an effect comparable to the maximum tolerable dose of cytarabine. Flow cytometer analysis of bone marrow cells revealed decreased number of human CD45(+) cells. Human IL-8 level was also reduced by JTE-607. Our results indicate that JTE-607 has potential to be a new class of antileukemic drug that exerts

inhibitory activities against both the proliferation and proinflammatory cytokine production of AML cells.

6. JTE-607, a multiple cytokine production inhibitor, induces apoptosis in AML cells via upregulation of $p21^{waf1/cip1}$ and prolongs mouse survival in a SCID xenograft leukemia model

Fukui K, Uesato N, Tajima N, Tojo A

Proinflammatory cytokines and growth factors have been thought to play crucial roles in the pathology of acute myeloid leukemia (AML) by supporting the proliferation and survival of AML cells in autocrine and paracrine manner. JTE-607 was originally identified as a multiple proinflammatory cytokine inhibitor that suppresses production of proinflammatory cytokines from lipopolysaccharide-stimulated peripheral blood mononuclear cells. Herein, we report that JTE-607 exhibits its unique biological activities on the functions of AML cell line, and prolonged survival time in a SCID mouse xenograft leukemia model. JTE-607 suppressed expression and production of cytokines, which are spontaneously upregulated in AML cell lines. JTE-607 also abrogated proliferation of AML cell lines and blasts from AML patients in a concentration range at which colony formation of normal bone marrow cells was not affected. In U-937 cell, JTE -607 sequentially induced cell-cycle arrest at S-phase and apoptosis, being accompanied with the decrease of c-Myc and increase of $p21^{waf1/cip1}$. In the leukemia model that engrafted with U-937 cell, JTE-607 significantly prolonged the mice survival and reduced human cytokine mRNA levels in the bone marrow. These results suggest that JTE-607 may provide a new strategy in chemotherapy of AML patients by its combined effects against proliferation and cytokine production of AML cells.

7. Light emission requires exposure to the atmosphere in ex vivo bioluminescence imaging.

Inoue Y, Izawa K, Sekine R, Tojo A,

The identification of organs bearing luciferase activity by in vivo bioluminescence imaging (BLI) is often difficult, and ex vivo imaging of excised organs plays a complementary role. This study investigated the importance of exposure to the atmosphere in ex vivo BLI. Mice were inoculated with murine pro-B cell line Ba/F3 transduced with firefly luciferase and p190 BCR-ABL. They were killed following in vivo BLI, and whole-body imaging was done after death and then after intraperitoneal air injection. In addition, the right knee was exposed and imaged before and after the adjacent bones were cut. Extensive light signals were seen on in vivo imaging. The luminescence disappeared after the animal was killed, and air injection restored the light emission from the abdomen only, suggesting a critical role of atmospheric oxygen in luminescence after death. Although no substantial light signal at the right knee was seen before bone cutting, light emission was evident after cutting. In conclusion, in ex vivo BLI, light emission requires exposure to the atmosphere. Bone destruction is required to demonstrate luciferase activity in the bone marrow after death.

8. In vitro validation of bioluminescent monitoring of disease progression and therapeutic response in leukaemia model animals.

Inoue Y, Izawa K, Sekine R, Soda Y, Kobayashi S, Tojo A

The application of in vivo bioluminescence imaging to non-invasive, quantitative monitoring of tumour models relies on a positive correlation between the intensity of bioluminescence and the tumour burden. We conducted cell culture studies to investigate the relationship between bioluminescent signal intensity and viable cell numbers in murine leukaemia model cells. Interleukin-3 (IL-3)-dependent murine pro-B cell line Ba/F3 was transduced with firefly luciferase to generate cells expressing luciferase stably under the control of a retroviral long terminal repeat. The luciferase-expressing cells were transduced with p190 BCR-ABL to give factor-independent proliferation. The cells were cultured under various conditions, and bioluminescent signal intensity was compared with viable cell numbers and the cell cycle stage. The Ba/F3 cells showed autonomous growth as well as stable luciferase expression following transduction with both luciferase and p190 BCR-ABL, and in vivo bioluminescence imaging permitted external detection of these cells implanted into mice. The bioluminescence intensities tended to reflect cell proliferation and responses to imatinib in cell culture studies. However, the luminescence per viable cell was influenced by the IL-3 concentration in factor-dependent cells and by the stage of proliferation and imatinib concentration in factor-independent cells, thereby impairing the proportionality between viable cell number and bioluminescent signal intensity. Luminescence per cell tended to vary in association with the fraction of proliferating cells. Although in

vivo bioluminescence imaging would allow non-invasive monitoring of leukaemia model animals, environmental factors and therapeutic interventions may cause some discrepancies between tumour burden and bioluminescence intensity.

Publications

1. Zhang X, Soda Y, Takahashi K, Bai Y, Mitsuru A, Igura K, Satoh H, Yamaguchi S, Tani K, Tojo A, Takahashi TA. Successful immortalization of mesenchymal progenitor cells derived from human placenta and the differentiation abilities of immortalized cells. Biochem Biophys Res Commun. 2006; 351: 853-9
2. Uesato N, Fukui K, Maruhashi J, Tajima N, Tojo A, Watanabe Y. JTE-607, a multiple cytokine production inhibitor, ameliorates disease in a SCID mouse xenograft acute myeloid leukemia model. Exp Hematol. 2006; 34(10): 1385-92.
3. Inoue Y, Izawa K, Tojo A, Sekine R, Okubo T, Ohtomo K: Light emission requires exposure to the atmosphere in ex vivo bioluminescence imaging. Mol Imaging. 2006; 5(2): 53-6.
4. Inoue Y, Tojo A, Sekine R, Soda Y, Kobayashi S, Nomura A, Izawa K, Kitamura T, Okubo T, Ohtomo K: In vitro validation of bioluminescent monitoring of disease progression and therapeutic response in leukaemia model animals. Eur J Nucl Med Mol Imaging. 2006; 33 (5): 557-65.
5. Izawa K, Sekine R, Kobayashi S, Tojo A: Development of a unique *in vitro* and *in vivo* model system of Philadelphia chromosome-positive acute lymphoblastic leukemia (Ph-ALL) with emphasis on cell to cell interaction. Blood 2006; 108(11): 522a
6. Futami M, Hatano T, Soda Y, Kobayashi S, Miyagishi A, Tojo A: shRNA targeting p190 successfully eliminates Ph-ALL cells with or without ABL kinase domain mutation and cooperates with imatinib. Blood 2006; 108(11) 520a

Advanced Clinical Research Center

Division of Cellular Therapy
細胞療法分野

Professor	Toshio Kitamura, M.D., D.M.Sc.	教　授	医学博士	北　村　俊　雄
Associate Professor	Kohichiro Tsuji, M.D., D.M.Sc.	助教授	医学博士	辻　　浩一郎
Research Associate	Toshiyuki Kawashima, M.D., D.M.Sc.	助　手	医学博士	川　島　敏　行
Research Associate	Jiro Kitaura, M.D.	助　手		北　浦　次　郎

Our major projects are (1) the roles of small GTPases and GAPs in the control of cell division and differentiation, (2) Molecular therapy targeting signal transduction pathways, (3) molecular mechanism regulating hematopoiesis, (4) molecular mechanisms of MLL-septin 6 and MLL-AF5q31-induced leukemias, (5) characterization of hematopoietic stem cells, (6) adhesion of mast cells, (7) characterization of new PIR (paired Ig receptors) family proteins and (7) molecular mechanism of leukemogenesis and myelodysplastic syndromes (MDS).

1. Co-ordinate control of cell division and cell differentiation of by the Rho family small GTPases.

Toshiyuki Kawashima, Yukinori Minoshima, Ying Chun Bao, Tomonori Hatori, Yasushi Nomura, Takaya Satoh[1], Yoshito Kaziro[2], Hideaki Nakajima[3], Tetsuya Nosaka, David Williams[4] and Toshio Kitamura: [1]Kobe University, [2]Biochemistry and Cell Biology Unit, HMRO, Kyoto University Graduate School of Medicine, [3]Project of mesenchymal stem cells, The 21st century center of excellence program, Institute of Medical Science, The University of Tokyo, [4]Cincinnati Children's Hospital Medical Center, USA

In the search for key molecules that prevent murine M1 leukemic cells from undergoing IL-6-induced differentiation into macrophages, we isolated an antisense cDNA that encodes full-length mouse MgcRacGAP through functional cloning. In human HL-60 leukemic cells, overexpression of the human MgcRacGAP induced growth suppression and macrophage differentiation. Interestingly, MgcRacGAP localized to the nucleus in interphase, accumulated to the mitotic spindle in metaphase, and was condensed in the midbody during cytokinesis. These findings indicate that MgcRacGAP dynamically moves during cell cycle progression and plays critical roles in cytokinesis. Moreover, the experiment using a GAP-inactive mutant showed that the GAP activity of MgcRacGAP was required for completion of cytokinesis. We also found that MgcRacGAP is phosphorylated by Aurora B at the midbody. Intriguingly, this phosphorylation induced the Rho-GAP activity of MgcRacGAP, which was critical for completion of cytokinesis. We identified S387 as a phosphorylation site responsible for the acquirement of Rho-GAP activity during cytokinesis at the midbody. On the other hand, MgcRacGAP mainly localizes in the nucleus in the interphase. Recently, we have found that MgcRacGAP directly binds transcription factors STAT3 and STAT5, and enhances transcriptional activation of STAT proteins probably as a Rac GAP. In summary, our results suggest that MgcRacGAP plays distinct roles depending on the cell cycle thereby co-ordinating cell division and cell differentiation/proliferation.

2. Molecular therapy targeting signal transduction pathways using small molecule compounds

Toshiyuki Kawashima, Akiho Tsuchiya, Yukinori Minoshima, Ken Murata and Toshio Kitamura:

Internal tandem duplications of the juxtamembrane region of the *Flt-3* (ITD-Flt3) are found in about 30% of the human acute myeloid leukemia patients. We previously identified small molecule compound GTP14565, a specific inhibitor of ITD-Flt3. GTP14564 preferentially inhibited the growth of the Ba/F3 cells transformed by the mutant *Flt-3*, but not Ba/F3 cells driven by the Flt-3 ligand/wild type Flt-3. Based on the in vitro results, we found that ITD-Flt3-induced cell growth was dependent on STAT5 activation while wild-type Flt3-induced cell growth was dependent on ERK and MAPK activation, suggesting the difference in signaling between phathological and physiological conditions. However, GTP14564 is unstable and insoluble, and cannot be used for preclinical trials.

