Advanced Clinical Research Center

Division of Novel Therapy for Cancer
癌制御分野

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The main theme of our research is to find out novel therapeutic tools for cancer of the liver, the pancreas, the stomach and the colon. This year we focused on the hepatocellular carcinoma (HCC). HCC is the most commonly occurring primary liver cancer and ranks as the fifth most frequently occurring cancer, overall, and the third leading cause of cancer deaths, worldwide. At present, there is no effective treatment for HCC; consequently, the prognosis for these patients is poor. Our aim in the present study was to identify a novel target for antibody therapy against HCC. We tested the efficacy of combined treatment with anti-FGFR1 monoclonal antibody and interferon-α/β in a murine xenograft model of human HCC. We found that interferon-α/β induces expression of FGFR1 in human HCC cell lines, and that an anti-FGFR1 monoclonal antibody (mAb) targeting of the induced FGFR1 can effectively inhibit growth and survival of HCC cells in vitro and in vivo. Moreover, the combination of interferon-α, anti-FGFR1 mAb and peripheral blood mononuclear cells (PBMCs) exerted a significant antitumor effect in vivo. Our results suggest that the combined use of an anti-FGFR1 antibody and interferon-α/β is a promising approach to the treatment of HCC.

1. Interferon-αIβ and Anti-Fibroblast Growth Factor Receptor 1 Monoclonal Antibody Suppress Hepatic Cancer Cells in Vitro and in Vivo

Kohzoh Imai, Shigeru Sasaki, Tadao Ishida, Hiroshi Yasui, Minoru Toyota

Hepatocellular carcinoma (HCC) is the most commonly occurring primary liver cancer and ranks as the fifth most frequently occurring cancer, overall, and the third leading cause of cancer deaths, worldwide. At present, surgery, percutaneous therapies such as ethanol injection and radiofrequency ablation, and transcatheter therapies such as arterial chemoembolization are employed in the treatment of HCC. These approaches can selectively remove and kill cancer cells, which makes them useful for control of the local tumor; however, they are not sufficient to improve the prognosis of HCC patients, as the disease readily recurs due to blood-born metastases (e.g., intrahepatic metastasis and vascular infiltration) or the development of new HCCs (multicentric carcinogenesis). Consequently, the 1-year and 3-year survival rates for HCC in Japan are only 36% and 17%, respectively. The weaknesses of the current HCC treatments include incomplete inhibition of multicentric carcinogenesis, difficulties in controlling intraportal infiltration, and the inability to prevent deterioration of hepatic functional reserve or foster its restoration. Thus development of new treatments that improve the prognosis of HCC patients and which can also be used in elderly and advanced stage patients would be highly desirable.

Targeting cell surface molecules using mAbs is an emerging strategy in cancer therapy, and mAbs against cancer-related surface molecules

such as EGFR, HER2 and CD20 have been successfully employed. However, cell surface expression of antigenic molecules is often weak and heterogeneous, which prevents the efficient targeting of tumors and, to date, only a few pilot studies examining expression of HCC-associated antigens have been carried out.

Interferons (IFNs), which are widely used for the treatment of neoplasias and viral diseases, enhance expression of several cell surface molecules both *in vitro* and in xenograft tumor models. Induction of gene expression by IFN is a complex phenomenon that involves activation of target genes via phosphorylation of STATs by JAK kinase. In addition, IFNs can induce expression of interferon regulatory factors (IRFs) and transcription factors, which then induce genes involved in apoptosis and immune responses. IFNs are already being used to treat most hepatitis patients, and their effects suggest targeting cell surface molecules induced by IFN may be a useful strategy for treating HCC. Our aim in the present study was to use HCC cell lines and a murine xenograft model of human HCC to examine the changes in gene expression induced by IFN and to identify potential targets for antibody therapy. Our findings suggest IFN-α/β-induced fibroblast growth factor receptor 1 (FGFR1) could be a novel therapeutic target for the treatment of HCC.

More specifically, combined treatment with IFN-α/β and an anti-FGFR1 mAb (A2C9-1) showed strong growth suppressive effects on human HCC cells *in vitro* and *in vivo.* Five isoforms of the transmembrane receptor FGFR (FGFR1-4 and FGFR5/1L) are known to be expressed in mammals. Each consists of three extracellular immunoglobulin-like domains, a transmembrane domain, and two intracellular tyrosine kinase domains. FGF binds to the FGFR via two of the immunoglobulin-like domains (II and III). During FGFR expression, alternative splicing of *FGFR* transcripts produces multiple splice variants with different tissue-specific ligand specificities. Among them, FGFR1 has been shown to be expressed in HCC and is known to promote the development of HCC in response to carcinogenic stimulation. FGFR1 is not expressed in noncancerous hepatocytes. FGFR1 mediated signaling is involved in cancer cell growth and infiltration, as well as in angiogenesis, which is already a target for antitumor therapies. In addition, previous studies have shown elevated expression of FGFR ligands, including FGF1 and FGF2, in primary HCC tissues and hepatic cancer cell lines, strongly suggesting FGF signaling plays a key role in the development of HCC. These characteristics make FGFR1 an attractive molecular target for treating HCC.

