Advanced Clinical Research Center

Division of Molecular Therapy
分子療法分野

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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. We also try to develop novel therapies in the field of regenerative medicine using bone marrow-derived mesenchymal stem cells.

(1) Molecular and cellular analysis of BCR-ABL-induced leukemias
Leukemia-specific genetic rearrangements often result in chimeric transcription factors and tyrosine kinases, which appear to be the primary cause of those leukemias. We are studying the molecular and cellular aspects of BCR-ABL-induced acute and chronic leukemia as a model system.

(2) Analysis of the putative role of miRNAs in the pathogenesis of hematological malignancies:
miRNAs are a recently discovered class of small noncoding RNAs that are 18-24 nucleotides long and that downregulate target genes at a post-transcriptional level. Downregulation of specific miRNAs in certain cancers implies that some miRNAs may act as tumor suppressors. We are investigating the role of certain miRNAs in the pathogenesis of hematological malignancies as well as the possibility that miRNAs may be promising targets for therapy and diagnosis.

(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) Multidisciplinary analyis of ATL cells and HTLV-1-infected cells on the basis of multicolor flow cytometry and quantitative monitoring viral load:
ATL cells are morphologically diverse and it is sometimes difficult to estimate ATL cells in peripheral blood exactly. To detect ATL cell specifically, we developed a new analytical procedure.

(5) Translational research on tissue engineering:
To accomplish this goal, we are focusing on the issues including a) identification and characterization of somatic stem cells, b) search for molecules to af fect the growth and differentiation of stem cells, and c) search for suitable

biomaterials as the scaffold to assemble these stem cells on.

1. Conditional activation of Bcr-Abl kinase recapitulates a diverse array of transforming signals in a time-dependent manner.

Harnprasopwat R, Izawa K, Kobayashi S, Kotani A, Tojo A

To revisit the mechanism of Bcr-Abl-induced leukemogenesis and especially to delineate early events upon Bcr-Abl activation, we applied this fusion technology to construct ΔCCp190ER, a p 190Bcr-Abl mutant including ER-LBD at the C-terminus but not CC domain at the N-terminus. Cytokine-dependent human TF-1 cells were retrovirally transduced with ΔCCp190ER as well as wild-type p190 (wt), ΔCCp190 and vector control, respectively. Such a series of transformants were subjected to not only biological assays but also biochemical analysis. ΔCCp190ER, but not ΔCCp190, can support cytokine-independent growth of both Ba/F3 and TF-1 cells in a dose-dependent manner on 4-HT, a synthetic ER-ligand, although excess of 4-HT is rather toxic to these cell lines. Optimal concentration of 4-HT has been determined as 0.5μM. The growth stimulatory effect of 4-HT on ΔCCp190ER -transduced cells was efficiently canceled by imatinib. Expectedly, upon 4-HT binding, a number of phosphotyrosil proteins could be detected. The overall protein profiles of ΔCCp190 ER-induced tyrosine phosphorylation including CrkL and Stat5 resembled p190wt- induced those, but a significant time-interval beyond a day was required for the former to be comparable to the latter in signal intensity. Curiously this holds true for autophosphorylated ΔCCp190ER. It is evidenced that Bcr-Abl constitutively activates NF-κB signaling pathway via unknown mechanism. TF-1/ΔCCp190ER cells were lentivirally transduced with kB/Luc reporter construct and were subjected to monitoring Luc activity upon ligand stimulation. Unstimulated TF-1/ΔCCp190ER-kB/Luc cells revealed a weak but significant Luc activity over the background. GM-CSF, but not 4-HT, moderately enhanced their Luc activities after 24 hrs of stimulation. On the contrary, 4-fold up-regulation of Luc activity was observed after 6 days culture with 4-HT, suggesting the delayed cellular transformation by ΔCCp190ER, which is compatible with delayed enhancement of tyrosine phosphorylation. The present results suggested that Bcr-Abl kinase may evoke early transforming signals in a unique time-dependent manner, leading to established transformed phenotype.

2. Immunophenotype signature of CD8+ T cells indicate impaired development of CD 26high memory subset on imatinib treatment.

Yokoyama K, Tojo A

Based on the expression level of CD26, CD4+ and CD8+ T cells can be divided into 3 (high/int/low) subsets. The role of each subset of CD 8+ T cells has remained to be elucidated. In light of these, we hypothesized that CD8+CD26 high subset might represent memory subset and IM might alter this subset. To test this hypothesis, firstly, we sought to define each CD8+CD26 subset based on their 13 immunophenotype antigen expression via multi-parameter flow cytometry analysis (FACs). To this aim, FACs data of each CD8+CD26 subset from control group (n=20) were subjected to unsupervised hierarchical clustering. As a result, according to the expression level of CD26, we could clearly categorize CD8+ T cells as follows: CD26highCD 8+ T cells are defined as memory T cells which has a phenotype of CD127++, CD28++, CCR 5+ and CD45RO+, CD26intCD8+ T cells as naïve T cells of CD27++, CCR7++, CD62L+ and CD45RA+, and CD26lowCD8+ T cells as effector T cells of IFNγ++, perfolin++, granzymeB++, CD57+ and CD11a++, respectively. We next investigated the effects of IM on 3 distinct subsets during CD8+ T cell differentiation program. We compared immunophenotype signature between IM group and control group according to CD26 subset, and observed a significant decrease of CD8+CD26high subset in IM group. Altered phenotypic profile (decreased CD127 and CD28 expression) was also noted in this subset from IM group (n=17). Lastly, we want to check the impact of IM on primed CD 8+ T cells in vitro. To this aim, purified CD8+ T cells from control were primed with various stimuli and subjected to the grading doses of IM, followed by FACs. CFSE labeling was used for monitoring cell proliferation. We found that human purified CD8+T cells in the presence of therapeutic concentrations of IM showed a reduced activation status and proliferation not

only in response to aCD3 triggering, but also in response to IL7 and IL15 stimuli, which are essential cytokines in the development and/or survival of memory CD8+T cells. Our result not only provides a possible usage of CD26 as a classification marker for CD8+ T cells but also offers another evidence for immunomodulatory effects of IM or the the critical role of Abl kinase in memory CD8+ T cell development, possibly via γc cytokines (IL-7/IL-15) dependent manner.

3. miR-128 is a potent steroid sensitizer and the novel mutation on the miR-128 gene blocks processing of miR-128 in MLL-AF4 ALL

Kotani A, Toyoshima T, Tojo A

MLL-AF4 Acute Lymphocytic Leukemia has a poor prognosis, and the mechanisms by which these leukemias develop are not understood despite intensive research based on well-known concepts and methods. MicroRNAs (miRNAs) are a new class of small noncoding RNAs that post-transcriptionally regulate expression of target mRNA transcripts. We reported that ectopic expression of miR-128b together with miR-221, two of the miRNAs downregulated in MLL-AF4 ALL, restores glucocorticoid resistance through downregulation of the MLL-AF4 chimeric fusion proteins MLL-AF4 and AF4-MLL that are generated by chromosomal translocation t(4; 11). Farthermore, we identified new mutations in miR-128b in RS4; 11 cells, derived from MLL-AF4 ALL patient. One novel mutation significantly reduces the processing of miR-128b. Finally, this base change occurs in a primary MLL-AF4 ALL sample as an acquired mutation. These results demonstrate that the novel mutation in miR-128 b in MLL-AF4 ALL alters the processing of miR-128b and that the resultant downregulation of mature miR-128b contributes to glucocorticoid resistance through the failure to downregulate the fusion oncogenes. The mechanism of the procesing block induced by the mutation cannot be explained by the canonical miRNA processing mahinary, indicating that the novel factor or novel mechanism is involved in the processing block caused by the mutation. The factors and mechanism are investigated.

4. MiR-126 induces B cell differentiation in leukemic and normal hematopoietic progenitor cells.

Harnprasopwat R, Kotani A, Toyoshima T, Tojo A

MLL-AF4 Acute Lymphocytic Leukemia (ALL) has a poor prognosis and shows both myeloid and B cell markers, which imply that common myeloid/B cell progenitor cells are transformed. Our analysis of data published by Lu etc 2005., showed that expression of miR-126 is downregulated in MLL rearranged ALL relative to other types of ALL. When we reexpressed miR-126 in MLL-AF4 ALL derived cell line, SEM, CD20 and CD19 is significantly upregulated, indicating that miR-126 induces differentiation of B cell in MLL-AF4 ALL. cDNA array of miR-126 overexpressing SEM cells showed that B cell related genes were upregulated compared with the control. Furthermore, we analyzed the function of miR-126 in vivo and found that it increases CD19+ cells in the peripheral blood to confirm that miR-126 induces B cell differentiation both in leukemia and normal hematopoietic cells. Finally the mechanism of B cell differentiation induced by miR-126 was analyzed by use of the coculture system of mouse fetal liver cells with TsT 4 stromal cells, showing that miR-126 works on uncommitted hematopoietic progenitor cells. The target genes of miR-126 are being searched by use of the system. These results indicate that miR-126 induces B cell differentiation in both leukemia and normal B cells and plays critical roles in cell fate determination of hematopoietic system.