STAT3 is frequently activated in many cancers and leukemias, and is required for transformation of NIH3T3 cells. Therefore, we have started searching for STAT3 inhibitors. We already established an efficient screening protocol for identification of STAT3 inhibitors, and identified several compounds that inhibit STAT3 activation. We will characterized these molecules.

3. Molecular mechanisms of hematopoietic stem cell-supportive activities of ISF a subunit of proton pump-associated ATPases.

Hideaki Nakajima[3], Fumi Shibata, Yumi Fukuchi, Yuko Goto-Koshino, Miyuki Ito, Atsushi Urano, Tatsutoshi Nakahata[2], Hiroyuki Aburatani[5], and Toshio Kitamura: [5]Research Center for Advanced Science and Technology, The University of Tokyo

In the search for stromal-derived growth factors, we have identified a novel secreted short form of immune suppressor factor (ISF) using a combination of a genetic approach and retrovirus-mediated functional screening. This protein was isolated based on its ability to support proliferation of a mutant clone S21, which was established from Ba/F3 cells that are usually interleukin-3-dependent but became dependent on a stroma cell line ST2 after chemical mutagenesis. ISF is a membrane protein harboring six transmembrane domains, and turned out to be a subunit of vacuolar H (+)-ATPase subunit. When overexpressed in bone marrow stroma cells, ISF conferred the cells with an ability to support the growth of S21 cells as well as hematopoietcn stem cells (HSCs). To elucidate the molecular mechanisms, we analyzed the expression profiles using DNA chips, and found that ISF overexpression resulted in the up-regulation of MMP3, and down-regulation of TIMP3 and SFRP-1. We also demonstrated that down-regulation of TIMP3 and SFRP-1 could lead to maintainance of HSCs.

4. Functional characterization of the septin family genes that are fused to MLL in infant leukemias with chromosomal translocations, and molecular analysis on the mechanism of leukemogenesis mediated by MLL-SEPT6.

Ryoichi Ono, Hideaki Nakajima[3], Yasuhide Hayashi[6], Katsutoshi Ozaki, Hidetoshi Kumagai, Toshiyuki Kawashima, Tomohiko Taki[7], Toshio Kitamura, and Tetsuya Nosaka:, [6] Gunma Children's Medical Center, [7]Kytoto Prefectural University of Medicine Graduate School of Medical Science.

We identified a human ortholog of mouse Septin6 as a fusion partner of MLL in three cases of de novo infant acute myeloid leukemia with complex chromosomal abnormalities involving 11q23 and Xq22-24 (Ono, R. et al., Cancer Res. 62, 333-337, 2002). Septins comprise a eukaryotic GTPase subfamily and are involved in cytokinesis. Among septin family genes, nearly complete ORFs of SEPT5, SEPT6, SEPT9, and SEPT11 were fused to 5′ half of MLL in leukemic patients, resulting in formation of chimeric proteins. It was controversial whether the MLL fusion protein is sufficient to induce acute leukemia without additional genetic alterations. We demonstrated that the fusion partner-mediated homo-oligomerization of MLL-SEPT6 is essential to immortalize hematopoietic progenitors in vitro. MLL-SEPT 6 induced myeloproliferative disease with long latency in mice, but not acute leukemia, implying that secondary genotoxic events are required to develop leukemia. We developed in vitro and in vivo model systems of leukemogenesis by MLL fusion proteins, where activated FMS-like receptor tyrosine kinase 3 (FLT3) together with MLL-SEPT6 not only transformed hematopoietic progenitors in vitro but also induced acute biphenotypic or myeloid leukemia with short latency in vivo. In these systems, MLL-ENL, another type of the fusion product that seems to act as a monomer, also induced the transformation in vitro and leukemogenesis in vivo in concert with

activated FLT3. These findings show direct evidence for a multistep leukemogenesis mediated by MLL fusion proteins and may be applicable to development of direct MLL fusion-targeted therapy.

5. Disruption of sept6, a fusion partner gene of MLL, does not affect ontogeny, leukemogenesis induced by MLL-SEPT6, or phenotype induced by the loss of sept4.

Ryoichi Ono, Masafumi Ihara[2], Hideaki Nakajima[3], Katsutoshi Ozaki, Yuki Kataoka-Fujiwara[8], Tomohiko Taki[7], Koh-ichi Nagata[9], Masaki Inagaki[10], Nobuaki Yoshida[8], Toshio Kitamura, Yasuhide Hayashi[8], Makoto Kinoshita[3], and Tetsuya Nosaka.: [8]Laboratory of Gene Expression and Regulation, Center for Experimental Medicine, Institute of Medical Science, The University of Tokyo, [9]Department of Molecular Neurobiology, Institute for Developmental Research, Aichi Human Service Center, [10]Divisions of Biochemistry and Virology, Aichi Cancer Center Research Institute.

Septins are evolutionarily conserved GTP-binding proteins that can heteropolymerize into filaments. Recent studies have revealed that septins are involved in not only diverse normal cellular processes but also the pathogenesis of various diseases, including cancer. SEPT6 is ubiquitously expressed in tissues and one of the fusion partner genes of MLL in the 11q23 trunslocations implicated in acute leukemia. However, the roles of Sept6 in vivo remain elusive. We have developed Sept6-deficient mice that exhibited neither gross abnormalities, changes in cytokinesis, nor spontaneous malignancy. Sept6 deficiency did not cause any quantitative changes in any of the septins evaluated in this study, nor did it cause any additional changes in the Sept4-deficient mice. Even the depletion of Sept11, a close homolog of Sept6, did not affect the Sept6-null cells in vitro, thus implying a high degree of redundancy in the septin system. Furthermore, a loss of Sept6 did not alter the phenotype of myeloproliferative disease induced by MLL-SEPT6, thus suggesting that Sept6 does not function as a tumor suppressor. To our knowledge, this is the first report demonstrating that a disruption of the translocation partner gene of MLL in 11q23 translocation does not contribute to leukemogenesis by the MLL fusion gene.

6. Functional characterization of the AF5q31 gene that is fused to MLL in infant acute lymphoblastic leukemia by chromosomal translocation.

Atsushi Urano, Masaki Endoh[11], Tadashi Wada[11], Yoshihiro Morikawa[12], Miyuki Itoh, Yuki Kataoka[8], Tomohiko Taki[7], Hideaki Nakajima[3], Nobuaki Yoshida[8], Yasuhide Hayashi[8], Hiroshi Handa[11], Toshio Kitamura, and Tetsuya Nosaka: [11]Graduate School of Bioscience and Biotechnology, Tokyo Institute of Technology, [12]Department of Anatomy and Neurobiology, Wakayama Medical University.

We identified a gene AF5q31 from an infant early pre-B acute lymphoblastic leukemia (ALL) with ins (5; 11) (q31; q13q23) as a fusion partner of MLL. The AF5q31 gene encodes a protein of 1163 amino acids, and is homologous to AF4-related genes, including AF4, LAF4, and FMR2 (Taki T et al., PNAS 96, 14535, 1999). The AF4 is directly involved in infant ALL with t (4; 11) (q 21; q23) of poor prognosis, and AF4 knockout mice display altered lymphoid development. The LAF4, a lymphoid-restricted nuclear protein, is expressed at the highest level in pre-B cells and is suggested to play a regulatory role in early lymphoid development. On the other hand, FRM2 was identified as a gene associated with FRAXE mental retardation. Although MLL is known to play an essential role in leukemogenesis by regulating HOX family gene expression, the fusion partner of MLL also appears to be important to modify the MLL function, thereby determining the phenotype of the leukemia. To clarify the role in leukemogenesis and the biological function of AF5q31, we have generated the AF5q31 knockout mice and found that AF5q31 is essential for spermatogenesis. Although most AF5q31-deficient mice died in utero and neonatally, some AF5q31-deficient mice survived. The survived male mice were sterile with azoospermia. Histological examinations revealed the arrest of germ cell development at the stage of spermiogenesis, and virtually no spermatozoa were seen in the epididymis. AF5q31 was preferentially expressed in Sertoli cells. Furthermore, mutant mice displayed severely impaired expression of protamine 1, protamine 2, and transition protein 2, which are indispensable to compact the haploid genome within the sperm head, and an increase of apoptotic cells in seminiferous tubules. Thus, AF5q31 seems to function as a transcriptional regulator in testicular somatic cells and is essential for male germ cell differentiation and survival. These results may have clinical implications in the understanding of human male infer-

tility. Clarification of the AF5q31-mediated gene regulation in testes will also help us to elucidate the molecular mechanism by which the fusion converts normal MLL into the leukemogenic form.

7. Integrin αIIbβ3 induces the adhesion and activation of mast cells through interaction with fibrinogen.

Toshihiko Oki, Jiro Kitaura, Koji Eto[12], Yang Lu, Yoshinori Yamanishi, Hideaki Nakajima[3], Hidetoshi Kumagai, and Toshio Kitamura

Integrin αIIb, a well-known marker of megakaryocyte-platelet lineage, has been recently recognized on hemopoietic progenitors. We demonstrate that integrin αIIbβ3 is highly expressed on mouse and human mast cells and that mast cells, with exposure to various stimuli, adhere to extracellular matrix proteins such as fibrinogen and von Willebrand factor in an integrin αIIβ3-dependent manner. In addition, the binding of mast cells to fibrinogen enhanced proliferation, cytokine production and migration and induced the uptake of soluble fibrinogen, implicating integrin αIIbβ3 in a variety of mast cell functions. Our goal is to delineate the biological significance of integrin αIIbβ3 on mast cells by in vivo allergy and inflammation models using integrin αIIb knockout mice.

8. Identification and characterization of a new pair of immunoglobulin-like receptors, leukocyte mono-Ig-like receptors (LMIRs).