One major problem with antibody therapy against cancer is the weak and heterogeneous expression of cell surface antigens. To overcome this problem, we examined genes up-regulated by IFN in HCC xenografts. We found that expression of FGFR1 is induced by IFN-α/β and that treating HCC cells with a combination of IFN-α/β and an anti-FGFR1 mAb effectively inhibits the growth and survival of HCC cells. Thus, one reason for the insufficient therapeutic effect of anticancer drugs targeting FGFR1 appears to be is that, without induction, expression of FGFR1 on cancer cells is not sufficient for effective treatment. Consistent with this idea, our immunohistochemical analysis showed expression of FGFR1 to be very low in untreated HCC cells. Notably, epidermal growth factor receptor (EGFR) is also up-regulated by IFN, and this up-regulation of EGFR is a crucial factor underlying the susceptibility of affected cancer cells to anti-EGFR antibody therapy. Taken together, these findings suggest treatment with a combination of IFN and an antibody may be an effective therapeutic strategy against various types of cancer.

The molecular mechanism by which IFN-α/β induces FGFR1 expression remains unknown. It is known, however, that the antitumor and antiviral effects of IFN involve changes in the transcriptional regulation of various genes, and that IFN-inducible genes contain an interferon response element (ISRE) in their promoter regions. By using a transcription factor search program, we identified several putative ISREs in the 5′ UTR of FGFR1, suggesting that FGFR1 could be a direct target of type I IFN. Further study will be necessary to determine precisely how interferon induces FGFR1.

It was previously reported that the binding of an antibody to a growth factor receptor results in the internalization of the antibody-receptor complex, and the down-regulation of downstream signaling. However, antibodies against growth factor receptors also exert growth suppressing effects via the immune system. Here, for example, we showed that IFN-α/β enhances the surface expression of FGFR1, perhaps enabling an anticancer effect based on antibody-dependent cell-mediated cytotoxicity to accompany the binding of anti-FGFR1 mAb to the receptor. The results of our *in vivo* experiment showing the importance of PBMCs to the antitumor effects of A2C9-1 is consistent with the idea that this antibody strongly stimulates antibody-dependent cell-mediated cytotoxicity.

In summary, we found that IFN-α/β induces expression of FGFR1 and that treatment with a combination of IFN-α/β and an anti-FGFR1

mAb suppresses HCC cell growth *in vitro* and *in vivo.* We also confirmed that IFN-α/β enhances the accumulation of the anti-FGFR1 mAb within tumors. This treatment protocol selectively inhibits the growth of HCC cells without affecting normal cells, which suggests it could be used in the treatment of HCC without reducing hepatic preliminary performance. We therefore suggest that our results may provide the basis for a novel approach to the treatment of HCC, for which there is no effective therapy at the moment.

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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. Hormone-conditional activation of Bcr-Abl kinase highlights the Stat5 anti-apoptotic pathway integrating multiple gene expression.

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

A ligand-controllable p190Bcr-Abl, p190ΔccER, was created by deletion of the Bcr coiled-coil domain and fusion with the estrogen receptor ligand-binding domain, and used to transform cytokine-dependent TF-1 cells. Following the replacement of GM-CSF by 4-hydroxytamoxifen (4-HT), TF-1/p190ΔccER cells were alive without cell growth for a few days and then proliferated vigorously. In a steady state, the profile of phosphotyrosine-containing proteins was similar in 4-HT-treated TF-1/p190ΔccER cells and TF-1/p190WT cells. Unexpectedly, stable detection of autophosphorylated p190ΔccER occurred after several hours of ligand stimulation, as did the detection of phospho-CrkL, because ligand-free p190ΔccER was quite unstable and its stability increased, in a time-dependent manner, only upon ligand binding. By contrast, 4-HT-induced tyrosine phosphorylation of Stat5 was observed within 10 min, suggesting its dominant role in the initial anti-apoptotic phase triggered by p190 ΔccER. Seven genes were significantly up-regulated by 4-HT in a time-dependent manner. Doxycycline-inducible expression of constitutively active Stat5A in TF-1 cells caused the suppression of apoptosis after GM-CSF withdrawal and significantly up-regulated five of seven candidate genes (BCL-XL, HIF-1A, HSPA1A, WT1, PRAME). Collectively, the Bcr-Abl/Stat5 pathway is likely to integrate multiple effector molecules to prevent apoptosis. These molecules may, in turn, serve as therapeutic targets in Ph-positive leukemias.