5. Analysis of the function of AID in CML and Ph+ALL for overcoming "Imatinib resistance"

Kawamata T, Kotani A, Toyoshima T, Tojo A

AID is the master gene of immunoglobulin class switch recombination and somatic hypermutation. Its expression is tightly regulated. However, in cancer, aberrant expression of AID is often found not only in the hematological malignancy but also gastric tumor and seminoma etc. Imatinib is a potent molecular target drug for CML and Ph1ALL. Recently AID was reported to express in Ph1ALL and CML blast crisis and involved in the mutagenesis of Abl, which causes Imatinib resistance. We analyze elaborately the mechanism of the mutanogenesis induced by AID in Ph1+ALL or CML by use of mouse bcr-abl ALL model.

6. Identification of the novel and helpful biomarkers of CNS involvement of hematological malignancy

Ohno N, Kotani A, Tojo A

The diagnosis of CNS involvement of hematological malignancy usually relies on the cyto-

logical or image examination. However, it is not sensitive enough to find it in the early stage. We investigate the novel biomarkers which is sensitive and specific to detect the early stage of CNS involvement of hematological malignancy.

7. Bioimaging analysis of stem cell signal activity in cancer cells using lentiviral reporter vector system

Kobayashi S, Izawa K, Tojo A

Accumulating findings suggest a hierarchical organization of developmental potential, so called stem cell system, in normal as well as malignant tissues. Cancer stem cells have been identified mainly on the basis of cell surface marker and/or side population phenotype, but not of functional parameters they present. We introduce a novel strategy to detect stem cell signals including Tert, Wnt, Notch and NF-κB, which are important for development and/or maintenance of stem cell function. Cancer cells were transduced with lentiviral reporter vectors harboring yellow fluorescent protein (YFP) analogue (Venus) or firefly luciferase (Luc) driven by responsive elements to individual stem cell signals, and then subjected to FACS or CCD camera for visualizing as fluorescence or bioluminescence intensity, respectively. This reporter assay enabled us to evaluate each stem cell signal activity, particularly in a single living cell. High transduction efficiency of VSV-G pseudotyped lentiviral vectors made it possible to analyze primary samples such as leukemia cells. Such a bioimaging analysis combined with the standard procedures will contribute to elucidate the nature of cancer stem cells and to identify target molecules for therapy.

8. Multi-color flow cytometric analysis of ATL cells and HTLV-1 infected cells in the peripheral blood of asymptomatic carriers.

Tian Y, Kobayashi S, Ohno N, Uchimaru K, Tojo A

ATL cells are morphologically diverse and it is sometimes difficult to estimate ATL cells in peripheral blood exactly. To detect ATL cell specifically, we developed a new analytical procedure using multi-color flow cytometry. CD4-positive cells from peripheral blood of 7 acute type ATL patients were preliminarily analyzed by this procedure. Combination of CD3 and CD 7 expression clearly discriminated ATL cells from HTLV-1 infected non-ATL cells and normal CD4 T cells. Furthermore we detected other CD4-positive cell population than ATL cells and normal CD4-positive T cells by this analytical system. These cells might be HTLV-1 infected cells to progress into ATL and we are now characterizing these cells. The diagnosis of smoldering type ATL is sometimes difficult because of morphologically subtle abnormality. We analyzed CD4-positive cells in the peripheral blood of 26 asymptomatic carriers and smoldering type ATL patients using this multi-color FACS system. Our FACS system detected abnormal pattern of CD3 and CD7 expression on CD4-positive cells in peripheral blood of smoldering type ATL patients and might be useful for screening smoldering type ATL patients.

9. Clinical study on bone tissue engineering

Kagami H, Agata H, Nagamura-Inoue T, Chinzei M, Tojo A.

Bone marrow stromal cells (BMSCs) have been widely used for bone tissue engineering. Although bone tissue engineering using BMSCs is considered feasible, little is known about the clinical efficacy of this novel treatment. We have conducted a clinical trial to test if BMSCs will be clinically effective in regenerating bone in patients with severe maxillary and/or mandibular bone absorption who undergo dental implant surgery. Ten patients (2 males and 8 females, average age 52.3) were enrolled in this clinical study. Two of them were dropped because of possible contamination risk to autoserum or insufficient number of cultured cells. The remaining eight patients underwent cell transplantation and all of them had implant installation. The results from X-P, CT and histological analyses showed bone regeneration in all patients who underwent cell transplantation and the average bone area was 41.9% at 6 months after transplantation, though significant deviations were observed in the parameters such as cell numbers, ALP activities and regenerated bone area among individuals. Bone regeneration using autologous BMSC-derived osteogenic cells was feasible and considered safe during the observation period. While the clinical study seems promising, it also highlights some problems, such as the unstable nature of somatic stem/progenitor cells and individual variation. Currently, we are planning a next clinical study on bone tissue engineering aiming to overcome those problems.

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Advanced Clinical Research Center

Division of Cellular Therapy
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Our major projects are (1) Co-ordinated control of cell division and differentiation by a crosstalk between JAK/STAT and small GTPases, (2) Molecular therapy targeting signal transduction pathways, (3) Characterization of a PIR (paired Ig receptor) family (LMIR/MAIR/CLM) and (4) Elucidation of molecular basis of leukemia, myelodysplastic syndromes, and myeloproliferative neoplasms.

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

Toshiyuki Kawashima, Toshihiko Oki, Yukinori Minoshima, Kohtaro Nishimura, Ying Chun Bao, Tomonori Hatori, Yasushi Nomura, Noriko Takahashi, Takaya Satoh[1], Yoshito Kaziroh[2], Tetsuya Nosaka, David Williams[3] and Toshio Kitamura: [1]Kobe University, [2]Biochemistry and Cell Biology Unit, HMRO, Kyoto University Graduate School of Medicine, [3]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 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. We also demonstrated that MgcRacGAP directly bound transcription factors STAT3 and STAT5, and enhanced transcriptional activation of STAT proteins as a Rac GAP. Recently, we have shown that MgcRacGAP harborsa functional NLS and works as a nuclear chaperon together with Rac1.

In addition, we have recently shown using an MgcRacGAP-GFP fusion protein that expression of MgcRacGAP increases in the early G1 phase in parallel with Geminin, suggesting that

MgcRacGAP may play some roles in G1 check point. In summary, our results suggest that MgcRacGAP plays distinct roles depending on the cell cycle thereby co-ordinating control of cell division and determination of cell fate.

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 pathological 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. Through the screening of a library of small molecule compounds, we found the compounds RJSI-1 and RJSI-2 that inhibited STAT3 activation. RJSI-2 also inhibited activation of STAT1, STAT5, JAK1 and JAK2, however RJSI-2 is not a kinase inhibitor. On the other hand, RJSI-1 inhibited nuclear transport of phosphorylated STAT proteins, implicating a novel mechanism in inhibiting STAT proteins. We are now in the process of analyzing molecular basis of RJSI-1 and 2 inhibition of STAT proteins, and evaluating its effects in a tumor-burden model. In addition, we have started collaboration with a Japanese pharmaceutical company and a US biotech venture company to modify these compounds for optimization to develop anti-cancer drugs.

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

Hideaki Nakajima[4], Fumi Shibata, Yumi Fukuchi[4], Yuko Goto-Koshino, Miyuki Ito, Atsushi Urano, Tatsutoshi Nakahata[2], Hiroyuki Aburatani[5], and Toshio Kitamura: [4]Keio University School of Medicine, [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. Integrin αIIbβ3 induces the adhesion and activation of mast cells through interaction with fibrinogen.

Toshihiko Oki, Jiro Kitaura, Koji Eto[6], Yang Lu, Yoshinori Yamanishi, Hideaki Nakajima[4], Hidetoshi Kumagai, and Toshio Kitamura: [6]Laboratory of Stem Cell Therapy, Institute of Medical Science, The University of Tokyo

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 mod-

els using integrin αIIb knockout mice.