Yoshinori Yamanishi, Jiro Kitaura, Kumi Izawa, Toshihiko Oki, Fumi Shibata, Kaori Tamitsu, Si-Zhou Feng, Hideaki Nakajima[3], Hidetoshi Kumagai, and Toshio Kitamura

We originally identified and characterized two mouse cDNAs in a mouse bone marrow-derived mast cell cDNA library. They encode type I transmembrane proteins including a single variable immunoglobulin (Ig) motif in the extracellular domain with about 90% identity of amino acids. LMIR1 contains immunoreceptor tyrosine-based inhibition motif (ITIM) in the intracellular domain, while LMIR2 harbors a short cytoplasmic tail associating with immunoreceptor tyrosine-based activation motif (ITAM)-bearing molecules such as DAP12. In addition to LMIR1 /2, related genes were identified by homology search in the close proximity on the same chromosome 11: LMIR3 is an inhibitory type receptor like LMIR1, and LMIR4, 5, 6 are activation type receptors like LMIR2. We are now trying to outline the biological role of LMIRs on immune cells by finding the ligands and analyzing deficient mice of LMIRs.

9. Molecular basis of acute leukemia, myelodysplastic syndromes (MDS), MDS overt leukemia, and myeloproliferative disorder (MPD).

Naoko Watanabe, Toshihiko Oki, Koichiro Yuji, Hironori Harada[13], Toshiya Inaba[13], Hideaki Nakajima[3], Tetsuya Nosaka, Jiro Kitaura, and Toshio Kitamura

To elucidate the molecular mechanisms of leukemia, MDS, and MPD, we attempted to establish mouse model using bone marrow transplant (BMT); we transduce mouse bone marrow cells with genes of leukemogenic mutations such as MLL-fusions or AML-1 using retroviruses. In the result, we are now able to reproduce acute leukemia, MPD and MDS-like symptoms in mice, and are now in the process of characterizing these mouse models. We also establish bone marrow-derived immature cell lines transduced with MLL fusions and AML-1 with mutations. While the differentiation of these cell lines are blocked probably through the dominant negative effects of MLL-fusions and the mutated AML-1, they still remain dependent on cytokines including SCF, IL-3, and Flt-3 ligand. In the mouse BMT model, we are beginning to understand that leukemogenesis (acute leukemia as well as MDS overt leukemia) require multiple mutations; mutations that block differentiation, and mutations that block apoptosis or induce factor-independent proliferation. Based on the mouse BMT model, we assume that there are the second hit mutations in addition to mutations such as MLL-fusions and AML-1 in patients' leukemic cells. To identify such mutations, we use retrovirus-mediated expression cloning method. To this end, we make cDNA libraries of patients' leukemic cells, and will isolate cDNAs that give rise to the autonomous growth of the cytokine-dependent cell lines established as stated above. In this way, we isolated ITD-Flt3 (constitutively activated Flt3 mutant found in 30% of patients with acute myeloid leukemia) and some proteins that activate Ras pathways.

Publications

Matsubara, A., Iwama, A., Yamazaki, S., Fruta, C., Hirasawa, R., Morita, Y., Osawa, M., Motohashi, T., Eto, K., Ema, H., Kitamura, T., Vestweber, D., and Nakauchi, H. (2006) Endomucin, a CD34-like sialomucin, marks stem cells throughout development. J. Exp. Med. 202: 1483-1492.

Oki, T., Kitaura, J., Eto, K., Maeda-Yamamot, M., Inagaki, N., Nagai, H., Yamanishi, Y., Kumagai, H. and Kitamura, T. (2006) Integrin αIIbβ3 induces the adhesion and activation of mast cells through interaction with fibrinogen. J. Immunol. 176: 52-60.

Nakajima, H., Shibata, F., Fukuchi, Y., Goto-Koshino, Y., Ito, M., Urano, A., Nakahata, T., Aburatani, H., and Kitamura, T. (2006) Immune suppressor factor confers stromal cell line with enhanced supporting activity for hematopoietic stem cells. Biochem. Biophys. Res. Commun. 340: 35-42.

Nakajima, H., Watanabe, N., Shibata, F., Kitamura, T., Ikeda Y., and Handa, M. (2006) N-terminal region of CCAAT/enhancer binding protein a is critical for cell cycle arrest, apoptosis and functional maturation during myeloid differentiation. J. Biol. Chem. 281: 14494-14502, 2006.

Nakajima, H., Shibata, F., Kumagai, H., Shimoda, K., and Kitamura, T. (2006) Tyk2 is dispensable for induction of myeloproliferative disease by mutant FLT3. Int. J. Hematol. 84: 54-59.

Fukuchi, Y., Shibata, F., Ito, M., Goto-Koshino, Y., Sotomaru, Y., Ito, M., Kitamura, T., and Nakajima, H. (2006) Comprehensive analysis of myeloid lineage conversion using mice expressing an inducible form of C/EBPα. EMBO J. 25: 3398-3410.

Kawashima, T., Bao, Y.C., Moon, Y., Tonozuka, Y., Minoshima, Y., Hatori, T., Kiyono, M., Nosaka, T., Nakajima, H., Williams, D.A., and Kitamura, T. (2006) Rac1 and a GTPase activating protein MgcRacGAP are required for nuclear translocation of STAT transcription factors. J. Cell Biol. 175: 937-946.

Huang, H., Ryu, R., Chang, E-J., Kim, H.J., Kim, H-M., Kitamura, T., Lee, Z.H., and Kim, H-H. (2006) Osteoclast differentiation requires TAK 1 and MKK6 for NFATc1 induction and NF-κB transactivation by RANKL. Cell Death and Differentiation 13: 1879-1891.

Kawakami, Y., Inagaki, N., Salek-Ardakani, S., Kitaura, J., Tanaka, H., Nagao, K., Kawakami, Y., Xiao, W., Nagai, H., Croft, M., and Kawakami, T. (2006) Regulation of dendritic cell maturation and function by Bruton's tyrosine kinase via IL-10 and Stat3. Proc Natl Acad Sci USA. 103: 153-158.

Kawakami, Y., Kitaura, J., and Kawakami, T. (2006) Techniques to study Fc epsilonRI signaling. Methods Mol Biol. 315: 175-189.

Kitaura, J., Kawakami, Y., Maeda-Yamamoto, M., Horejsi, V., and Kawakami, T. (2007) Dysregulation of Src family kinases in mast cells from epilepsy-resistant ASK versus epilepsy-prone EL mice. J Immunol. 178: 455-462.

Sugiyama, D. and Tsuji, K. (2006) Definitive hematopoiesis from endothelial cells in mouse embryo; A simple guidance. Trends Cardiovas Med 16: 45-49.

Yabe, H., Inoue, H., Matsumoto, M., Hamanoue, S., Koike, T., Ishiguro, H., Kike, H., Suzuki, K., Kato, S., Kojima, S., Tsuchida, M., Mori, T., Adachi, S., Tsuji, K., Koike, K., Morimoto, A., Sako, M., and Yabe, M. (2006) Allogeneic hematopoietic cell transplantation from alternative donors with a conditioning regimen of low-dose irradiation, fludarabine and cyclophosphamide in Fanconi anemia. Br. J. Haematol. 134: 208-212.

Sugiyama, D., Ogawa, M., Nakao, K., Ma, F., Osumi, N., Nishikawa, S., Nishikawa, S., Katsuki, M., Asano, S., Arai, K., Nakahata, T., and Tsuji, K. B cell can be obtained from precirculatory yolk sac, but with low frequency. Dev Biol, in press.

Ma, F., Wang, D., Hanada, S., Ebihara, Y., Kawasaki, H., Zaike, Y., Heike, T., Nakahata, T., and Tsuji, K. Novel method for efficient production of multipotential hematopoietic progenitors from human embryonic stem cells. Int J Haematol, in press.

Advanced Clinical Research Center

Division of Infectious Diseases
感染症分野

Professor	Aikichi Iwamoto, M.D., D.M.Sc.	教 授	医学博士	岩 本 愛 吉
Assistant Professor	Takeshi Fujii, M.D., D.M.Sc.	助 手	医学博士	藤 井 毅
Assistant Professor	Ai Kawana-Tachikawa, D.M.Sc.	助 手	医学博士	立川(川名) 愛

The long-term goal of our division is medical sciences on infectious diseases in two directions, from clinic to bench and from bench to clinic. We, therefore, assist patient-care in the Department of Infectious Diseases and Applied Immunology in the affiliated hospital. Our current main subjects are immuno-pathogenesis and drug resistance in HIV.

Analysis of the induction of HIV-1 epitope-specific CTLs after peptide-loaded dendritic cell vaccination

Ai Kawana-Tachikawa, Mariko Tomizawa, Mutsunori Iga[1], Testuya Nakamura[2], Takashi Odawara[2], Aikichi Iwamoto: [1]International Research Center for Infectious Diseases, [2]Department of Infectious diseases and Applied Immunology

Immune response enhanced by therapeutic HIV-1 vaccine may control viral proliferation after discontinuation of potent antiretroviral therapy (PART). Although which strategies for therapeutic vaccination are feasible remains controversial, application of dendritic cells (DCs) as a vaccine adjuvant represents a promising approach to improve deteriorated immune function in HIV-1-infected individuals. The safety and efficacy of DC-based vaccine loaded with HIV-1-derived CTL-epitope peptides were thus investigated in the Research Hospital of IMSUT.

Autologous DCs loaded with seven CTL-epitope peptides with HLA-A*2402 restriction were immunized to four HIV-1-infected individuals under PART. We examined CTL responses to each epitope peptide in the vaccinated individuals. ELISPOT assay to detect IFN-γ production in CD8+ lymphocytes revealed a limited breadth of responses to the immunized peptides in two participants, but no responses in the other two. The differences in immunological response could be attributed to two factors: (1) responders displayed higher nadir CD4 cell counts before starting PART, and (2) responders were immunized with a larger number of DCs per reactive peptide than non-responders. (1)

Analysis of HIV-1 sequence drift around syphilis infection

Ichiro Koga, Takashi Odawara[2], Tetsuya Nakamura[2], Wataru Sugiura[3] and Aikichi Iwamoto: [3]National Institute for Infectious Diseases

Increasing syphilis incidence among men who have sex with men has been reported. Sexually transmitted infections (STIs) such as syphilis not only imply practice of unsafe sex but may contribute to the transmission of HIV-1. Infection with drug-resistant HIV-1 is also increasing in large cities in the world. Although superinfec-

tion may be less frequent than initially concerned, superinfections with drug-resistant HIV-1 have actually been reported.