2. Bcr-Abl impairs T cell development from murine induced pluripotent stem cells

Bidisha C, Izawa K, Harnprasopwat R, Takahashi K, Kobayashi K, Tojo A

Chronic myeloid leukemia (CML) is a clonal myeloproliferative disorder generally believed to originate from a hematopoietic stem cell carrying the BCR-ABL fusion gene, which encodes 210 kD and 190 kD constitutively active tyrosine kinases termed as p210 and p190, respectively. In spite of the stem cell origin and the competence for differentiation even toward mature B cells, there is a longstanding consensus that CML never involves the T cell lineage at least in chronic phase. We used *in vitro* T cell differentiation model from murine induced pluripotent stem (iPS) cells. C57BL/6 mouse embryonic fibroblasts (MEF) were reprogrammed using a polycistronic, self-inactivating (SIN) lentiviral Tet-On vector encoding Oct4, Sox2 and Klf4, which were tandemly linked via porcine teschovirus-1 2A peptides, together with another lentiviral vector expressing rtTA driven by the EF-1α promoter. To delete almost all the vector sequences including the transgenes after derivation of iPS cells, a loxP site was inserted into the truncated 3′ LTR of each vector. Doxycycline (DOX)-inducible iPS cell colonies were picked up after 3 weeks of culture on a MEF feeder layer. Pluripotency of these cells were confirmed by their expression of embryonic stem cell signatures as well as formation of teratomas containing all 3 germ layer-derived tissues in NOD-SCID mice. A clone of MEF-3FiPS cells were further transduced with p190ΔccER by infection of murine stem cell virus-based retroviral vector. Finally, adenovirus-mediated expression of Cre recombinase successfully excised the lentiviral vector components and left only remnant 291-bp SIN LTRs containing a single loxP site. Stability and tyrosine kinase activity of p190ΔccER is absolutely dependent on a ligand, 4-hydroxytamoxyfen (4-HT). For T cell lineage differentiation, MEF-3FiPS/p190ΔccER cells were recovered from a feeder-free culture supplemented with LIF and plated onto a subconfluent OP9-DL1 monolayer in the presence of Flt3L and IL7 with or without 0.5 μM 4-HT. After 3 weeks of T lineage differentiation, iPS cell-derived blood cells were collected and subjected to FACS analysis for their lineage determination. Approximately 70% of lymphocyte-like cells from the 4-HT(−) culture expressed CD3, but only 20% of counterparts from the 4-HT(+) culture expressed CD3, suggesting that in this culture system Bcr-Abl impairs T cell development

possibly through interfering with Notch signaling. The precise mechanism underlying impaired T lymphopoiesis by Bcr-Abl is under investigation.

3. 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 posttranscriptionally regulate expression of target mRNA transcripts. We recently 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 MLLAF4 chimeric fusion proteins *MLLAF4* and *AF4-MLL* that are generated by chromosomal translocation t (4; 11). We report the identification of 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 MLLAF4 ALL sample as an acquired mutation. These results demonstrate that the novel mutation in *miR-128b* 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.

4. Alteration of processing induced by a single nucleotide polymorphism in pri-miR-126

Harnprasopwat R, Kotani A, Toyoshima T, Tojo A

MicroRNAs (miRNAs) are small non-coding RNAs that inhibit expression of specific target genes at the post-transcriptional level. Sequence variations in miRNA genes, including pri-miRNAs, pre-miRNAs and mature miRNAs, could potentially influence the processing and/or target selection of miRNAs. In this study, we have found the single nucleotide polymorphism (SNP) at the twenty-fourth nucleotide (+24) of the mature miR-126 in the genome of RS4; 11 cells, derived from a MLL-AF4 ALL patient. Through a series of in vivo analyzes, we found that this miR-126 SNP significantly blocks the processing of pri-miRNA to mature miRNA, as well as reduces miRNA-mediated translational suppression. Moreover, its frequency is different among races. Thus, our study emphasizes the importance of identifying new miRNA SNP and its contribution to miRNA biogenesis which is possible link to human genetic disease.

5. Imatinib mesylate directly impairs class switch recombination through down-regulation of AID: its potential efficacy as an AID suppressor

Kawamata T, Kotani A, Toyoshima T, Yokoyama K, Tojo A

Activation-induced cytidine deaminase (AID) is essential for class-switch recombination (CSR) and somatic hypermutation (SHM). Its deregulated expression acts as a genomic mutator that contributes to development of various malignancies. During treatment with Imatinib mesylate (IM), patients with chronic myeloid leukemia (CML) often represent hypogammaglobulinemia, the mechanism of which has not yet been clarified. Here, we showed some evidence that CSR upon B cell activation is apparently inhibited by IM through downregulation of AID. Furthermore, expression of E2A, one of the key transcription factors for AID induction, was dramatically suppressed by IM. These results shed light on not only the underlying mechanism of IM-induced hypogammagloblinemia but also its potential efficacy as an AID suppressor.