5. Identification and characterization of a novel family of paired Ig (immunoglobulin-like) receptors LMIRs.

Yoshinori Yamanishi, Jiro Kitaura, Kumi Izawa, Masahiro Sugiuchi, Ayako Kaitani, Yutaka Enomoto, Toshihiko Oki, Fumi Shibata, Kaori Tamitsu, Si-Zhou Feng, Hideaki Nakajima[4], Jiro Kitaura, and Toshio Kitamura

We originally identified and characterized two mouse cDNAs from a mouse bone marrow-derived mast cell cDNA library. They encoded 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 LMIR4-8 are activation type receptors like LMIR2 (LMIR4 and LMIR8 are missing in humans). LMIRs are also called CLMs or MAIRs. Those receptors are mainly expressed in cells involved in innate immunity including mast cells, granulocytes, monocytes, dendritic cells. In addition, some of the receptors are also expressed in some cells in colon, trachea, and lung, indicating that these receptors play important roles in innate immunity. We have recently identified a ligand for LMIR5, and have established gene-disrupted mice lacking LMIR3, LMIR4 or LMIR5. Investigation to elucidate the biological roles of LMIRs on immune cells is now underway.

6. Molecular basis of acute leukemia, myelodysplastic syndromes (MDS), MDS overt leukemia, and myeloproliferative neoplasm (MPN).

Naoko Watanabe, Yukiko Komeno, Naoko Kato, Yutaka Enomoto, Toshihiko Oki, Koichiro Yuji, Yuka Harada[7], Hironori Harada[7], Toshiya Inaba[8], Hideaki Nakajima[4], Tetsuya Nosaka, Jiro Kitaura, and Toshio Kitamura: [7]Department of Hematology/Oncology and [8]Department of Molecular Oncology, Research Institute for Radiation Biology and Medicine, Hiroshima University

To elucidate the molecular mechanisms of leukemia, MDS, and MPN, we established mouse model using bone marrow transplant (BMT); we transduced mouse bone marrow cells with genes of leukemogenic mutations derived from patients including MLL-fusions and BCR-Abl, and mutant forms of AML1 and C/EBPa using retroviruses. The bone marrow cells transduced with these mutant genes derived from patients were transplanted to irradiated mice. Using this mouse BMT model, we have shown several interesting things; 1) Combination of class I (MLL-Sept6) and class II mutations lead to development of acute leukemia; 2) A class II mutation (AML1 mutations) induced MDS-like disease, and some of the mice progressed to acute leukemia with additional mutations such as overexpression of Evi1; 3) Combination of BCR-Abl and Hes1 expression induced CML blast crisis (BC) like disease. In fact, overexpression of Hes1 was demonstrated in 8 of 20 patients with CML-BC but not in patients with CML-chronic phase; 4) Two classes of C/EBPa mutations (N-terminal and C-terminal mutations) collaborate with each other in inducing acute leukemia in mouse BMT models, probably working as class I and class II mutations.

We also establish bone marrow-derived immature cell lines transduced with MLL fusions and Hes1 overexpression. While the differentiation of these cell lines is blocked probably by MLL-fusions and Hes1 overexpression, they still remain dependent on IL-3. In the mouse BMT model, we are beginning to understand that leukemogenesis (acute leukemia as well as MDS/AML and CML-BC) 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 RasGRP4 that activate Ras pathways. We are now testing the effects of RasGRP4 in mouse BMT models.

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Advanced Clinical Research Center

Division of Infectious Diseases
感染症分野

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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. Our current main subject is immune-pathogenesis of HIV-1 infection. We wish to clarify how cellular immune responses can control HIV-1 infection in some patients but not in others. We work together with the staffs in the Department of Infectious Diseases and Applied Immunology in the IMSUT hospital and apply the research results to the people living with HIV-1/AIDS.

HIV-1 viral burden has an impact on Th1- and Th17-related cytokines/chemokines production of T cells

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In HIV-1 infection, plasma viral load (VL) stabilize after a period of acute infection. The stable VL, known as viral set point, is an important clinical parameter related to disease progression. Although high VL is known to associate with rapid progression to AIDS, the mechanisms of immune system collapse has not been elucidated. To find out how HIV-1 affects comprehensive immunologic feature, we examined production of large variety of cytokines/chemokines from peripheral blood mononuclear cells (PBMCs) in HIV-infected subjects with different set point. Treatment-naive HIV-1-infected subjects with Low VL (LVL) and high VL (HVL) were recruited in this study. The median VL was 1,200 copies/ml (range 53 to 3,600) and 62,000 copies/ml (range 25,000 to 500,000) in LVL and HVL groups, respectively. PBMCs were stimulated with Phytohemagglutinin (PHA) for 48hrs, and levels of 25 cytokines/chemokines in culture medium were evaluated using microbeads array system. We also performed multi-color flow cytometric analysis to reveal phenotypic profiles (differentiation, activation, and exhaustion status) of T cells. Production levels of MIP-1a, MIP-1b, RANTES, soluble IL-2R, IFNγ, IL-7, and IL-17 were significantly low in HVL subjects compared to LVL subjects. In contrast, there was no difference in production of Th2 cytokine (IL-4, IL-5, and IL-13) and IL-10. In central memory (CM) and effector memory (EM) CD4+ and CD8+ T cells, frequencies of activated (CD38+) and exhausted (PD-1+, CTLA-4+) cells were significantly higher in HVL subjects than LVL subjects. Levels of these cytokines/chemokines production were inversely correlated to percentage of CD38+, PD-1+ and CTLA-4+ of T cells. These data suggest that high viral burden causes dysfunction of Th1- and Th17- related, but not Th2 and Treg, immune response in HIV-1 chronic infection. Persistent activation of T cells by a large amount of HIV-1 may cause aberrant signal

pathway specific for Th1- and Th17-related response.

1. Influence of polymorphism in dendritic cell-specific intercellular adhesion molecule-3-grabbing nonintegrin-related (DC-SIGNR) gene on HIV-1 trans-infection.

Dayong Zhu, Ai Kawana-Tachikawa, Aikichi Iwamoto, Yoshihiro Kitamura[1]: [1]Research Center for Asian Infectious Diseases, the Institute of Medical Science, the University of Tokyo, Tokyo, Japan

The dendritic cell-specific intercellular adhesion molecule-3-grabbing nonintegrin (DC-SIGN) and DC-SIGN-related (DC-SIGNR) molecules on the cell surface are known to enhance human immunodeficiency virus type 1 (HIV-1) infection by capturing the virions and transmitting them to CD4+ T-cell, a process termed trans-infection. The neck region and carbohydrate recognition domain of the two proteins are important for efficient binding to the HIV-1 envelope protein. DC-SIGNR is polymorphic in Exons 4 and 5 that encode the neck region and carbohydrate recognition domain, respectively; the former contains a variable number of tandem repeats, and the latter the SNP (rs2277998). Since it remains unclear whether the DC-SIGNR polymorphism is related to the risk of HIV-1 infection, we tested possible effects of the polymorphism on HIV-1 trans-infection efficiency, by constructing six kinds of cDNAs encoding DC-SIGNR variants with various numbers of repeat units and various SNP. We were able to express the variants on the surface of Raji cells, a human B cell line. Flow cytometry showed that all the tested DC-SIGNR molecules were efficiently expressed on the cell surface at various levels; the assay for HIV trans-infection efficacy showed that all the tested variants had that activity with different efficacy levels. We found a correlation between the HIV trans-infection efficiency and the mean fluorescent intensity of DC-SIGNR expression ($R(2)=0.95$). Thus, our results suggest that the variation of the tested DC-SIGNR genotypes affects the efficacy of trans-infection by affecting the amounts of the protein expressed on the cell surface.

2. Generation of recombinant monoclonal antibodies against an immunodominant HLA-A*2402-restricted HIV-1 CTL epitope

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Cellular immune response by cytotoxic T lymphocytes (CTLs) is a critical line of host defense against human immunodeficiency virus type 1 (HIV-1). The first step in the CTL-based immune response is CTL activation, which is triggered through antigen recognition by a clonotypic T cell receptor (TCR). Therefore, molecular interaction between the peptide/MHC class I complexes (pMHCs) and TCR is fundamental to the effector function of CTLs. To understand cellular immune responses against HIV-1 infection, both antigen presentation and cellular responses should be analyzed. A decade ago, Altman et al. developed a system to analyze and evaluate the phenotype of antigen specific T lymphocytes using pMHC tetramers. However, relatively few studies have been performed to examine antigen presentation at cellular and molecular levels. Lack of a suitable reagent has precluded the direct visualization and quantification of pMHCs derived from infecting HIV-1. Antibodies against HIV-1 specific pMHCs could be a useful tool to analyze antigen presentation both qualitatively and quantitatively, and the molecular interaction between pMHC and their ligands. However, it is notoriously difficult to isolate monoclonal antibodies against pMHCs by the conventional hybridoma technique.