An HIV-1-infected patient who had been treated successfully with PART presented with syphilitic skin rash, and coincidentally his HIV-1 viral load exploded abruptly to 6.7×10^4/ml. His rebounded plasma HIV-1 was found to contain multiple drug-resistant mutations (DRMs). Therefore, a possibility of superinfection with drug resistant HIV-1 was considered in this index case. Since syphilis infection has been reported to increase HIV-1 viral load, it may also be a driving force of new mutations in HIV-1. We analyzed HIV-1 sequences before and after syphilis infection in four HIV-1-positive patients including the index case to explore DRMs and a possibility of HIV-1 superinfection. Two patients who had already some DRMs at baseline revealed VL increase and further accumulation of mutations around syphilis infection, whereas the other two that were devoid of any DRMs before syphilis infection did not show VL increase or mutation accumulation. No evidence of superinfection was observed in any patient. The accumulation of DRMs in two cases was probably due to loosened adherence to ARVs. The importance of strict adherence should be re-emphasized. (4)

Analysis of HIV-1 specific CD8+T cell response

Eriko Miyazaki, Ai Kawana-Tachikawa, Mriko Tomizawa, Junichi Nunoya and Aikichi Iwamoto

HIV-1 specific CD8+ T cells are thought to play a critical role in controlling HIV-1. Although it is well known that HIV-1 easily mutate and escape from CD8+ T cell recognition in HIV-infected patients, how host immune response fight against the mutated HIV-1 remained unknown. In this study, we analyzed qualitatively HIV-1-specific CD8+ T cells which recognized variant peptides.

We have found a common variant in HLA-A*2402-restricted CTL epitope Nef 138 (RYLPTFGWCF) in previous studies. Nef138 was an immunodominant epitope, and the same amino acid substitution occurred in 80% of HLA-A*2402+ patients. We made two HLA-A 24 tetramers labeled with different fluorochromes: one presented original Nef138 peptide, and the other presented the variant peptide.

Cultured PBMCs from HIV-infected patients were stained with these tetramers and we found there were some populations with different cross-reactivity among Nef138-specific CD8+T cells. Now we are analyzing TCR repertoire of each population to elucidate qualitative difference between them.

Genetic analysis of HIV-1 propagated in Japan

Sayaka Watanabe, Ai Kawana Tachikawa, Tetsuya Nakamura[2] and Aikichi Iwamoto

The error-prone replication of HIV-1 enables it to adapt to host environment. Cytotoxic T lymphocytes (CTLs) are considered to be one of the major driving force of HIV-1 genetic diversity. To elucidate an influence of CTL pressure on HIV-1 adaptation, we have analyzed HIV-1 genomic RNA sequences in HIV-1-infected patients focusing on CTL epitope-coding regions. Especially, we are making a comparison of the sequences between corresponding sexual partners.

Development of an early detection method for pathogens causing acute respiratory infection

Takeshi Matsumura, Takeshi Fujii, Yoshihiro Kitamura[4], Tetsuya Nakamura[2] and Aikichi Iwamoto: [4]Center for Asian Infectious Diseases

Immunochromatography (IC) has recently become a useful method for rapid detection of influenza at clinics, but it cannot detect the infection very early when the amount of viral antigens is still low. PCR can detect viruses earlier, but it is not done easily at every clinic. To develop a rapid detection method for pathogens causing acute respiratory infection, we have employed Loop-mediated isothermal amplification (LAMP) and elucidated appropriate primers for the detection of each pathogen *in vitro*. This year we tested the feasibility of this method for clinical samples.

During the 2005-2006 winter season, nasal swabs (remnants of IC test for influenza at clinics) were collected and frozen-stocked. 236 samples from 230 patients were collected and 91 samples (38.5%) of them were positive for influenzavirus antigen by IC test. To these samples we applied RT-LAMP that can detect influenzavirus A/B, RSV A/B, or metapneumovirus. We could detect influenzavirus A or B from 137 samples (58.1%), which was 1.5 times more than IC detection. RSV could be detected only from 4 samples, and metapneumovirus was not detected from any of them, probably because the samples were collected from otherwise healthy adults who visited clinics with flu-like symptoms. Of note was earlier detection of influenza

infection by LAMP than IC; in two out of three patients whose nasal swabs were taken twice, LAMP revealed positive for both earlier and later samples, whereas IC test was positive only for the later one. Thus LAMP is considered superior to IC in early detection of influenza infection.

Publications

1. Ide, F., Nakamura, T., Tomizawa, M., Kawana -Tachikawa, A., Odawara,T., Hosoya, N., Iwamoto, A. Peptide-loaded dendritic-cell vaccination followed by treatment interruption for chronic HIV-1 infection: A phase 1 trial. J. Med. Virol. 78: 711-718, 2006.
2. Shinoe、T., Wanaka, A., Nikaido, T., Kakuta, Y., Masunaga, A., Shimizu, J., Duyckaerts, C., Imaizumi, K., Iwamoto, A., and Kanazawa, I. The pro-apoptotic human BH3-only peptide harakiri is expressed in cryptococcus-infected perivascular macrophages in HIV-1 encephalitis patients. Neuroscience Letters 393: 102-107, 2006.
3. Hishima, T., Oyaizu, N., Fujii, T., Tachikawa, N., Ajisawa, A., Negishi. M., Nakamura, T., Iwamoto, A., Hayashi, Y., Matsubara, D., Sasao, Y., Kimura, S., Kikuchi, Y., Teruya, K., Yasuoka, A., Oka, S., Saito, K., Mori, S., Funata, N., Sata, T., Katano, H. Decrease in Epstein-Barr virus-positive AIDS-related lymphoma in the era of highly active antiretroviral therapy.Microbes Infect. 8: 1301-7, 2006.
4. Koga, I., Odawara, T., Matsuda, M., Goto, M., Nakamura, T., and Iwamoto, A. Analysis of HIV-1 sequences before and after co-infecting syphilis. In press. Microbes and Infection, 2006.
5. Hoshino, S., Sun, B., Konishi, M., Shimura, M., Segawa, T. Hagiwara, Y., Koyanagi, Y., Iwamoto, A., Mimaya, J., Terunuma, H., Kano, S., and Ishizaka, Y. Vpr in plasma of HIV-1-positive patients is correlated with the HIV-1 RNA titers. In press. AIDS Research and Human Retroviruses.
6. Takahashi, H., Yotsuyanaggi, H., Yasuda, K., Koibuchi,T., Suzuki, M., Kato, T., Nakamura, T., Iwamoto, A., Nishioka, K., Iino, S., Koike, K., and Itoh, F. Molecular epidemiology of hepatitis A virus in the Metropolitan Area in Japan. J. Gastroenterology In press.
7. Wichukchinda, N., Kitamura, Y., Rojanawiwat, A., Nakayama, EE., Song, H., Pathipvanich, P., Auwanit, W., Sawanpanyalert, P., Iwamoto, A., Shioda, T., and Ariyoshi, K. The polymorphisms in DC-SIGNR affect susceptibility to HIV-1 infection. AIDS Research and Human Retroviruses. In press. 2007.
8. Maeda, T., Fujii, T., Matsumura, T., Endo, T., Odawara, T., Itoh, D., Inoue, Y., Ohkubo, T., Iwamoto, A., and Nakamura, T.AIDS-related cerebral toxoplasmosis with hyperintense foci on T1-weighted MR images: A case report. Journal of Infection 53: e167-e170, 2006.
9. Maeda, T., Oyaizu, N., Endo, T., Odawara, T., Nakamura, T., Iwamoto, A., Fujii, T. Pneumocystis jiroveci pneumonia in an AIDS patient: Unusual manifestation of multiple nodules with multiloculated cavities. European Journal of Radiology, In Press.

Advanced Clinical Research Center

Division of Bioengineering
臓器細胞工学分野

Professor	Hideaki Tahara, M.D., Ph.D.	教　授	医学博士	田　原　秀　晃
Lecturer	Takuya Takayama, M.D., Ph.D.	講　師	医学博士	高　山　卓　也
Research Associate	Marimo Sato, Ph.D.	助　手	学術博士	佐　藤　まりも

Our department has been focusing on the development of innovative cancer therapy using immunologic approaches and gene therapy strategies for clinical trials.

Development of innovative cancer therapy using immunologic approaches

a. Potent anti-tumor effects of combination therapy using Interleukin-23 and α-galactosylceramide

Hide Kaneda, Marimo Sato, Yoko Nango, Takeshi Kusafuka, Hideaki Tahara

Co-administration of α-galactosylceramide (α-GalCer) with Interleukin (IL)-12 augmented the anti-metastatic effects in mice. This treatment was associated with the elevation of Natural killer (NK) activity though the increase of Interferon (IFN)-γ activity. IL-23 is a relatively novel cytokine, which consists of a heterodimer of the IL-12p40 subunit and a novel p19 subunit. IL-23 has biological activities similar to but distinct from IL-12. IL-23 can enhance the proliferation of memory T cells and the production of IFN-γ, IL-12 and tumor necrosis factor (TNF)-α from activated CD4+ T-cells. IL-23 is produced by dendritic cells and possesses potent anti-tumor and anti-metastatic activity in murine tumor models. In this study, the combined therapeutic effect of IL-23 and α-GalCer against highly metastatic B16F10 melanoma was investigated. The combined treatment of tumor-bearing mice with α-GalCer plus IL-23 induced the elevation of serum IFN-γ concentration but at the much lower level compared with that of IL-12 treatment. However IFN-γ production was observed in CD 4+ and CD8+T-cells in the lymph nodes obtained from the mice treated with the combination upon stimulation with immobilized anti-CD 3 mAb. In parallel with the augmented IFN-γ production, the tumor-specific cytotoxic and NK activities were significantly augmented with the combined therapy. Furthermore, lymph nodes cells cultured with anti-CD3 mAb alone or α-GalCer and anti-CD3 mAb produced high amount of IFN-γ and IL-17. These findings suggest that the combination treatment using IL-23 and α-GalCer may activate both NKT cells and NK cells, and show potent anti-metastatic effects.

b. Development of anti-angiogenic cancer therapy with vaccination using epitope peptides derived from human vascular endothelial growth factor receptor 1 (VEGFR 1)

Ishizaki H, Tsunoda T, Wada S, Yamauchi M, Shibuya M, Tahara H.