6. A search for adult-T cell leukemia stem cells using patient samples.

Kobayashi S, Tian Y, TojoA, Uchimaru K.

Adult T-cell leukemia (ATL) is a malignant disorder caused by human T-cell leukemia type 1 (HTLV-1). We reported that leukemic T cells are specifically enriched in a unique $CD3^{dim}$ $CD7^{low}$ subpopulation of $CD4^{+}$ T cells in acute-type ATL (Tian et al, cancer science, in press). In the study, we unexpectedly found that in the $CD4^{+}$ population, there are three distinct subpopulations on CD3 vs CD7 plot: $CD3^{high}CD7^{high}$, $CD3^{dim}CD7^{dim}$ and $CD3^{dim}CD7^{low}$ subpopulations. Analysis of clonality by inverse long PCR revealed that $CD3^{dim}CD7^{dim}$ and $CD3^{dim}CD7^{low}$ subpopulations contain the same clone. However cells in the two population were different in terms of morphology. These results suggest transformation through multi-step oncogenesis in the ATL clone and led us speculate that finding biological difference among these subpopulation lead to identify ATL stem cells. Using the cobblestone-area-forming cell assay which re-

flects the ferequency of stem/progenitor cells, we plan to search for the subpopulation which has a high cobblestone forming ability.

7. Leukemic T cells are specifically enriched in a unique $CD3^{dim}CD7^{low}$ subpopulation of $CD4^+$ T cells in acute-type adult T cell leukemia.

Tian Y, Kobayashi S, Ohno N, Isobe M, Ohfuchi-Tsuda M, Tojo A, Uchimaru K,

The morphological discrimination of leukemic from non-leukemic T cells is often difficult in adult T cell leukemia (ATL) as ATL cells show morphological diversity, with the exception of typical "flower cells." As defects in expression of CD3 as well as CD7 are common in ATL cells, we applied multi-color flow cytometry to detect a putative leukemia-specific cell population in the peripheral blood from ATL patients. $CD4^+CD14^-$ cells subjected to two-color analysis based on a CD3 vs. CD7 plot clearly demonstrated the presence of a $CD3^{dim}CD7^{low}$ subpopulation in each of nine patients with acute-type ATL. The majority of sorted cells from this fraction showed a flower cell-like morphology, and carried a high proviral load for human T cell leukemia virus (HTLV-I). Genomic integration site analysis (inverse long-range PCR) and analysis of the T cell receptor Vb repertoire by flow cytometry indicated that the majority of leukemia cells were included in the $CD3^{dim}CD7^{low}$ subpopulation. These results suggest that leukemic T cells are specifically enriched in a unique $CD3^{dim}CD7^{low}$ subpopulation of $CD4^+$ T cells in acute-type ATL.

8. 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.

Publications

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Division of Cellular Therapy
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Our major projects are (1) Co-ordinate 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 receptors) family (LMIR/MAIR/CLM) and (4) Elucidation of molecular basis of leukemia, myelodysplastic syndromes, myeloproliferative disorders.

1. Co-ordinate 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 Kaziro[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 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. 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 harbors 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 a 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 Ras 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 searched 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. The in vivo function of Integrin αIIbβ3 in mast cells

Toshihiko Oki, Jiro Kitaura, Koji Eto[4], Yang Lu, Yoshinori Yamanishi, Hideaki Nakajima[5], Hidetoshi Kumagai, and Toshio Kitamura: [4]Laboratory of Stem Cell Therapy, Institute of Medical Science, The University of Tokyo, [5]Keio University School of Medicine

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. Interestingly, soluble FB promoted cytokine production of BMMCs in response to Staphylococcus aureus with FB-binding capacity, through integrin alpha IIb beta 3-dependent recognition of this pathogen. Analysis of integrin αIIb-deficient mice showed that integrin αIIb deficiency strongly suppressed chronic inflammation with the remarkable increase of mast cells induced by continuous intraperitoneal administration of FB, although it did not affect acute allergic responses or mast cell numbers in tissues in steady states. Collectively, integrin αIIbβ 3 in mast cells plays an important part in FB-associated, chronic inflammation and innate immune responses.