To isolate monoclonal antibodies against an immunodominant HIV-1-derived CTL epitope in the nef gene, we panned phage clones from a human scFv phage display library with biotinylated Nef 138-10 / HLA-A*24 (A 24) and streptavidin-coated magnetic beads. We repeated the panning three times with decreasing amount of antigen. Finally, eight Nef138-10/A24-specific scFv clones were able to be isolated and two of them (scFv#3 and scFv#27) were selected for further analysis. The clones specifically stained A24-positive cells pulsed with Nef138-10 peptides. We tried to reconstitute humanized immunoglobulin Gs (IgGs) using a Baculovirus expression system. All of the reconstituted IgGs kept the original specificities of the parental scFvs. The dissociation constants were 23 μM and 20 μM by Biacore, respectively.

In this study, we successfully isolated scFvs directed against an HIV-1-specific pMHC, Nef138-10/A24, using a panning procedure with magnetic beads to select the specific antibodies from phage display libraries. Also, we success-

fully reconstituted the human IgGs directed against the HIV-1 Nef138-10 CTL epitope loaded on an HLA-A24 molecule using the baculovirus expression system. All the clones that reconstituted using this system kept the same specificity of parental scFvs and were easily obtained at a high amount and purity after one-step purification. These systems will provide us with a rapid generation of monoclonal antibodies that is difficult to generate using conventional hybridoma technology. Ours is the first report to describe the generation of monoclonal antibodies against an HIV-1 CTL epitope loaded on an MHC class I molecule.

3. HLA-Associated Alterations in Replication Capacity of Chimeric NL4-3 Viruses Carrying *gag-protease* from Elite Controllers of Human Immunodeficiency Virus Type 1

Toshiyuki Miura, Mark A. Brockman[1], Zabrina L. Brumme[1], Chanson J. Brumme[1], Florencia Pereyra[1], Alicja Trocha[1], Brian L. Block[1], Arne Schneidewind[1], Todd M. Allen[1], David Heckerman[2], Bruce D. Walker[1]: [1]The Ragon Institute of MGH, MIT and Harvard, Charlestown, Massachusetts, USA, [2]Microsoft Research, Redmond, Washington, USA

RNA/ml without treatment have been termed elite controllers (EC). Factors contributing to durable control of HIV in EC are unknown, but an HLA-dependent mechanism is suggested by overrepresentation of "protective" class I alleles, such as B*27, B*51, and B*57. Here we investigated the relative replication capacity of viruses (VRC) obtained from EC (n=54) compared to those from chronic progressors (CP; n=41) by constructing chimeric viruses using patient-derived gag-protease sequences amplified from plasma HIV RNA and inserted into an NL4-3 backbone. The chimeric viruses generated from EC displayed lower VRC than did viruses from CP ($P<0.0001$). HLA-B*57 was associated with lower VRC ($P=0.0002$) than were other alleles in both EC and CP groups. Chimeric viruses from B*57+EC (n=18) demonstrated lower VRC than did viruses from B*57+CP (n=8, $P=0.0245$). Differences in VRC between EC and CP were also observed for viruses obtained from individuals expressing no described "protective" alleles ($P=0.0065$). Intriguingly, two common HLA alleles, A*02 and B*07, were associated with higher VRC ($P=0.0140$ and 0.0097, respectively), and there was no difference in VRC between EC and CP sharing these common HLA alleles. These findings indicate that cytotoxic T-lymphocyte (CTL) selection pressure on gag-protease alters VRC, and HIV-specific CTLs inducing escape mutations with fitness costs in this region may be important for strict viremia control in EC of HIV.

4. HLA-B57/B*5801 Human Immunodeficiency Virus Type 1 Elite Controllers Select for Rare Gag Variants Associated with Reduced Viral Replication Capacity and Strong Cytotoxic T-Lymphotye Recognition

Toshiyuki Miura, Mark A. Brockman, Arne Schneidewind, Michael Lobritz[1], Florencia Pereyra, Almas Rathod[2], Brian L. Block, Zabrina L. Brumme, Chanson J. Brumme, Brett Baker[2], Alissa C. Rothchild[2], Bin Li[2], Alicja Trocha, Emily Cutrell[2], Nicole Frahm[2], Christian Brander[2], Ildiko Toth[2], Eric J. Arts[1], Todd M. Allen, Bruce D. Walker: [1]The Case Western Reserve University, Cleavleand, Ohio, USA, [2]The Ragon Institute of MGH, MIT and Harvard, Charlestown, Massachusetts, USA

Human immunodeficiency virus type 1 (HIV-1) elite controllers (EC) maintain viremia below the limit of commercial assay detection (<50 RNA copies/ml) in the absence of antiviral therapy, but the mechanisms of control remain unclear. HLA-B57 and the closely related allele B*5801 are particularly associated with enhanced control and recognize the same Gag240-249 TW10 epitope. The typical escape mutation (T242N) within this epitope diminishes viral replication capacity in chronically infected persons; however, little is known about TW10 epitope sequences in residual replicating viruses in B57/B*5801 EC and the extent to which mutations within this epitope may influence steady-state viremia. Here we analyzed TW10 in a total of 50 B57/B*5801-positive subjects (23 EC and 27 viremic subjects). Autologous plasma viral sequences from both EC and viremic subjects frequently harbored the typical cytotoxic T-lymphocyte (CTL)-selected mutation T242N (15/23 sequences [65.2%] versus 23/27 sequences [85.1%], respectively; $P=0.18$). However, other unique mutants were identified in HIV controllers, both within and flanking TW10, that were associated with an even greater reduction in viral replication capacity in vitro. In addition, strong CTL responses to many of these unique TW10 variants were detected by gamma interferon-specific enzyme-linked immunospot assay. These data suggest a dual mechanism for durable control of HIV replication, consisting of viral fitness loss resulting from CTL escape mutations together with strong CD8 T-cell immune responses to the arising variant epitopes.

5. HLA-Associated Viral Mutations Are Common in Human Immunodeficiency Virus Type 1 Elite Controllers

Toshiyuki Miura, Chanson J. Brumme, Mark A. Brockman, Zabrina L. Brumme, Florencia Pereyra, Brian L. Block, Alicja Trocha, Mina John[1], Simon Mallal[1], P. Richard Harrigan[2], Bruce D. Walker: [1]Centre for Clinical Immunology and Biomedical Statistics, Royal Perth Hospital and Murdoch University, Perth, Australia, [2]British Columbia Centre for Excellence in HIV/AIDS, Vancouver, British Columbia, Canada

Elite controllers (EC) of human immunodeficiency virus type 1 (HIV-1) maintain viremia below the limit of detection without antiretroviral treatment. Virus-specific cytotoxic CD8_ T lymphocytes are believed to play a crucial role in viral containment, but the degree of immune imprinting and compensatory mutations in EC is unclear. We obtained plasma *gag*, *pol*, and *nef* sequences from HLA-diverse subjects and found that 30 to 40% of the predefined HLA-associated polymorphic sites show evidence of immune selection pressure in EC, compared to approximately 50% of the sites in chronic progressors. These data indicate ongoing viral replication and escape from cytotoxic T lymphocytes are present even in strictly controlled HIV-1 infection.

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Our division has been conducting basic research projects related to the cancer and transplantation immunology. The reagents, modalities, and concepts developed in this division have been clinically applied as translational research projects by the clinicians of Department of Surgery in our research hospital. We believe that bidirectional information exchange between the bench and the bed side would be one of the most important requirements for the successful development of novel and effective therapies.