Antiangiogenic therapy is now considered to be one of promising approaches to treat various types of cancer. In this study, we examined the possibility of developing antiangiogenic cancer vaccine targeting vascular endothelial growth factor receptor 1 (VEGFR1) overexpressed on endothelial cells of newly formed vessels in the tumor. Epitope-candidate peptides were predicted from the amino acid sequence of VEGFR1 based on their theoretical binding affinities to the corresponding HLAs. The A2/Kb transgenic mice, which express the alpha1 and alpha2 domains of human HLA-A*0201, were immunized with the epitope candidates to examine their effects. We also examined whether these peptides could induce human CTLs specific to the target cells in vitro. RESULTS: The CTL responses in A 2/Kb transgenic mice were induced with vaccination using identified epitope peptides restricted to HLA-A*0201. Peptide-specific CTL clones were also induced in vitro with these identified epitope peptides from peripheral blood mononuclear cells donated by healthy volunteers with HLA-A*0201. We established CTL clones in vitro from human peripheral blood mononuclear cells with HLA-A*2402 as well. These CTL clones were shown to have potent cytotoxicities in a HLA class I-restricted manner not only against peptide-pulsed target cells but also against target cells endogenously expressing VEGFR1. Furthermore, immunization of A2/Kb transgenic mice with identified epitope peptides restricted to HLA-A*0201 was associated with significant suppression of tumor-induced angiogenesis and tumor growth without showing apparent adverse effects. These results strongly suggest that VEGFR1 is a promising target for antiangiogenic cancer vaccine and warrants further clinical development of this strategy.

c. Genome-wide exploration of tumor-associated antigens using cDNA microarray profiling

Takako Suda, Takuya Tsunoda, Takeshi Watanabe, Naotaka Uchida, Satoshi Wada, Yoichi Furukawa, Yusuke Nakamura, Hideaki Tahara

It has been demonstrated that $CD8^+$ cytotoxic T lymphocytes (CTLs) recognize epitope peptides derived from tumour-associated antigens (TAAs) presented on MHC Class I molecules and then lyse the tumor cells. After the discovery of MAGE family as the first example of TAAs, other TAAs have been discovered using similar immunological approaches. Although the significant progress has been made in the development of cancer vaccine using specific epitope peptides, usefulness of this strategy is still greatly hampered by the fact that limited numbers of TAAs are currently available for the treatment of cancer patients. Although new TAAs have been successfully identified after the extensive efforts using these measures, one might need to admit that useful new TAAs are still in need for clinical application. Development of cDNA microarray technologies, coupled with genome information, has enabled us to obtain comprehensive profiles of gene expressions of malignant cells comparing with those of normal cells. This approach discloses the complex nature of cancer cells and leads to identification of genes of which expression patterns are different in tumors when compared with those of non -transformed cells. Since TAAs should theoretically be expressed excessively and preferentially by the tumor cells but not by the normal tissues, gene expression profiling using cDNA microarray technologies is useful to identify TAAs. We analyzed the expression profiles of the newly identified genes with a genome-wide cDNA microarray technology, selected TAA candidates from these genes using the information, and examined whether they contain antigenic T-cell epitope peptides to prove that they are indeed TAAs. We previously performed gene expression profile analyses of 20 intestinal-type gastric cancers, and identified a set of genes whose expression levels were elevated in the cancer tissues compared to their corresponding non-cancerous tissues. Among them, we in this study focused on *Immunoglobulin superfamily 11 (IGSF11)*. Its expression was also elevated in colorectal cancers and hepatocellular carcinomas as well as intestinal-type gastric cancers. Northern blot analysis showed that it showed abundant expression in testis and ovary. These data suggest that IGSF11 is a good candidate of cancer-testis antigen (CTA). Furthermore, suppression of *IGSF11* by siRNA retarded the growth of gastric cancer cells. To investigate the possibility of clinical application of peptide vaccine to IGSF11, we synthesized candidate epitope peptides for IGSF11 and tested whether the peptides elicit IGSF11-specific CTLs. As a result, we successfully established oligo-clonal CTLs by the stimulation with IGSF11-9-207. In addition,

we also established additional CTLs by IGSF-9V, anchor-modified peptides of IGSF11-9-207. These peptides showed IGSF11-specific cytotoxic activity in an HLA-A*0201-restricted fashion, suggesting that these peptides may be applicable for cancer immunotherapy. These findings have brought novel insight into carcinogenesis of stomach, colon, and liver, and will be helpful for the development of novel therapeutic strategies to a wide range of human cancers.

d. CCL21 rapidly promotes the generation and expansion of tumor-specific cytotoxic T cell in local tumor site

Shigenori Nagai, Takuya Takayama, Tetsuya Saito, Hideaki Tahara

Chemokines and receptors are controlling chemotaxis. CCL21, which is a group of CC chemokine, controls the recruiting of naïve T cells and mature DCs by corresponding receptor CCR7 to secondary lymphoid tissue. Several in vivo studies have demonstrated that local elaboration of CCL21 induced anti-tumor responses accompanied by the infiltration of dendritic cells (DCs) and T cells into local tumor site. However, it has not been fully understood about the functional roles of CCL21 on T cell immunity. In this study, we examined the role of CCL21 on T cell immunity using in vitro co-culture system. We found that CCL21 induced T-helper type 1 (Th1) skewing capacity and promoted the rapid generation of tumor-specific CTLs. These results indicated that T cells stimulated by CCL21 play a significant role in inducing tumor specific immunity through promoting Th1 skewing and CTL generation.

Development of innovative cancer therapy using gene therapy strategies

a. Potent and systemic anti-tumor immunity enhanced by intra-tumoral gene delivery of IL-18 in combination with dendritic cells mobilization in situ by Flt3 ligand therapy.

Tetsuya Saito, Takuya Takayama, Shigenori Nagai, Hideaki Tahara

Interleukin-18 (IL-18), which induces IFN-γ production and enhances the cytolytic activity of NK cells, is considered to be a good candidate to be used for cancer immunotherapy. However, we have already found that treatment with systemic or local administration of IL-18 alone was not enough to induce a potent systemic anti-tumor immunity. On the other hand, we also have reported that dendritic cells (DCs) can capture tumor antigens from tumor cells killed by NK cells activated with IL-18 and efficiently induce tumor-specific CTL in vitro. In order to enhance the systemic anti-tumor response induced by local administration of IL-18 in vivo, we examined the effects of the combination therapy with IL-18 and Flt3 ligand (Flt3L). The Flt3L is a hematopoietic cytokine which increases and mobilizes both DCs and NK cells in vivo.

The C57BL/6 female mice received intradermal inoculation of MCA205 fibrosarcoma on day 0 in the bilateral flanks. On day 5 and 12, mice were treated with in vivo electroporation (IVE) with DNA plasmids carrying cDNA of human Flt3L or EGFP to bilateral hind legs. As the combination therapy, some of the mice were also treated with intra-tumoral injection of adenoviral vector carrying IL-18 gene (Ad.IL-18) or EGFP gene (Ad.EGFP) on day 9 and 16. In the treated tumors, significant anti-tumor effect was observed in mice treated with Ad.IL-18 alone and the ones treated with combination therapy of Flt3L-IVE and Ad.IL-18 when compared to those mice with control ($p<0.01$). The combination treatment with Ad.IL-18 resulted in the more potent anti-tumor response when compared to Flt3L-IVE treatment alone ($p<0.01$), and the complete eradication was observed more frequently (100% vs 33%: $p<0.05$) in mice treated with the combination therapy when compared to ones with Ad.IL-18 treatment alone. In the un-injected tumors, only the combination therapy showed significant anti-tumor. Lymphoid cells in regional lymph nodes of the mice treated with the combination therapy showed a significant cytolytic activity against MCA205. Moreover, cytolytic activity of the combination therapy against YAC-1 (NK target) was significantly higher than that of Ad.IL-18 treatment alone ($p<0.05$). Flow cytometric analysis about tumor infiltrating cells showed that the combination therapy induced a significant increase in number of both NK cells and DCs compared to the control ($p<0.01$). These results suggested that local gene transfer of IL-18 combined with DCs mobilization in situ with Flt3L may enhance the anti-tumor effect and induce a potent systemic anti-tumor immunity.

Publications

Suda T, Tsunoda T, Uchida N, Watanabe T, Hasegawa S, Satoh S, Ohgi S, Furukawa Y,

Nakamura Y, Tahara H. Identification of secernin 1 as a novel immunotherapy target for gastric cancer using the expression profiles of cDNA microarray. Cancer Sci. 97: 411-9, 2006

Shimokawa T, Matsushima S, Tsunoda T, Tahara H, Nakamura Y, Furukawa Y. Identification of TOMM34, which shows elevated expression in the majority of human colon cancers, as a novel drug target. Int J Oncol. 29: 381-6, 2006

Ishizaki H, Tsunoda T, Wada S, Yamauchi M, Shibuya M, Tahara H. Inhibition of tumor growth with antiangiogenic cancer vaccine using epitope peptides derived from human vascular endothelial growth factor receptor 1. Clin Cancer Res. 12: 5841-9, 2006

Advanced Clinical Research Center

Division of Clinical Immunology
免疫病態分野

Professor	Chikao Morimoto, M.D., D.M. Sc.	教　授	医学博士	森　本　幾　夫
Associate Professor	Hirotoshi Tanaka, M.D., D.M. Sc.	助教授	医学博士	田　中　廣　壽
Research Associate	Satoshi Iwata, M.D., D.M. Sc.	特任助手	医学博士	岩　田　哲　史
Research Associate	Hiroto Yamazaki, M.D., D.M. Sc.	特任助手	医学博士	山　崎　裕　人
Research Associate	Tadanori Yamochi, Ph. D.	特任助手	医学博士	矢　持　忠　徳

Our long term goal is to define the molecular and structural basis for the mechanisms of the immune abnormalities observed in various immune-mediated disorders such as autoimmue disease as well as to cure patients suffering from the above immune-mediated disorders. To accomplish this goal, we have focused on defining the structure and function of cell surface and intracellular molecules expressed in human T cells and other cells and on understanding how the immune regulatory system works in normal and disease conditions. Moreover, we will establish the translational research to cure such diseases. Our study will provide new insights into understanding the precise molecular mechanisms that underlie immune abnormalities found in various autoimmune diseases as well as other immune-mediated disorders and will lead to the development of new rational therapy for the manipulation of the abnormalities found in such diseases.