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

Yoshinori Yamanishi, Kumi Izawa, Masahiro Sugiuchi, Ayako Kaitani, Mariko Takahashi, Akie Maehara, Yutaka Enomoto, Toshihiko Oki, Fumi Shibata, Kaori Tamitsu, Si-Zhou Feng, Hideaki Nakajima[5], 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 ho-

mology search in the close proximity on the same chromosome 11: LMIR3 is an inhibitory type receptor like LMIR1, and LMIRs 4-8 are activation type receptors like LMIR2. It is of note that LMIR3 has a unique property to associate with FcRγ and thereby functions as an activating receptor in concert with TLR4 stimulation. LMIRs are also called CLMs or MAIRs. Those receptors are mainly expressed in cells involved in innate immunity including mast cells, neutrophils, monocytes, and dendritic cells. In addition, some of the receptors are also expressed in some cells in colon, trachea, and lung, suggesting that these receptors play important roles in innate immunity.

LMIR5 is a DAP12-coupled activating receptor predominantly expressed in myeloid cells. We have identified T cell Ig mucin 1 (TIM1) as a possible ligand for LMIR5 by retrovirus-mediated expression cloning. TIM1 interacted only with LMIR5 among the LMIR family, whereas LMIR5 interacted with TIM4 as well as TIM1. Stimulation with TIM1 or TIM4 induced LMIR5-mediated activation of mast cells. Notably, LMIR5 deficiency suppressed TIM1-Fc-induced recruitment of neutrophils in the dorsal air pouch, and LMIR5 deficiency attenuated neutrophil accumulation in a model of ischemia/reperfusion injury in the kidneys in which TIM1 expression is up-regulated. In that model, LMIR5 deficiency resulted in ameliorated tubular necrosis and cast formation in the acute phase. Collectively, our results indicate that TIM 1 is an endogenous ligand for LMIR5 and that the TIM1-LMIR5 interaction plays a physiological role in immune regulation by myeloid cells.

We have established gene-disrupted mice lacking LMIR3, LMIR4 or LMIR5. Investigation to elucidate the biological roles of LMIRs on immune cells is now underway.

5. 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[6], Hironori Harada[6], Toshiya Inaba[7], Hideaki Nakajima[5], Tetsuya Nosaka, Jiro Kitaura, and Toshio Kitamura: [6]Department of Hematology/Oncology and [7]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.

6. The function of TSC-22 as a tumor suppressor.

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Transforming growth factor-beta (TGF-beta)-stimulated clone-22 (TSC-22) was originally isolated as a TGF-beta-inducible gene. We identified TSC-22 as a potential leukemia suppressor. Two types of FMS-like tyrosine kinase-3 (Flt3) mutations are frequently found in acute myeloid leukemia: Flt3-ITD harboring an internal tandem duplication in the juxtamembrane domain associated with poor prognosis and Flt3-TKD harboring a point mutation in the kinase domain. Comparison of gene expression profiles between Flt3-ITD- and Flt3-TKD-transduced Ba/F3 cells revealed that constitutive activation of Flt3 by Flt3-TKD, but not Flt3-ITD, upregulated the expression of TSC-22. Forced expression of TSC-22 suppressed the growth and accelerated the differentiation of several leukemia cell lines into monocytes, in particular, in combination with differentiation-inducing reagents. On the other hand, a dominant-negative form of TSC-22 accelerated the growth of Flt3-TKD-transduced 32 Dcl. 3 cells. Collectively, these results suggest that TSC-22 is a possible target of leukemia therapy.

We are now analyzing the function of TSC-22 as a tumor suppressor by using TSC-22-deficient mice.

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Advanced Clinical Research Center

Division of Infectious Diseases
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Summary

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.

1. Impaired replication capacity of acute/early viruses in persons who become HIV controllers

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Human Immunodeficiency Virus type-1 (HIV-1) controllers maintain viremia at ＜2,000 RNA-copies/ml without antiretroviral therapy. Viruses from controllers with chronic infection were shown to exhibit impaired replication capacity, in part associated with escape mutations from cytotoxic T lymphocyte (CTL) responses. In contrast, little is known about viruses during acute/early infection in individuals who subse-

quently become HIV controllers. Here we examine viral replication capacity, HLA types and virus sequences from 18 HIV-1 controllers identified during primary infection. *Gag-protease* chimeric viruses constructed using the earliest post-infection samples displayed significantly lower replication capacity than isolates from the persons who failed to control viremia ($p=0.0003$). Protective HLA class I alleles were not enriched in these early HIV controllers, but viral sequencing revealed significantly higher prevalence of drug resistance mutations associated with impaired viral fitness in controllers than noncontrollers [6/15(40.0%) vs 10/80(12.5%), $p=0.018$]. Moreover, of two B57+controllers identified, both harbored signature escape mutations within Gag at the earliest time point tested that likewise impair viral replication capacity. Only five controllers did not express "protective" alleles or harbor drug resistance mutations; intriguingly two of them displayed typical B57 signature mutations (T242N), suggesting the acquisition of attenuated viruses from B57+ donors. These data indicate that acute/early stage viruses from the persons who become controllers have evidence of reduced replication capacity during the initial stages of infection, which likely associated with transmitted or acquired CTL escape mutations or transmitted drug resistance mutations. These data suggest that viral dynamics during acute infection have a major impact on HIV disease outcome.