I. Basic research to develop innovative immunologic therapy against cancer.

a. The role of molecules involved in apoptotic cell phagocytosis in compromising antitumor immunity and promoting tumorigenesis; the role of milk-fat globule EGF8 (MFG-E8) in melanoma progression

Masahisa Jinushi, Marimo Sato, Akira Kanamoto, Hideaki Tahara

Carcinogenesis reflects the dynamic interplay of transformed cells and normal host elements, but cancer treatments typically target each compartment separately. Within the tumor microenvironment, the secreted protein milk fat globule epidermal growth factor-8 (MFG-E8) stimulates disease progression through coordinated $\alpha_v\beta_3$ integrin signaling in tumor and host cells. MFG-E8 enhances tumor cell survival, invasion, and angiogenesis, and contributes to local immune suppression. We show that systemic MFG-E8 blockade cooperates with cytotoxic chemotherapy, molecularly targeted therapy, and radiation therapy to induce destruction of various types of established mouse tumors. The combination treatments evoke extensive tumor cell apoptosis that is coupled to efficient dendritic cell cross-presentation of dying tumor cells. This linkage engenders potent antitumor effector T cells but inhibits $FoxP3^+$ T reg cells, thereby achieving long-term protective immunity. Collectively, these findings suggest that systemic MFG-E8 blockade might intensify the antitumor activities of existing therapeutic regimens through coordinated cell-autonomous and immune-mediated mechanisms.

b. Significant enhancement of antigen-specific antitumor effects of DNA vaccine with systemic administration of interleukin-23

Kimiyasu Yoneyama[1], Marimo Sato, Hideaki Tahara: [1]Research Fellow

The DNA vaccination is a promising approach as a cancer immunological therapy but is still in the developmental phases. In this study, we examined whether co-administration of systemic interleukin (IL)-23 using *in vivo* electroporation

(IVE) could enhance the anti-tumor effects of the vaccine with the DNA plasmid carrying cDNA of tyrosinase-related protein-2 (TRP-2), a tumor associated antigen of murine B16F10 melanoma. Systemic administration of IL-23, a cytokine which is composed of the p40 subunit shared with IL-12 and the IL-23-specific p19 subunit, has been shown to induce potent antitumor effects with the characteristics immune responses mediated by the functions of $CD4^+$ T cells. In prophylactic setting, intra-muscular administration of DNA plasmid carrying TRP-2 combined with IL-23-IVE showed significant anti-tumor effects to suppress the tumor growth. The lymphocytes of treated mice showed specific and potent cytotoxic activities against the B16F10 tumor cells in mixed lymphocyte-tumor reaction (MLTR). Furthermore, the significant involvement of Interferon (IFN)-gamma was strongly suggested by the results of the MLTR *in vitro* and by the results of *in vivo* anti-tumor effects in IFN-gamma-knockout mice. Thus, systemic IL-23 administration using IVE could serve as a cytokine adjuvant to increase the specific anti-tumor immunity induced with the vaccination using DNA plasmids carrying tumor associated antigens.

II. Clinical trials for cancer patients using immunologic approaches

a. Phase I/IIa clinical trial of melanoma vaccine using gp100 derived peptides restricted to HLA-A*2402.

Akira Kanamoto, Marimo Sato, Kimiyasu Yoneyama[1], Akihiko Ito[2], Hideaki Tahara: [2]Department of Surgery, Research Hospital. IMSUT

From the results of phase I clinical trial of melanoma vaccine using gp100 derived peptides, phase I/IIa clinical trial of melanoma vaccine using gp100 derived peptides were performed. HLA-A*2402-restricted gp100 derived peptide (gp100-int4)was used with IFA and interleukin (IL-2) in order to augment for anti-tumor immunity. Our goals in this clinical trial are to examine these clinical efficacy, furthermore, safety and immune responses associated with the peptide vaccination. We have enrolled 30 melanoma patients until 2009. So far, the protocols were well tolerated, and no cardiac, hematological, hepatic, or renal toxicity was noted.

b. Cancer immunotherapy using monocyte-derived DCs stimulated with OK-432 and prostaglandin E_2 pulsed with gp100 epitope peptide in malignant melanoma (Phase I clinical trial)

Marimo Sato, Akira Kanamoto, Hisako Katano[3], Takafumi Nakamura[3], Kimiyasu Yoneyama, Akihiko Ito[1], Hideaki Tahara: [3]Core Facility for Therapeutic Vectors, IMSUT

We have been involved in development of cancer immunotherapy using dendritic cells (DCs) manipulated to induce better immune responses. Our strategies include the usage of agents to induce desirable maturation of DCs in culture of DCs to have better function in situ. In order to obtain DCs suitable for the vaccination with class I-restricted melanoma-associated antigen gp100, we have been using monocyte-derived DCs stimulated with OK-432 and prostaglandin E_2 (OK-P-DCs). We have shown that OK-P-DCs have phenotypic characteristics of matured DCs, ability to successfully induce antigen specific CTLs in vitro, and capability to migrate (Sato M et al, Cancer Sci. 2003). Based on these preclinical results, we initiated phase I clinical protocol to treat stage IV melanoma patients (n=7) with OK-P-DCs pulsed with gp100 epitope peptide restricted to HLA-A*2402. In this study, we have evaluated peptide-specific immunological responses in the enrolled patients using the methods established for the analysis of PBMCs. All the patients enrolled have well tolerated the treatment with no serious adverse events related to the treatment. The migration of the administered OK-P-DCs pulsed with gp100 was confirms with the imaging for the radio-labeled DCs in the patients. Significant immune responses to gp100 were detected as early as 2 weeks after the 1st injection of OK-P-DCs pulsed with gp100 in all patients. These results warrant further development of our vaccination strategy using OK-P-DCs pulsed with gp100. The information related to these trials would be useful to develop effective immunotherapy against malignant melanoma.

c. Emerging concepts in biomarker discovery

Hideaki Tahara, Marimo Sato, Akira Kanamoto

Supported by the Office of International Affairs, National Cancer Institute (NCI), the "US-Japan Workshop on Immunological Biomarkers in Oncology" was held in March 2009. The workshop was related to a task force launched by the International Society for the Biological

Therapy of Cancer (iSBTc) and the United States Food and Drug Administration (FDA) to identify strategies for biomarker discovery and validation in the field of biotherapy. The effort will culminate on October 28th 2009 in the "iSBTc-FDA-NCI Workshop on Prognostic and Predictive Immunologic Biomarkers in Cancer", which will be held in Washington DC in association with the Annual Meeting. The purposes of the US-Japan workshop were a) to discuss novel approaches to enhance the discovery of predictive and/or prognostic markers in cancer immunotherapy; b) to define the state of the science in biomarker discovery and validation. The participation of Japanese and US scientists provided the opportunity to identify shared or discordant themes across the distinct immune genetic background and the diverse prevalence of disease between the two Nations. Converging concepts were identified: enhanced knowledge of interferon-related pathways was found to be central to the understanding of immune-mediated tissue-specific destruction (TSD) of which tumor rejection is a representative facet. Although the expression of interferon-stimulated genes (ISGs) likely mediates the inflammatory process leading to tumor rejection, it is insufficient by itself and the associated mechanisms need to be identified. It is likely that adaptive immune responses play a broader role in tumor rejection than those strictly related to their antigen-specificity; likely, their primary role is to trigger an acute and tissue-specific inflammatory response at the tumor site that leads to rejection upon recruitment of additional innate and adaptive immune mechanisms. Other candidate systemic and/or tissue-specific biomarkers were recognized that might be added to the list of known entities applicable in immunotherapy trials. The need for a systematic approach to biomarker discovery that takes advantage of powerful high-throughput technologies was recognized; it was clear from the current state of the science that immunotherapy is still in a discovery phase and only a few of the current biomarkers warrant extensive validation. It was, finally, clear that, while current technologies have almost limitless potential, inadequate study design, limited standardization and cross-validation among laboratories and suboptimal comparability of data remain major road blocks. The institution of an interactive consortium for high throughput molecular monitoring of clinical trials with voluntary participation might provide cost-effective solutions.

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免疫病態分野

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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/NEDD9

Satoshi Iwata, Shunsuke Kondo, Tomonori Katayose , Satoshi Murakami , Yoshiko Kichikawa, 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 that β1 integrins are costimulatory molecules of T cells. Pp105 was first described in our laboratory as a protein predominantly tyrosine phosphorylated by the ligation of β1 integrins in H9 T cells. By cDNA cloning, we demonstrated that pp105 was a homologue of p130Cas (Crk-associated substrate)/BCAR1 (Breast Cancer Antiestrogen Resistance 1), and designated as Cas-L (Cas lymphocyte type). It has been shown that Cas-L, HEF1 (human enhancer of filamentation), and Nedd9 (neural precursor cell expressed, developmentally down-regulated 9) are identical gene products. We found that transfection of Cas-L cDNA into Jurkat T cells restored β1 integrin-mediated costimulation and cell migration, indicating that Cas-L plays a key role in the β1 integrin-mediated T cell functions.