I. β1 integrins and Cas-L (Crk-associated substrate lymphocyte type)

Satoshi Iwata, Teruo Inamoto, Sakiko Inamoto, Yasuyo Urasaki, Yutaka Hashizume, Koji Yo, Shunsuke Kondo, Yukiko Nakamura, Akiko Souta-Kuribara, Osamu Hosono, Hiroshi Kawasaki, Hirotoshi Tanaka and Chikao Morimoto.

β1 integrins play crucial roles in a variety of cell processes such as adhesion, migration, proliferation, and differentiation of lymphocytes. Previously we showed that co-immobilized anti-β1 integrin mAbs or its ligand with a submitogenic dose of anti-CD3 mAb induced a marked increase of IL-2 secretion and proliferative response of T cells, indicating the role of β1 integrins in T cell activation. Next we showed that the ligation of β1 integrins induced protein tyrosine phosphorylation of FAK (focal adhesion kinase), paxillin, and pp105 in H9 T cell line and peripheral T cells. Pp105 was first described in our laboratory as a protein that is predominantly tyrosine phosphorylated by the ligation of β1 integrins in H9 cells. We demonstrated that pp105 is a hematopoietic variant of p130Cas (Crk-associated substrate) by cDNA cloning, and designated Cas-L (Cas lymphocyte type). We found that transfection of Cas-L cDNA into Jurkat T cell line restored β1 integrin-mediated costimulation as well as cell migration, indicating that Cas-L plays a key role in the β1 integrin-mediated T cell functions.

Recently, we have demonstrated that Cas-L is overexpressed and tyrosine phosphorylated in vivo in HTLV-I (human T lymphotropic virus type I) Tax transgenic mice, which develop polyarthritis similar to rheumatoid arthritis. Furthermore, we have found the possible involve-

ment of Cas-L in the pathogenesis of ATL (adult T cell leukemia) through Tax-mediated overexpression and hyper-phosphorylation of Cas-L, which results in markedly enhanced motility of lymphocytes. Furthermore, we have shown that the interaction of Tax and Cas-L may result in the modulation of Tax-mediated transactivation of NF-κB.

Our present projects aim at identifying associating molecules of Cas-L, investigating its biological significance in vitro, and evaluating the clinical relevance of Cas-L-mediated signaling pathways in inflammatory diseases and malignancies.

a. Cas-L and TGF-β (transforming growth factor-β) pathway

In the present study, we demonstrate that Cas -L potentiates TGF-β signaling pathway by interacting with Smad6 and Smad7. Immunoprecipitation experiments revealed that single domain deletion of full length Cas-L completely abolished its docking function with Smad6 and Smad7, suggesting that the natural structure of Cas-L is necessary for its association with Smad 6 and Smad7. On the other hand, both N-terminal and C-terminal deletion mutants of Smad6 and Smad7 still retained their docking ability to Cas-L, suggesting that Smad6 and Smad7 possess several binding motifs to Cas-L. Moreover, Cas-L interaction with MH2 domain, but not with MH1 domain of Smad6 or Smad7, ameliorated TGF-β-induced signaling pathway. Finally, depletion of Cas-L by siRNA oligo attenuated TGF-β induced growth inhibition of Huh-7 cells, with a concomitant reduction in phosphorylation of Smad2 and Smad3. These results strongly suggest that Cas-L is a potential regulator of TGF-β signaling pathway.

b. Cas-L associates with adaptor protein Nck. Role of Cas-L/Nck interaction in β1 integrin - and TCR-mediated cell signaling and migration

In the present study, we show that Cas-L associates with adaptor protein Ncks (Nck1 and Nck2) by β1 integrin- and TCR-mediated stimulation. Co-precipitation experiments revealed that the association of Ncks and Cas-L was dependent of tyrosine phosphorylation of Cas-L, and that Ncks bound to the substrate domain of Cas-L. Co-localization study showed that phosphorylated Cas-L merged with Ncks in the cytoplasm. In addition, it was shown that endogenous tyrosine-phosphorylated Cas-L associated with Ncks in H9 cells upon the stimulation with fibronectin or anti-CD3 mAb. Furthermore, we demonstrated that Cas-L localizes in the lipid raft, where tyrosine-phosphorylated Cas-L interacts with Nck in H9 cells upon stimulation with anti-CD3 mAb. Finally, the depletion of Cas-L by small interfering RNA reduced IL-2 production and migration of H9 cells. Based on the recent study showing that Nck is an important component for the immunological synapse conformation in the lipid raft following the TCR-mediated signaling, our data thus suggest that Cas-L/Nck may play a pivotal role at the immunological synapse in the β1 integrin- and TCR-mediated signaling and cell migration.

c. SHP-2 regulates beta-1 integrin signaling by dephosphorylating Cas-L

Cas-L plays an important role in cell migration and cytokine production. We have reported that protein level of Cas-L is elevated in affected tissues in rheumatoid arthritis or its model mouse (HTLV-I Tax transgenic mice), and that Cas-L/p130Cas may be involved in the RANKL-induced differentiation of osteoclasts.

It has been shown that Cas-L is tyrosine phosphorylated by FAK and Src family tyrosine kinase, however, little is known about the dephosphorylation process of Cas-L. Here we show that SHP-2, a non-receptor tyrosine phosphatase containing SH2 domains, interacts and co-localizes with Cas-L, and dephosphorylates the tyrosine residues of Cas-L. These results suggest the possibility that SHP-2 negatively regulates the tyrosine phosphorylation of Cas-L, and β1 integrin/TCR-mediated cell migration and cytokine production.

II. Structural basis for CD26 mediated T cell costimulation and function in normal and disease conditions.

Kei Ohnuma, Tadanori Yamochi, Shinichiro Kina, Wakae Fujimaki, Nozomu Takahashi, Satoshi Iwata, Osamu Hosono, Hiroshi Kawasaki, Hirotoshi Tanaka and Chikao Morimoto (in collaboration with Nam H Dang, Nevada Cancer Institute, USA).

CD26 is a 110-kDa cell surface glycoprotein that posseses dipeptidyl peptidase IV(DPPIV) (EC. 3.4.14.5) activity in its extracellular domain and a primary marker of activated T cells. In the resting state, CD26 is preferentially expressed on a subset of CD4 memory T cells where they account for the majority of IL-2 secretory capabilities and help for B cell Ig production and are the primary responders to recall antigen such as tetanus toxoid. CD26 is also capable of providing a potent costimulatory or "second" signal

which can augment other activation pathways leading to proliferation, cytokine production and effector functions. The mechanism of costimulation remains unclear since the cytoplasmic domain consists of only 6 amino acid and lacks a phosphorylation site, leading to the conclusion that CD26 interacts with other cell surface molecules. We have already shown that CD26 may interact with CD45RO which modulates TCR/CD3 activity through its intracellular tyrosine phosphatase domain. Recently, we have detected another CD26 binding protein, the mannose-6-phosphate/insulin-like growth factor II receptor (M6P/IGFIIR) as being critical for this interaction for CD26 mediated T cell costimulation in addition to adenosine deaminase (ADA). More recently, we have shown that CD26 localizes into lipid rafts, and targeting of CD26 to rafts is necessary for signaling events through CD26. Importantly, aggregation of CD26 by anti-CD26 mAb crosslinking also causes coaggregation of CD45 into rafts. In addition, we have demonstrated that recombinant soluble CD26 (sCD26) has an enhancing effect on T cell proliferation in the presence of the recall antigen, tetanus toxoid. This enhancement resulted in an increase in the surface expression of the costimulatory molecule CD86 on monocytes following uptake of sCD26.

Currently we are focusing on the molecular and structural basis for CD26-mediated T cell activation signaling and are searching for its ligand directly involved in CD26-mediated T cell costimulation. Furthermore we are focusing on the translational research of utilization of anti-CD26 mAb as well as recombinant soluble CD26 for treatment of malignant tumors, immune-mediated disorders and immune deficiency diseases. Hopefully we will perform phase I clinical trial utilizing humanized CD26 antibody for the treatment of the above diseases, this year.

a. Caveolin-1 triggers T-cell activation via CD 26 in association with CARMA1

CD26 is a widely distributed 110-kDa cell surface glycoprotein, having an important role in T -cell costimulation. We previously demonstrated that CD26 binds to caveolin-1 in antigen-presenting cells (APC), and that following exogenous CD26 stimulation, Tollip and IRAK-1 disengage from caveolin-1 in APC. IRAK-1 is then subsequently phosphorylated to upregulate CD86 expression, resulting in subsequent T cell proliferation. However, it is unclear whether caveolin-1 is a costimulatory ligand for CD26 in T-cell. Using soluble caveolin-1-Fc fusion protein, we now showed that caveolin-1 is the costimulatory ligand for CD26, and that ligation of CD26 by caveolin-1 induces T-cell proliferation and NF-κB activation in a TCR/CD3-dependent manner. We also demonstrated that the cytoplasmic tail of CD26 interacts with CARMA1 in T cells, resulting in signaling events that lead to NF-κB activation. Ligation of CD26 by caveolin-1 recruits a complex consisting of CD26, CARMA1, Bcl10, and IKKβ to lipid rafts. Taken together, our findings hence provide novel insights into the regulation of T-cell costimulation via the CD26 molecule.

b. T cell activation via CD26 and caveolin-1 in rheumatoid synovium

CD26 is a T cell costimulatory molecule with dipeptidyl peptidase IV (DPPIV) activity in its extracellular region. We previously reported that recombinant soluble CD26 (rsCD26) enhances peripheral blood T cell proliferation induced by the recall antigen tetanus toxoid (TT). Recently, we demonstrated that CD26 binds Caveolin-1 on APC, and that residues 201 to 211 of CD26 along with the serine catalytic site at residue 630, which constitute a pocket structure of CD26 /DPPIV, contribute to binding to caveolin-1 scaffolding domain. In addition, following CD26 -caveolin-1 interaction on TT-loaded monocytes, caveolin-1 is phosphorylated, with linkage to NF -κB activation, followed by upregulation of CD 86. Finally, reduced caveolin-1 expression on monocytes inhibits CD 26-mediated CD 86 upregulation and abrogates CD26 effect on TT-induced T cell proliferation, and immunohistochemical studies revealed an infiltration of CD26 + T cells in the sublining region of rheumatoid synovium and high expression of caveolin-1 in the increased vasculature and synoviocytes of the rheumatoid synovium. Taken together, these results strongly suggest that CD26-caveolin-1 interaction plays a role in the upregulation of CD 86 on TT-loaded monocytes and subsequent engagement with CD28 on T cells, leading to antigen-specific T cell activation such as the T-cell-mediated antigen-specific response in rheumatoid arthritis.

c. Anti-CD26 monoclonal antibody-mediated G1-S arrest of human renal clear cell carcinoma caki-2 Is associated with retinoblastoma substrate dephosphorylation, cyclin-dependent kinase 2 reduction, p27kip1 enhancement, and disruption of binding to the extracellular matrix

CD26 is a 110-kDa cell surface glycoprotein with a role in tumor development through its association with key intracellular proteins. In

this report, we show that binding of soluble anti -CD26 monoclonal antibody (mAb) inhibits the growth of the human renal carcinoma cells in both in vitro and in vivo experiments.