2. A novel HIV-1 phenotypic tropism assay using Dual Split Protein (DSP) - mediated quick membrane fusion detection system

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Background: Coreceptor usage is one of the most fundamental steps in HIV-1 replication. Since the development of CCR5 inhibitors for clinical use, the viral tropism is a matter of great importance in the antiretroviral therapy. Both phenotypic (PTA) and genotypic assays (GTA) are available for viral tropism determination. Although PTA is a gold standard, it requires well-trained personnel, biosafety facilities and also is time-consuming. Here, we present the first HIV-1 PTA which does not use pseudovirus in tropism determination. Method: Dual split protein (DSP) composed of split green fluorescent protein (GFP) and split renilla luciferase (RL) was employed as a marker for cell fusion phenomenon. 70% of GFP and RL (DSP1) was stably expressed in NP2 cells expressing CD4/CXCR4 (N4X4-DSP1) or CD4/CCR5 (N4R5-DSP1). HIV-1 envelope gene from cloned reference strains or patients' plasma was ligated to an expression vector containing 30% of GFP and RL (DSP2), so called pRE11-env. pRE11-env was transfected to 293FT cells. Two days post-transfection, pRE11-env-transfected 293FT cells were overlaid to N4X4-DSP1 or N4R5-DSP1. After 6 h of cocultivation, the tropism could be determined by detection of either GFP signal (by In Cell Analyzer) or luciferase activity (by Enduren) resulted from re-association of DSP1 and DSP2 among fused cells. The results were compared to in-house pseudoviral tropism assay. Results: Using reference strains (BaL, HXB2, LAI, NL4-3, SF2) envelopes for assay validation, the tropisms were precisely determined. Fluorescent signals were proportionate with luciferase signals and completely concordant. A number of clinical isolates' clones were examined. The results were concordant with in-house pseudoviral tropism assay. Conclusion: This is the first phenotypic HIV-1 tropism assay without pseudovirus production. This novel assay offers the following advantages: rapid determination (turn-around time within 5 days) with simple manipulation and biosafety (no viral production). DSP offers fast and convenient tropism determination by two-way result confirmation. The assay can be used for basic research, epidemiologic study, diagnostic test, drug development, etc, in both resource-rich and -limited settings.

3. Imbalanced Production Of Cytokines By T Cells Associates With The Activation/Exhaustion Status Of Memory T Cells In Chronic HIV-1 Infection

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Plasma HIV-1 viral load (VL) is closely correlated to disease progression and the level of immune activation. However, the mechanism by

which the persistent presence of HIV-1 damages immune cells is still not fully understood. To evaluate how HIV-1 affects disruption of T cell-mediated immune responses during chronic HIV-1 infection we determined the functional profiles of T cells from subjects with chronic HIV-1 infection. We found T cell impairment during chronic HIV-1 infection is restricted on Th1-related and Th17 responses and directly affected by amount of HIV-1. And the function was recovered in high VL subjects soon after virus elimination by antiretroviral therapy. Our data suggest that HIV-1 levels directly affect the ability of memory T cells to produce specifically Th1- and Th17-related cytokines during chronic HIV-1 infection.

4. Development of a PCR-SSOP-Luminex Assay for HIV-1 Drug Resistance

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A simple, rapid genotypic assay to detect HIV-1 drug resistance (DR) mutations would be valuable for both clinical practice and epidemiological research. We developed a strategy that combines a PCR amplification- sequence-specific oligonucleotide probes (SSOP) protocol and Luminex 100^{IS} technology to detect 6 mutations in HIV-1 RT gene: M41L, K65R, K70R, K103N, M184V and T215Y/F. The system appears capable of detecting DR mutation if they comprise at least 10-20% of the population. We used the assay to determine the presence or absence of the 6 RT mutations in plasmas from 74 clade B HIV-1-infected patients, and compared the results to those obtained by cloning and sequencing. In 83.8%, 58.1%, 93.2%, 74.4%, 85.1%, and 90.5% samples for M41, K65, K70, K103, M184, and T215, respectively, PCR-SSOP-Luminex method and sequencing results were completely concordant. In 31 samples, we were unable to detect signals for K65 with the initial set of oligoprobes. We identified genetic polymorphisms in these 31 patients by sequencing within the target codons or their flanking regions. By introducing additional set of oligoprobes, we could improve the system to detect those (58.1%→91.9 %). The assay system specificity would be 95.5 %, 100%, 90.0%, 71.4%, 81.0%, and 95.7%, and the sensitivity 78.8%, 91.5%, 95.3%, 80.6%, 88.5 %, and 93.8% for M41L, K65R, K70R, K103N, M184V and T215Y/F, respectively. Our data suggest that PCR-SSOP-Luminex assay can be used effectively to detect clinically significant DR mutations in HIV-1. With the development of suitable primers and oligoprobes, this method could be applicable to other HIV-1 subtypes and possibly use in clinical practice and/or public health research, especially in the developing country.