Our present projects aim at investigating the biological significance of Cas-L/Nedd9 *in vitro* and *in vivo*. Our approach may shed a light on the clinical relevance of Cas-L/Nedd9-mediated signaling pathways in inflammatory diseases and malignancies.

a. Role of Cas-L and Nck association in cell migration and cytokine production of T cells

Cas-L/Nedd9 is a docking protein that is heavily tyrosine phosphorylated upon the engagement of β1 integrins and TCR in T cells. In the present study, we show that Cas-L associates with adaptor protein Nck upon β1 integrin- and TCR-mediated stimulation. Co-precipitation and co-localization studies revealed that Nck bound to the substrate domain of Cas-L/Nedd9 in a tyrosine phosphorylation-dependent manner. In addition, endogenous tyrosine-phosphorylated Cas-L associated with Nck in H9 cells following stimulation with fibronectin or anti-CD3 mAb. Furthermore, we demonstrated that Cas-L localized in the lipid raft in which tyrosine-phosphorylated Cas-L interacts with Nck in H9 T cells upon stimulation with anti-CD3 mAb. The depletion of Cas-L by shRNA resulted in the reduction of IL-2 production and migration of H9 T cells. Finally, we demonstrated that anti-CD3 mAb and SDF-1-induced translocation of Nck into the lipid raft was attenuated in splenic T cells of Cas-L null mice. Since Nck is an important component of the immunological synapse formation following TCR-mediated signaling, our data suggest that Cas-L and Nck may play a pivotal role in β1 integrin- and TCR-mediated signaling and cell migration.

b. Crk-associated substrate lymphocyte type promotes migration, invasion and recurrence in human lung cancer.

Lung cancer is the most common cause of cancer mortality throughout the world. Surgical intervention is currently the most effective treatment modality for non-small cell lung cancer (NSCLC) confined to the thorax. The relapse rate among patients with early-stage NSCLC is 40% within 5 years after potentially curative treatment. Multiple large randomized trials have demonstrated that adjuvant chemotherapy using modern cisplatin-based regimens can significantly improve five-year survival in carefully selected patients with NSCLC. But these survival benefits are by no means satisfactory. In addition to disease stage, several studies have examined gene expression profiles in NSCLC, identifying molecular subtypes associated with patient outcome. Prognostic factors are useful in determining which patients may benefit from adjuvant chemotherapy. Additional studies are required to determine whether patients can be selected for adjuvant platinum-based chemotherapy based upon predictive factor of recurrence.

The epidermal growth factor receptor (EGFR)/human epidermal growth factor receptor (HER) 1 is a tyrosine kinase receptor that is over-expressed in many tumor types, including NSCLC. Activation of the EGFR promotes tumor proliferation, angiogenesis, and metastasis. Monoclonal antibodies directed against EGFR, such as cetuximab, target the extracellular domain of EGFR to prevent ligand binding and subsequent receptor activation. Small-molecule tyrosine kinase inhibitors such as erlotinib and gefitinib bind the ATP-binding pocket of the receptor to prevent ligand-induced phosphorylation and downstream signaling.

We evaluated the potential role of Cas-L/Nedd9 in the phosphorylation and downstream signaling of EGFR in NSCLC. Subsequently, we found that EGFR stimulation promote tyrosine phosphorylation of Cas-L in human non small cell lung cancer cell lines (PC-9 and A549), which was abrogated by inactivation of EGFR using Gefitinib. Introduction of siRNA for Cas-L reduced the migratory activity of PC-9 and A549 cells. The extent of reduction was significantly higher in the case with siRNA for Cas-L compared to that of p130Cas. These results indicate that the crosstalk between EGF and integrin signaling pathways might be occurred at the level of Cas-L, and that Cas-L may be a therapeutic target for the treatment of malignancies such as lung cancer.

c. Analysis on collagen-induced arthritis of Cas-L/Nedd9 knockout mice.

Rheumatoid arthritis (RA) is an autoimmune polyarthritis that is accompanied by the infiltration of T cells into articular joints, proliferation of synovium, degradation of articular cartilage, and osteolytic changes in the affected joints. In the affected joints of RA patients, there have been reported an elevated expression of beta 1 integrins (VLAs) and their ligands such as VCAM-1. Previously, we found that the levels of Cas-L are markedly upregulated in various tissues of HTLV-I tax transgenic mice, a murine model of RA. In particular, large amount of Cas-L positive lymphocytes and leukocytes migrating into the inflamed joints, suggesting a role of Cas-L in pathophysiology of RA. To further evaluate the involvement of Cas-L in the development of RA, we employed collagen-induced arthritis model using Cas-L −/− mice on a C57BL6 (B6) background. We compared three groups of mice, Cas-L −/−, Cas-L −/+, and littermate controls (wild type) on the incidence and severity of CIA. As a result, the arthritis was found in all three groups, however, the severity of arthritis was lower in Cas-L

−/+ and Cas-L −/− mice compared to the littermate controls. X-ray analysis suggest that the joint space narrowing and bone destruction of hind limb is more apparent in wild type mice than that of Cas-L −/+ and Cas-L −/− mice. Further histological analysis and measurement of cytokines are required. We are now performing histological study and microarray analysis of mRNA obtained from the affected joints of those mice. Finally, we hope that our study might suggest a pathophysiological role of Cas-L in vulnerability to rheumatoid arthritis in mouse models.

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

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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 sCD26 binding to Caveolin-1 expressed on monocytes. Moreover, we showed that CD26 plays a role in Tumor growth and Invasion.

Currently we are focusing on the molecular and structural 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, Caveolin-1 fusion protein and recombinant soluble CD26 for treatment of malignant tumors, immune-mediated disorders and immune deficiency diseases. The phase I/II clinical trial utilizing humanized CD26 antibody for the treatment of malignant Mesothelioma has already been started at Gustave Roussy Institute in Paris.

a. A fine strategy for immunetolerance induction therapy using blockade of CD26-mediated T cell costimulation with soluble Caveolin-1-Ig fusion protein

Recently, we have demonstrated that caveolin-1 is a binding protein of CD26, and that CD26 on activated memory T-cells interacts with caveolin-1 on tetanus toxoid (TT)-loaded monocytes. Moreover, following CD 26-caveolin-1 interaction on TT-loaded monocytes, caveolin-1 is phosphorylated, with linkage to NF-κB activation, followed by upregulation of CD86. To examine the binding of caveolin-1 to CD26 in T-cells more precisely, we made soluble Fc fusion proteins containing the N-terminal domain of caveolin-1 (Cav-Ig), and found that ligation of CD26 by solid-phased Cav-Ig induces T-cell proliferation in the presence of TCR/CD3 costimulation.

Presentation of antigen to the T cell receptor (TCR) without costimulation results in a state of antigen-specific unresponsiveness on rechallenge, known as anergy in vitro and tolerance in vivo. However, blockade of costimulation via the CD40 or CD28 pathways, which effectively leads to a state of tolerance of naïve donor-specific T cells in allogeneic transplantation, may not be effective against donor-specific memory T cells elicited either by exposure to donor antigens or viral pathogens. Since CD26 is preferentially expressed on a specific population of lymphocytes, the subset of CD4+ CD45RO+memory T cells, and is unregulated

following T cell activation, blockade of CD26-mediated T cell costimulation may lead to effective T cell anergy in response to recall or allogeneic antigens. In this year, we therefore evaluated the effect of in vitro treatment of recombinant, soluble Cav-Ig as a surrogate ligand for CD26 binding in an effort to block CD26-related functions on CD4+lymphocytes, hypothesizing that this approach could prevent efficient T cell activation during autologous and allogeneic immune responses. Consistent with previous work involving antibody blocking studies, blockade of CD26 costimulation by Cav-Ig renders CD4+ T cells unresponsive not only to TT-pulsed autologous APC, but also to allogeneic APC. These results suggest that blockade of CD26-mediated costimulation provides immune tolerance to an allogeneic response, such as that found in transplantation. In this regard, therapeutic use of Cav-Ig in immune disorders may be tested in vitro and in vivo.

b. A novel mechanism of human CD8+ T cell function via CD26 molecule

CD26 is originally found as a T cell activation antigen, and contains dipeptidyl peptidase IV (DPPIV) enzymatic activity in its extracellular domain. Our laboratory has vigorously examined and found that CD26 molecule is functioned in T cell costimulation on human CD4+ T cells. Indeed, CD26 is preferentially expressed on human CD4+ memory T cells, and CD4+ CD45RO+CD26+ T cells exhibit maximal response to recall antigens. Human T-helper 1 (TH1) cells display a higher expression of CD26, and are much more sensitive to CD26-mediated costimulation than human TH2 cells. In contrast, the role of CD26 in human CD8+ T cells still remains to be elucidated, while CD8+ T cells, as well as CD4+ T cells, express CD26 molecules. In this year, we have examined the effector function of CD26-mediated costimulation of human CD8+ T cells. In comparison with CD28 -medaited costimulation, which is one of established T cell costimulation, CD26-mediated costimulation in human CD8+ T cells showed delayed proliferation than that of CD28 stimulation, but finally expanded to a similar extent. The secretion of inflammatory cytokines, TNF-α and IFN-γ, was strongly induced after CD26-mediated costimulation. In contrast, the secretion of IL-2 and IL-5 was significantly less as compared with CD28-mediated costimulation. Finally, we have shown that the expression of Granzyme B, one of the major effector molecules in the cytotoxic activity of CD8+ T cells, was markedly enhanced by CD26-mediated costimulation in dose dependent manner of anti-CD26 monoclonal antibody stimulation. Moreover, with CD26-mediated costimulation, CD8+ T cells were observed to kill target cells in a Granzyme B-dependent manner. Taken together, our data strongly suggest that CD26 plays an important role in CD8+ T cell dependent defense against viruses and tumor cells, and also involves in pathophysiology such as autoimmune diseases and graft-versus-host disease.