Growth inhibition by anti-CD26 mAb was assessed using proliferation assay and cell cycle analysis. Anti-CD26 mAb, chemical inhibitors, dominant-negative, or constitutively active forms of specific signaling molecules were used to evaluate CD26-associated pathways. The in vivo growth-inhibitory effect of anti-CD26 mAb was also assessed in a human renal carcinoma mouse xenograft model.

In vitro experiments show that anti-CD26 mAb induces G1-S cell cycle arrest associated with enhanced p27kip1 expression, down-regulation of cyclin-dependent kinase 2, and dephosphorylation of retinoblastoma substrate. Moreover, our data show that enhanced p27kip1 expression is dependent on the attenuation of Akt activity. Anti-CD26 mAb also internalizes cell surface CD26, leading to decreased binding to collagen and fibronectin. Experiments with a mouse xenograft model involving human renal carcinoma cells show that anti-CD26 mAb treatment drastically inhibits tumor growth in tumor -bearing mice, resulting in enhanced survival.

Taken together, our data strongly suggest that anti-CD26 mAb treatment may have potential clinical use for CD26-positive renal cell carcinomas.

d. Developmental process of CD25+CD4+ regulatory T cells in human thymocytes, cord and adult peripheral blood: cord blood regulatory T cells possess default function.

CD25+CD4+ regulatory T cells (Treg) suppress T cell activation in vitro and regulate multiple immune reactions in vivo. To explore the developmental process of Treg, we investigated the functional differences of Treg from thymocytes (Thy), cord blood (CB) and adult peripheral blood (APB). CB Treg showed weak suppressive activity compared to Treg from Thy or APB. The phenotype was mostly CD45RA+. The FOXP3 protein, typically expressed in Treg, was weak. In contrast, Treg from Thy or APB had strong suppressive activity. Most of APB Treg expressed CD45RA- phenotype with mixture of CD45RA+ phenotype. The features of CD45RA+ APB Treg were between those of CD 45RA- APB Treg and those of CB Treg, and were thought to maturate to CD45RA- Treg. Thymic Treg contained both CD45RA+ and CD 45RA- subsets and expressed FOXP3 protein in both subsets. Although CB Treg showed weak suppressive activity, expanded Treg restored strong suppressive activity with increased expression of FOXP3 protein. These results show the ability of Treg is down regulated after leaving thymus and restores the function to respond to acquired stimulation after birth with change of CD45 isotype. The default function in CB Treg may be important for the rejection of fetus at delivery.

III. Therapeutically Targetting Transcription Factors

Hirotoshi Tanaka, Noritada Yoshikawa (Rheumatology Clinic), Noriaki Shimizu, Chikao Morimoto (Division of Clinical Immunology), Hiroshi Handa (TokyoInstitute of Technology), Motoaki Sano, Keiichi Fukuda (Keio University School of Medicine)

We are interested in the mechanism of eukaryotic gene expression and development of novel therapy and/or drugs which target transcriptional machineries. For this purpose, our recent work is mainly focused on research for conditional regulation of transcription factors including the glucocorticoid receptor and hypoxia-inducible factor-1α.

a. Glucocorticoid receptor project

Glucocorticoid hormones are effective in controlling inflammation and immunity, but underlying mechanisms are largely unknown. It has been shown that both positive and negative regulation of gene expression are necessary for this process. The genes whose activity is negatively modulated in the anti-inflammatory process code for several cytokines, adhesion molecules. Most of them do not carry a classical binding site for regulation by the glucocorticoid receptor (GR), but have instead regulatory sequences for transcription factors such as AP-1 or NF-κB. Considering various severe side effects of glucocorticoids, it may be pharmacologically important to dissociate these negative regulatory function of the GR from induction of genes for metabolic enzymes, expression of which have been shown to be positively regulated by the GR. We propose that a certain class of compounds (surprisingly, some of them are non-steroidal chemicals) may dissociate transactivation and transrepression function of the GR and offer opportunities for the design of such compounds that could function more effectively as antiinflammatory drugs. In this line, we are developing novel therapeutic strategy.

(i) Redox Regulation of the Glucocorticoid Receptor

Redox regulation is currently considered as a mode of signal transduction for coordinated regulation of a variety of cellular processes. Transcriptional regulation of gene expression is also influenced by cellular redox state, most possibly through the oxido-reductive modification of transcription factors. The glucocorticoid receptor belongs to a nuclear receptor superfamily and acts as a ligand-dependent transcription factor. We demonstrate that the glucocorticoid receptor function is regulated via redox-dependent mechanisms at multiple levels. Moreover, it is suggested that redox regulation of the receptor function is one of dynamic cellular responses to environmental stimuli and plays an important role in orchestrated crosstalk between central and peripheral stress responses.

(ii) Development of Dissociating Ligand for the Glucocorticoid Receptor

The GR function could be differencially regulated by ligands. We have recently shown that not only synthetic glucocorticoids but also certain bile acids could differentially modulate GR function. Moreover, the effects of those compounds are indicated to be ascribed to the ligand binding domain of the receptor. In this line, we are going to isolate the dissociating ligand that preferencially promotes transrepression function of the GR. Recently we have demonstrated that certain ligands can modulate interdomain communication of the GR, which will eventually contribute to isolation of novel category of ligands.

On the other hand, receptor specificity is another important aspect of novel GR regulator. In this line, we have shown that cortivazol is extremely specific for GR and does not bind to MR. We are studying the molecular basis for this receptor specificity of the ligand using cortivazol as a model. Recent microarray analyses revealed that GR and MR have distinct effects on the repertoire of target genes in rat cardiomyocyts. We will clarify novel mechanism for regulation of gene expression by GR and MR.

(iii) Molecular biology of small nuclear RNA binding protein HEXIM1

Expression of HEXIM1 is induced by treatment of vascular smooth muscle cells with a differentiation inducer hexamethylane bisacetamide. It is shown that HEXIM1 binds 7 SK snRNA and inhibits P-TEFb-mediated transcriptional elongation process. On the other hand, we have found that HEXIM1 directly associates with the GR in the absence of 7SK and represses GR-mediated transcription. We are currently working on regulation of HEXIM1 expression, physiological role of HEXIM1 in GR action.

b. Hypoxia-inducible Factor (HIF)-1α project

HIF-1α is essential for not only angiogenesis but also development of certain organs. In this line, molecular biology of HIF-1α will provide us possible advantage to characterize and manipulate such processes.

Peripheral T cells encounter rapid decrease in oxygen tension as they are activated by antigen recognition and migrate into inflammatory sites or tumors. Activated T cells, therefore, are thought to have such machineries that enable them to adapt to hypoxic conditions and execute immune regulation in situ. We have recently shown that survival of CD3-engaged human peripheral blood T cells is prolonged under hypoxic conditions and HIF-1 and its target gene product adrenomedullin play a critical role for the process. It is also shown that hypoxia alone is not sufficient but TCR-mediated signal is required for accumulation of HIF-1α in human peripheral T cells. In the present study, we show that TCR-engagement does not influence hypoxia-dependent stabilization but stimulates protein synthesis of HIF-1α, most possibly via PI3K/mTOR system, and that expression of HIF -1α and its target gene is blocked by treatment with rapamycin. Since some of those gene products, e.g., glucose transporters and phosphoglycerate kinase-1, are considered to be essential for glycolysis and energy production under hypoxic conditions and adequate immune reaction in T cells, this TCR-mediated synthesis of HIF-1α may play a pivotal role in peripheral immune response. Taken together, our results may highlight a novel aspect of downstream signal from antigen recognition by TCR with giving insight of a unique pharmacological role of rapamycin. We are currently working with the mechanism of translational regulation of HIF-1α.

IV. Cancer Stem Cells

Hiroto Yamazaki, Hiroyuki Kayo, Shiho Matsumoto, Hiroko Nishida, Nam H. Dang, and Chikao Morimoto

a. Characterization of Cancer Stem Cell Properties in Adult T-Cell Leukemia (ATL)

The existence of cancer stem cells (CSC) has been recently demonstrated in myeloid leukemia and solid tumors. Adult T-cell leukemia (ATL) is a peripheral T-cell neoplasm caused by the human T-cell leukemia virus 1 (HTLV-1) with generally very poor prognosis. The fact that ATL patients can have tumor cells with diverse

appearance and resistance to chemotherapy suggests that ATL cells contain a population of CSC. Since hematopoietic stem cells (HSC) and some CSC are enriched in the side population (SP) fraction, we examined the potential existence of SP cells in ATL cell lines and found 5 of 11 ATL-derived cell lines containing SP cells. However, culture assays following cell sorting revealed that both SP and non-SP cells reconstituted the original cell line pattern, suggesting that the SP fraction of ATL was quite different from other CSC. We also performed extensive analysis of surface antigen markers and found that several HSC-related markers were found on ATL cells. In addition, the level of surface expression for some of the markers were quite variable in range, with proliferative activity being associated with expression levels. In particular, the observed difference in proliferative activity was due mainly to apoptosis of the slow proliferating cells, indicating that the ATL cells consisted of heterogeneous cellular populations. Although we could not identify a small subpopulation of "ATL stem cells", these data suggest that ATL cells exhibit common characteristics with stem cells.