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Advanced Clinical Research Center

Division of Bioengineering
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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.

Development of innovate cancer therapy using immunologic approaches

I. Cancer immunotherapy using monocyte-derived DCs stimulated with OK-432 and prostaglandin E2 pulsed with gp100 epitope peptide in malignant melanoma

Marimo Sato-Matsushita, Akira Kanamoto, Hideaki Tahara

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 gp 100. The information related to these trials would be useful to develop effective immunotherapy against malignant melanoma.

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

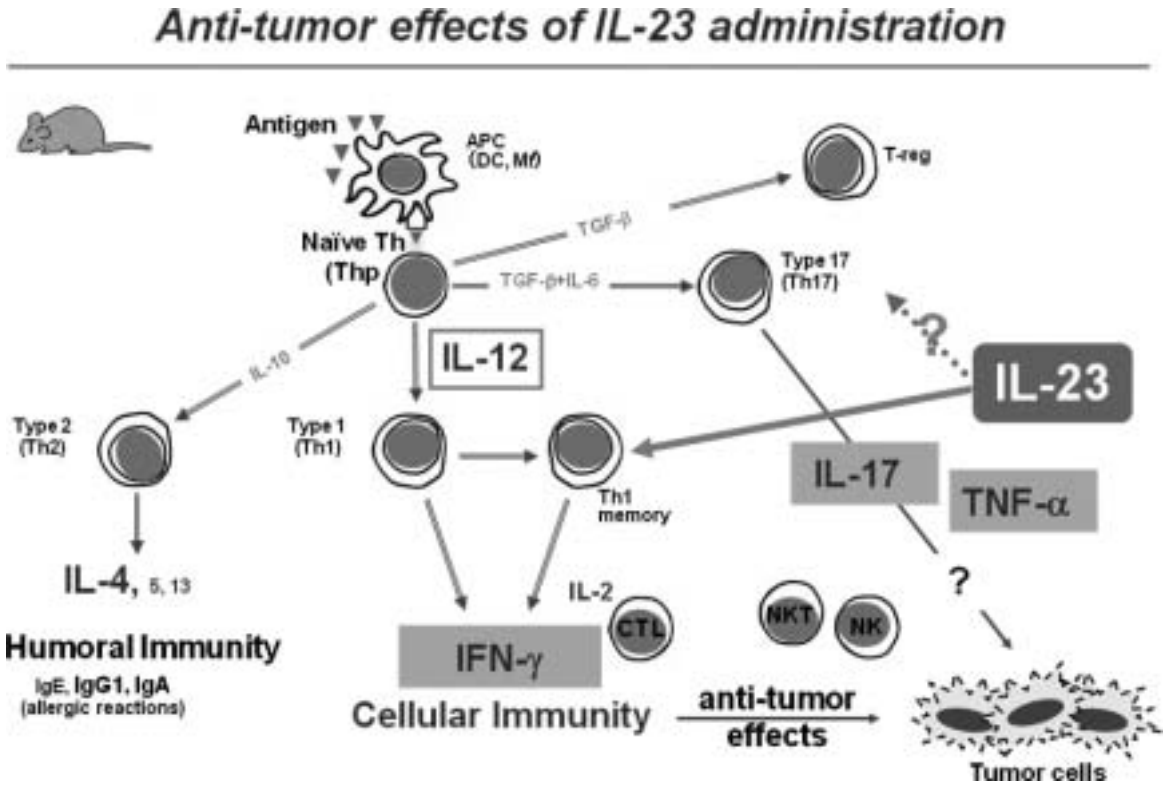
Marimo Sato-Matsushita, Hideaki Tahara

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.

III. Induction of systemic and therapeutic antitumor immunity using dendritic cells genetically modified to express interleukin-23

Marimo Sato-Matsushita, Hideaki Tahara

We have reported that the systemic administration of IL-23 induces potent antitumor immunity primarily mediated when the Th1-type response. In this study, we investigated whether bone marrow- derived dendritic cells (BM-DCs) adenovirally transduced with genes encoding murine IL-23 have therapeutic benefits for antitumor immunotherapy. We made RGD fiber-mutant adenovirus (Ad) vectors encoding IL-23 or EGFP. The MCA205 fibrosarcoma intradermally inoculated to C57BL/6 on day 8, the mice were intratumorally injected with BM-DCs transduced with Ax3CAmIL23/RGD (Ad-IL-23-DCs). The tumors of mice treated with AD-IL-23-DCs resulted in insignificant growth suppression when compared to that with BM-DCs transduced Ad-EGFP-F/RGD. Ad-IL-23-DCs treatment induced MCA-205-specific and potent CTL responses. In addition, The significant induction of IFN-γand IL-17 and decrease of Tregs in TIL were strongly suggested in the mice injected with Ad-IL-23-DCs. This strategy designed to deliver genetically modified DCs to tumor sites is associated with systemic and therapeutic antitumor immunity and could be an alternative approach to those using delivery of DCs loaded with defined tumor antigens.