c. CD26-based molecular target therapy for graft-versus-host disease in hematopoietic stem cell transplantation

Graft-versus-host disease (GVHD) remains a major cause of morbidity and mortality in allogeneic hematopoietic stem cell transplantation (alloHSCT). In GVHD, mature donor T cells that accompany the stem cell graft attack of recipient tissues, especially the skin, liver, gastrointestinal tract, and lung. Therefore, all patients undergoing alloHSCT receive GVHD prophylaxis to impair T cell function; however, treatment to prevent GVHD can be deleterious since mature donor T cells play a critical role in mediating reconstitution of the adaptive immune system. Recipients of alloHSCT are thus at great risk for infections, particularly when prolonged immunosuppression is required for treatment of GVHD. Although the role of CD26/DPPIV in GVHD needs to be studied in more detail, treatment with a murine antibody against human CD26 was reported to have an effect in patients with steroid-resistant acute GVHD following alloHSCT (Bacigalupo A., et al., Acta Haematol 1985: 73: 185, de Meester, et al., Immunobiology 1993: 188: 145). To examine the efficacy of CD26-targeting therapy in GVHD more profoundly, we established mouse GVHD model using human peripheral blood lymphocytes (huPBL) (xenograft GVHD mouse model; x-GVHD). After NOD/Cg-Prkdcscidil2rgtm1Sug/Jic mice were injected with appropriate numbers of huPBL, mice show symptoms of GVHD such as loss of weight, loss of hair, deterioration of activity, and thinning of ear pads. Histopathological examination revealed that CD3+ CD8+CD26+ human lymphocytes were infiltrated in the skin, intestinal mucosa, salivary gland, lung and liver of the x-GVHD mice. In this mouse model, humanized anti-CD26 monoclonal antibody (mAb) was injected two weeks later of onset of x-GVHD, and the symptoms of GVHD were improved after ten injections of humanized anti-CD26 mAb. Moreover, x-GVHD was observed to be suppressed when humanized anti-CD26 mAb was prophylactically administered. Taken together, it may be possible that the full therapeutic potential of alloHSCT

will be realized by approaches that aim to minimize GVHD by targeting CD26-mediated T cell regulation.

d. A role of endothelial CD26/DPPIV in inflammatory vascular injury

Human endothelial cells have been also reported to provide costimulatory signals to CD4+ T cells, leading to T cell migration into inflammatory site. Recently, chronic graft-versus-host disease (cGVHD) leads to a rarefaction of microvesseles caused by the infiltration of alloactivated T cells. In this year we have examined in vitro and in vivo whether CD26 expression plays a role on inflammatory response of microvascular endothelial cell. Normal human aortic endothelial cell (HAEC), coronary artery endothelial cell (HCAEC), and dermal microvascular endothelial cell (HMVEC) were used to determine CD26 expression, cell migration and wound closure assay in the presence or absence of CD26 expression with treatment of TNF-α, IL-1 or IL-6. For in vivo vasculization assay, matrigel plug assay was performed with or without CD26 expression in mice. CD26 expression in HMVEC reduced by TNF-α, and endothelial activity was affected in vitro and in vivo with changes of CD26 expression. Moreover, we observed that CD26 has a pivotal role of endothelial angiogenic activity in mice model. These findings strongly suggest that CD26 in endothelial cells plays an important role in inflammatory response.

e. CD26 expression on T cell lines increases SDF-1-alpha-mediated invasion.

CD26 is a multifunctional membrane-bound glycoprotein that regulates tumour growth in addition to its other activities. Because disease aggressiveness is correlated with CD26 expression in several T-cell malignancies, we decided to investigate the invasiveness of cells expressing different levels of CD26. To assess CD26 involvement in cell invasion, we performed in vitro invasion assays with human T cell lines expressing different levels of CD26. These included the parental CD 26-positive T-lymphoblast cell line HSB-2 and clones infected with a retrovirus expressing siRNA vectors that either targeted CD26 or encoded a missense siRNA, and the parental CD26-negative T-leukaemia cell line Jurkat and clones expressing CD26. CD26 expression in these cell lines was evaluated by flow cytometry and western immunoblotting. CXCR4 expression, phosphorylation of signalling kinases, and MMP-9 secretion were also evaluated by western immunoblotting, whereas MMP-9 activity and the effect of kinase and CD45 inhibitors on activity were measured by zymography of conditioned media. The presence of CD26 enhanced stromal-cell-derived factor-1-alpha (SDF-1-alpha)-mediated invasion of T cell lines. This process was regulated in part by the PI-3K and MEK1 pathways, as indicated by increased phosphorylation of p44/42 MAP kinase and Akt in the presence of SDF-1-alpha and the effect of their respective inhibitors on MMP-9 secretion and in vitro invasion. In addition, CD26-associated enhancement of SDF-1-alpha-induced invasion was decreased when CD45 was inhibited. Our results indicate that the expression of CD26 in T cell lines leads to increased SDF-1-alpha-mediated invasion in an in vitro system and that this is controlled in part by the PI-3K and MEK1 pathways. The data also suggest that CD26 enhancement of invasion may be mediated by CD45, however, more studies are required to confirm this involvement.

III. Therapeutically targetting transcription factors

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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 conditional regulation of transcription factors including the glucocorticoid receptor and hypoxia-inducible factor-1a.

a. Glucocorticoid receptor (GR) 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 regulations 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 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-steridal 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 GR

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 GR

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. Our recent microarray study demonstrated that GR and MR have differential role in homeostatic regulation in non-classical corticosteroid target tissues including the heart. Notably, collaboration with Professor Miyano's laboratory greatly contributed to development of this program.

(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 7SK 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. Indeed, HEXIM1 has differential roles in gene regulation in a context and gene specific fashion. We have recently characterized that HEXIM1 may play an important role in tissue-specific regulation of glucocorticoid-mediated gene expression. Physiological significance of HEXIM1 is being studied using newly generated transgenic mice.

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

HIF-1a is essential for not only angiogenesis but also development of certain organs. In this line, molecular biology of HIF-1a will provide us possible advantage to characterize and manupilate 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 plays 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-1a in human peripheral T cells. In the present study, we showed that TCR-engagement does not influence hypoxia-dependent stabilization but stimulates protein synthesis of HIF-1a, most possibly via PI3K/mTOR system, and that expression of HIF-1a 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-1a 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-1a.

IV. Cancer Stem Cells

Hiroto Yamazaki, Ghani Farhana Ishrat, Motohiko Naito, Toshihiro Okamoto and Chikao Morimoto

Stem cell signatures of human malignant mesothelioma cells

Malignant mesothelioma (MM) is an aggressive neoplasm arising from the mesothelial cells of the pleura and usually associated with the history of long-term asbestos exposure. As the prognosis is very poor, novel therapeutic strategies are needed to be developed. Recent works in stem cell biology suggest that some cancers contain stem-like cells (cancer stem cells, CSCs). In human cancers, CSCs have already been identified in most of the malignant tumors. As the stem cell characters of cancer are supposed to be the major reason for therapy-resistance, understanding these properties in MM cells is important. For this purpose, we first established mouse xenograft model of MM using patient samples. We established 6 mouse serial transplanation models and a new cell line TUM1. Next, we searched stem cell markers such as side population (SP) and CD markers using the existing and our new cell lines. We found that many cell lines contain SP cells, suggesting that they keep one of the stem cell properties. In CD marker analysis, we found that CD9 and CD24, formerly described CSC markers, are heterogeneously expressed in many lines. Sorting and culture assay by SP, CD9 or CD24 revealed that some of MM cell lines proliferated by asymmetric cell division-like manner. In transplantation assay, we also found that these marker-positive cells generated larger tumors than negative cells. In addition, analysis of cellular functions such as cell cycle and invasion activity revealed that the marker-positive cells demonstrated higher proliferation and invasive potentials. Moreover, stem cell culture of the cell lines revealed that the expressions of several stem cell genes were up-regulated significantly in every line and TUM1 formed spheroids colony. These results suggests that MM harbours several stem cell characters and they can be new therapeutic targets in the future.

IV. Immunomodulatory effects of RXM and its analogs

Noriko Otsuki, Emi kumagai, Chikao Morimoto

Roxithothromycin (RXM) is a macrolide antibiotic that is effective in treatment of chronic lower respiratory tract infection. Its mechanism of actions besides its antibacterial action remains unclear.