V. Immunobiology of inflammatory cytokines and chemokines

Hiroshi Kawasaki, Chikao Morimoto

We are investigating the linkage of innate immunity and acquired immunity to fully understand the pathogenesis of various immune-mediated disorders. Chemokines are interesting molecules in that they recruit immuocytes to resume immune response and inflammatory response in local tissue.

In allergic disorders, basophils migrate from the blood stream to inflamed tissue sites. Since transbasement membrane migration is an important step for local basophil accumulation, we performed a human basophil transmigration assay using a model basement membrane, Matrigel. IL-3 in the upper chamber was critical for basophil trans-basement membrane migration over baseline levels, since none of the chemoattractants placed in the lower chambers induced migration. RANTES, IL-8, 5-oxo-6E,8Z,11Z,14Z-eicosatetraenoic acid (5-oxo-ETE) and platelet-activating factor (PAF) significantly up-regulated the transmigration of IL-3-treated basophils. Neutralizing experiments indicated the involvement of β2 integrin and matrix metalloproteinase (MMP)-2/9 in basophil transmigration. Real-time quantitative PCR revealed that basophils constitutively expressed transcripts for MMP-9, and at lower levels, MMP-2, but cell-surface expression was only detected for MMP-9. MMP-9 was also detected in the cytoplasm and culture supernatant of the basophils. Treatment with IL-3 up-regulated the surface level of MMP-9 on the basophils. Our results suggest that basophils possess a unique regulatory mechanism for trans-basement membrane migration which is affected by cytokines, chemoattractants, β2 integrin and MMPs, especially MMP-9. MMP-9 may be critically involved in the pathogenesis of local basophil influx in allergic diseases.

In the T cell activation, Cas-L molecule identified by Morimoto et al. is implicated to work as an important adaptor in the receptor- downstream signaling upon the ligation of inflammatory cytokines. Cas-L also exerts trans-activation of various suraface molecules. In pararell with the various events associated with T-cell activation including IL-2 secretiom and upregulation of CD25 expression, the expression of chemokine receptor is also enhanced. We are interested in the pursuit of the possibility of Cas-L mediated trans-activation of chemokine receptor upregulation.

Publications

Ohnuma, K., Inoue, H., Uchiyama, M., Yamochi, T., Hosono, O., Dang, NH., Morimoto, C. T-cell activation via CD26 and caveolin-1 in rheumatoid synovium. Mod Rheumatol. 16: 3-13 (2006)

Inamoto, T., Yamochi, T., Ohnuma, K., Iwata, S., Kina, S., Inamoto, S., Tachibana, M., Katsuoka, Y., Dang, NH., Morimoto, C. Anti-CD 26 monoclonal antibody-mediated G1-S arrest of human renal clear cell carcinoma Caki-2 is associated with retinoblastoma substrate dephosphorylation, cyclin-dependent kinase 2 reduction, p27(kip1) enhancement, and disruption of binding to the extracellular matrix. Clin Cancer Res. 12: 3470-7 (2006)

Suzukawa, M., Komiya, A., Likura, M., Nagase, H., Yoshimura-Uchiyama, C., Yamada, H., Kawasaki, H., Ohta, K., Matsushima, K., Hirai, K., Yamamoto, K., Yamaguchi, M. Trans-basment membrane migration of human basophils : role of matrix metalloproteinase-9. International Immunology. 18: 1575-1583 (2006)

Advanced Clinical Research Center

Division of Medical Data Processing Network System
ゲノム医療情報ネットワーク分野

Professor Tetsuo Shimizu

教 授 清 水 哲 男

The purpose of Research Laboratory of Medical Data Processing Network System is to research and develop advanced system engineering methodology and computer technology suitable for the 21-th century type research hospital. The system is called "Infrastructure for Translational Research of Genome Based Medicine", which is expected to strongly support bi-directional translation between genome based life-science and clinical medicine. Our main research objectives are as follows;
-Construction of knowledge database system for translational research between genomic science and clinical medicine called "e-pathfinder",
-Development of clinical protocol management system using computer technologies called "e-protocol",
-Research and development of agent based simulation for epidemiological and biological analysis and application of "Logical Atomism" and "Hyper-cycle Theory" to systems medical sciences.

1. Integrated knowledge database system (E-pathfinder)

In post-genomic era, bridging genomic science and clinical medicine is the most important issue. To make bi-directional migration of knowledge in both fields efficient, it is necessary to integrate knowledge of genomic science and clinical science in a single architecture with Information Technology. To this end, we defined EBKA (Evidence Based Knowledge Architecture as the single architecture. EBKA has LEAHS (Logically Extended Anatomically Hierarchical Structure) as a knowledge backbone that enables to integrate knowledge from macro to micro. In LEAHS, knowledge is represented in knowledge unit that is a set of logical unit and its supportive evidence.

Focusing on hematology and immunology, we try to develop an integrated knowledge database system (E-pathfinder) prototype, which is able to be customized by users. This system is intended to bridges between clinical and biological knowledge under LEAHS and to represent the huge amount of knowledge using GUI (Graphical User Interface). For instance, it shows clinical information (epidemiology, pathogenesis, diagnosis, treatment and prognosis etc.), models of molecular mechanism of diseases, information of genes involved in diseases, treatment protocols, information of clinical examination and drugs, and so on, in an easy-to-understand way. Furthermore, we plan to incorporate E-pathfinder with other systems useful for translational research, including clinical protocol management system described as below.

In this year we have continued to develop the system for blood and immunological diseases.

We believe that many clinicians and basic scientists might have great insights from this knowledge database system in the future.

2. Clinical protocol management system for translational research

The realization of a clinical method that brings from molecular biological findings requires experiment "in human," that is, "experimental care." Because experimental care entails various risks in the process of the care, it should be done in the maximum of safety and efficiency, which is essential for the success of translational research. We are developing a protocol management system that supports experimental care. In this year we have designed the concept of the protocol management system and also have constructed a prototype of the system.

The system manages optimized operation of experimental care by means of the combination of (1) the protocol development support and (2) the implementation management of protocols. By the word "protocol," we mean a detailed procedure of experimental care, which should ensure the maximum of patient's safety. The first subsystem, protocol development support system, helps to construct protocols. A protocol for an experimental care is constructed by integrating new methods into an existing or standard care procedure. The integrated procedure should be well broken down so that it clarifies what each clinical staff should do in every situation of the care. We call such a concrete level of procedures, "working activity plan." The working activity plan should be developed considering various conditions, for example, personal health conditions and resources of a health care provider. The protocol development support system gives tools for the integration and the break-down task.

At the same time, the compliance with the constructed protocol is also important to accomplish the safe and efficient care. The second subsystem, protocol implementation management system, assists each clinical staff smoothly to comply with the protocol. Based on the working activity plan, the subsystem gives appropriate instructions to members of the staff at the proper timing. When a member completes a task, the result will be recorded on the electric medical record database. Making use of the working activity plan and the database, the subsystem manages and analyzes the experimental data, as well as the support for clinical decisions. These features enable the prevention of wrong care, and the early detection of patient's abnormal conditions. In this year we have developed the system especially for chord blood cell plantation protocol.

3. Research and development of agent based simulation for epidemiological and biological analysis and application of "Logical Atomism" and "Hyper-cycle Theory" to systems medical sciences

Agent based modeling is becoming more important for evidence based policy making. This modeling method is expected to provide traceability of the evidence for economical, social and organizational planning. We apply the method of agent based simulation for the analysis of epidemiological and biological issues.

We developed a new framework of agent based modeling named SOARS (Spot Oriented Agent Role Simulator). In this development, we first aimed at the modeling of the SARS (Severe Acute Respiratory Syndrome) infection in hospital where many kinds of human agents interact with each other. However, it was difficult to represent the complex social behavior of human agents properly by the present modeling platforms. So we developed SOARS as a multipurpose agent based modeling platform implemented in Java computational language.

In hospital, there are various persons who play each role such as "doctor", "nurse" and "patient". They act complex social behavior as their own rules and interact with each other. We regard the persons as autonomous agents with various interactions like cooperation, opposition, or unaware infection. We treat them as a kind of Complex Systems, and abstract the model of agents' rule actions and represent them as computational script language.

Also, in biological view, a human body consists of various organs, and the organs consist of numerous cells. The ordinary biological analyses handle the cells by quantitative methods mainly using differential equation, but the analyses of some diseases caused by functional abnormality of cells need representation of qualitative changes and may properly described by the method of agent based simulation. We are also developing hybrid simulation platform of continuous and discrete agent based system using SOARS. In this year we have developed prototype simulation system especially for blood cell interaction taking Hyper-cycle theory into consideration.

"Logical Atomism" is the basic language system proposed by Bertrand Russell for the foundation of all physical sciences. This theory combined with "Hyper-cycle Theory" will provides us a powerful tool to describe ontological system consisting of many types and classes of lives. We are planning and preparing its appli-

cation to genome based medical sciences through constructing “e -pathfinder” system.

Publications

Jun Nakaya, Tetsuo Shimizu, and Hiroshi Tanaka “Knowledge Platform to use Bio-Medical Semantic WEBs Individually: Bridging Gene Ontologies and Clinical Ontologies at Individual site”, IJCSNS International Journal of Computer Science and Security, Vol. 6, No. 6 No. 2A, February 2006.

Jun Nakaya, and Tetsuo Shimizu “Knowledge Architecture based on Evidence Based Logical Atomism for Translational Research”, IJCSNS International Journal of Computer Science and Security, Vol. 6, No. 6 No. 2A, February 2006.

Jun Nakaya, Tetsuo Shimizu, and Hiroshi Tanaka “A Translational Research (TR) Protocol Generator and Monitor”, IJCSNS International Journal of Computer Science and Security, Vol. 6, No. 6 No. 7B, July 2006.

清水哲男：「ゲノム医療の論理と倫理」，新医療 2006年 3 月号，pp 105−108