We have previously reported the Immunomodulatory effects of RXM, including inhibition of TNF-α and IL-6 production by activated human T cells and macrophages, and reduction of T cell trans-endothelial migration in vitro. Moreover, we showed that in vivo, RXM treatment of collagen-induced arthritis (CIA) mice inhibited the development of CIA, levels of serum IL-6, and the migration of leukocytes into affected joints.

In the present study, we synthesized 21 types of analog compounds to RXM that do not have antibacterial effects, and 2 compounds (5-I and 8-B) of them were roughly selected with inhibition of TNF-α and IL-6 production in activated PBMC. To evaluate potency of analogs in arthritis model, we compared the immunomodulatory effects of RXM with its analogs in parallel, and then attempted to elucidate the mechanism of these effects.

We examined cytokine production and transendthelial migration of activated PBMC or T cells. IL-2, IL-6, IL-17A, IFN-γ and TNF-α production were inhibited to similar levels by addition of RXM or 5-I, although IL-4, IL-5 and IL-10 production were not altered. These effects do not depend on the inhibition of proliferative responses of PBMC. Trans-endothelial migration of pre-activated T cells was inhibited in the presence of RXM or 5-I. In CIA model mice, intraperitoneal administration of RXM or 5-I inhibited the exacerbation of arthritis, even treatment was started after the onset of the disease. The amelioration of CIA by 5-I treatment appeared immunomodulatory effects similar to RXM. These data strongly suggest that 5-I is promising candidate for lead compound to treat rheumatoid arthritis without antibiotic effects.

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We have been working on the following three projects, 1) identification of novel therapeutic strategies of human cancer, 2) clarification of mechanisms underlying human cancer, and 3) development of diagnostic strategies for hereditary colorectal cancer. These projects are aimed to develop strategies for better diagnosis, effective treatment, and prevention of human cancer.

1. Identification of novel molecular targets for the treatment of human cancer

Kiyoshi Yamaguchi, Yusuke Nakamura[1], and Yoichi Furukawa: [1]Laboratory of Molecular Medicine, Human Genome Center

To identify novel molecular targets for diagnosis and treatment of human colorectal cancer (CRC), we earlier carried out global gene expression profiles of colorectal tumors using genome-wide cDNA microarray. Among the genes overexpressed in the tumors, we selected MRG-binding protein (*MRGBP*) or *C20orf20*, a subunit of TRRAP/TIP60-containing histone acetyltransferase complex, as a candidate gene for clinical application. Quantitative PCR and immunohistochemical staining confirmed that expression of *MRGBP* was frequently elevated in the great majority of the CRCs examined. Importantly, suppression of *MRGBP* by short interfering RNA (siRNA) resulted in growth suppression of CRCs. Furthermore, MRGBP-knockdown significantly reduced DNA synthesis and cell population in S-phase. These results suggested that MRGBP might play an essential role in proliferation and/or survival of colorectal cancer cells through regulation of cell cycle. We also found that MRGBP associates with bromodomain containing 8 (BRD8) by yeast two-hybrid screening. This interaction changed subcellular localization of BRD8 from cytoplasm to nucleus, and led to its accumulation in the nucleus in a post-transcriptional manner. Since *BRD8* siRNA also suppressed proliferation of CRC, BRD8 may be an important down-stream target of MRGBP.

These findings may contribute to the better understanding of colorectal carcinogenesis, and open a new avenue to the development of novel therapeutic and/or diagnostic approach to this type of tumor.

2. Functional analysis of SMYD3, a molecular target for CRC, hepatocellular carcinoma, and breast cancer

Tomoaki Fujii, Kiyoshi Yamaguchi, Sumiko Watanabe[2], Yoichi Furukawa: [2]Division of Molecular Developmental Biology, IMSUT

We earlier showed that SMYD3, a histone H3-Lysine 4-specific methyltransferase, is frequently up-regulated in human colorectal, liver, and breast cancers compared to their matched non-cancerous cells, and that its activity is associated

with the growth of these tumors. Among adult tissues that we examined, SMYD3 was abundantly expressed in the testis and skeletal muscle. Another report showed that it was ubiquitously expressed in zebrafish. However, the physiological role of SMYD3 in development remains unknown. To clarify the role of Smyd3 in development, we have studied its expression patterns in zebrafish embryos and the effect of its suppression on the development using smyd 3-specific antisense morpholino-oligonucleotides. We found that transcripts of *smyd3* were expressed in zebrafish embryos at all developmental stages examined and that knockdown of *smyd3* in embryos resulted in pericardiac edema and defects in the trunk structure. In addition, these phenotypes were associated with abnormal expression of *cmcl2*, *amhc* and *vmhc*, three heart chamber-specific markers, and that of *myod*, a skeletal muscle-specific marker. These data suggest that smyd3 plays an important role in the development of cardiac and skeletal muscles.

3. Analysis of fucosylation in CRC.

Yoichi Furukawa, Yusuke Nakamura[1]: [1]Laboratory of Molecular Medicine, Human Genome Center, IMSUT

Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) promotes apoptosis in cancer cells, but not normal cells, and is critically involved in tumor rejection through natural killer (NK) cell-mediated immune surveillance. Oligosaccharides are involved in various aspects in carcinogenesis, and fucosylation is one of the most important oligosaccharide modifications in cancer. To clarify the role of fucosylation in human carcinogenesis, we studied GMDS, an enzyme that plays a key role in fucosylation, in collaboration with Department of Molecular Biochemistry & Clinical Investigation and [2]Biochemistry, Osaka University Graduate School of Medicine. We performed mutational analysis for *GMDS* using several kinds of cancer cell lines, which identified a deletion of *GMDS* in HCT116 colon cancer cells. Importantly, the deletion led to a complete deficiency of cellular fucosylation, and transfection of the wild-type *GMDS* gene into HCT116 cells restored the cellular fucosylation. When mock and GMDS-rescued cells were transplanted into athymic mice, tumor growth and metastasis of the GMDS-rescued cells were dramatically suppressed through NK cell-mediated tumor surveillance. In addition, the GMDS-rescued cells showed high susceptibility to TRAIL-induced apoptosis, and anti-TRAIL blocking antibody suppressed the accelerated direct cell lysis of the GMDS-rescued cells by splenocytes. We further searched for *GMDS* mutation in a total of 100 colorectal cancer tissues and consequently identified two mutations; c.739 C>T, (pR247X) in exon 7, and c.345+5 G>A (IVS4+5) in intron 4. These data suggested that *GMDS* mutation should be involved in the progression of human CRC through the escape from NK cell-mediated tumor surveillance.

4. Genetic diagnosis of HNPCC

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Hereditary non-polyposis colorectal cancer (HNPCC) is an autosomal dominant hereditary disease accompanied by tumors arising mainly in the colon and other associated organs, such as stomach, renal pelvis, and endometrium. The frequency of HNPCC in Caucasian patients with colorectal cancer is estimated between two and five percent. However the frequency in Japanese patients with colorectal cancer remains undetermined. Therefore, we earlier performed genetic analyses of *MSH2*, *MLH1*, and *MSH6*, three responsible genes for HNPCC, as a collaborative project of registration and diagnosis of Japanese HNPCC patients conducted by Japanese Study Group for Colorectal Cancer. A total of 131 patients with familial colorectal cancer who fulfilled the modified Amsterdam's II criteria were registered, and the frequency of HNPCC in registered patients with colon was determined. For genetic diagnosis, we performed PCR-direct sequencing and Multiplex Ligation-dependent Probe Amplification for the three responsible genes. As a result, we identified pathogenic mutations in 69 of 131 cases. These mutations included missense and nonsense mutations, small insertions and deletions, and gross genetic alterations including large deletions and duplications. In addition to these genetic changes, mutations in introns were also involved in the pathogenesis. However it is sometimes difficult to interpret correctly the pathogenicity of variants in exons as well as introns.

To evaluate the effect of splice-site mutations in two patients, we carried out a functional splicing assay using minigenes. Consequently, this assay showed that the mutation of c.1731+5G>A in *MLH1* led to exon15 skipping, and that the mutation of c.211+1G>C in *MSH2* created an activated cryptic splice-site 17-nucleotides upstream in exon1. These aberrant splicing patterns were not observed when wild

type sequence was used for the assay. We also obtained concordant results by RT-PCR experiments with transcripts from the patients. Furthermore, additional functional splicing assays using two different intronic mutations described in earlier studies revealed splicing alterations that were in complete agreement with the reports. These results suggested that functional splicing assay is helpful for evaluating the effects of genetic variants on splicing.

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