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Advanced Clinical Research Center

Division of Clinical Immunology
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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, Tomonori Katayose, Yoshiko Kichikawa, Hiromi Ichihara, 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. 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. Crk associated substrate lymphocyte type (Cas-L) is docking protein that is heavily tyrosine phosphorylated by integrins and growth factor dependent signals. Cas-L has been expressed in lung cancer cells. Cas-L has a role of biological regulation of cell attachment, migration and invasion, apoptosis and cell cycle and also plays a role in the development of metastatic capability in cancer. Cas-L protein is identified with an essential switch for pro-metastatic behavior in tumors. Cas-L mRNA and protein expression were elevated in a significant percentage of metastatic melanomas and that this elevated Cas-L expression was required for the metastatic process. We showed here the potential role of Cas-L in the phosphorylation and downstream signaling of EGFR in lung cancer. Furthermore, elevated of Cas-L expression is associated with recurrence and poor prognosis in human lung cancer patients. It is hoped that, by demonstrating clinical significance by prospective patient accumulation and performing postoperative adjuvant chemotherapy by selecting patients or developing new drugs that molecularly target Cas-L, in the future it will be possible to prevent recurrences and suppress metastasis in NSCLC patients.

b. 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 pathophysiological role of Cas-L in the development of RA, we employed collagen-induced arthritis model using Cas-L knockout mice on a C57BL6 background.

We compared three groups of mice, Cas-L $-/-$ (homo), Cas-L $-/+$ (hetero), and littermate controls (wt) on the incidence and severity of CIA. Although the incidence of arthritis was unaltered, wt and hetero mice showed higher severity of arthritis compared to homo mice, which was evaluated by scoring and histology. 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. The serum levels of inflammatory cytokines, TNF-α, IL-17, and IL-6 were higher in wt and hetero mice, whereas that of anti-inflammatory cytokine, IL-10 was higher in homo mice. We are now performing 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.

c. A new derivative, 5-I of Roxithromycin ameliorates collagen-induced arthritis.

The macrolide antibiotics have immunomodulatory properties distinct from antibacterial functions. We synthesized 5-I, the new derivative of RXM with less antimicrobial activity, and studied immunomodulatory effects of 5-I in vitro and in vivo.

5-I specifically inhibited production of Th1, Th 17, and proinflammatory cytokines by T cells stimulated with anti-CD3 and anti-CD28 mAbs, and monocytes stimulated with LPS. 5-I also inhibited activated T cells migration. Finally, we found that the administration of 5-I to collagens-induced arthritis mice reduced severity of arthritis. Their effectiveness was also observed delayed treatment after onset of disease.

Our findings strongly suggest that 5-I may be useful for the therapy of rheumatoid arthritis.

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 costi-

mulation 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 CD 26 (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 CD 26-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 CD 26 antibody for the treatment of malignant mesothelioma has already been started at Gustave Roussy Institute and Cochin Hospital in Paris.

a. A fine strategy for immune-tolerance 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 CD26-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 T lymphocytes, the subset of CD4+CD45 RO+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. 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 a 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. We have shown that 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 sti-mulation. Moreover, with CD26-mediated costi-mulation, CD8+ T cells were observed to kill target cells in a Granzyme B-dependent manner. Taken together, our data strongly suggest that CD26 plays a very 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 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 alloSCT 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. A role of CD26/DPPIV in colon cancer

CD26 (dipeptidyl peptidase IV, DPPIV) is a 110 kDa surface glycoprotein expressed in most normal tissues, and is a potential novel therapeutic target for selected cancers. Our work

evaluates the mechanism involved in confluence-dependent CD26 expression in colon cancer.

We found that the colon cancer cell lines HCT-116 and HCT-15 exhibited a confluence-dependent increase in CD26 mRNA and protein, associated with decreased expression of c-Myc, increased USF-1 and Cdx 2 levels, and unchanged HIF-1α expression. Meanwhile, ectopic expression of c-Myc in both cell lines led to decreased CD26 expression. In contrast, transfection of a siRNA targeted to Cdx2 resulted in decreased CD26 level. Importantly, culturing of cells in serum-depleted media, but not acidic conditions, upregulated CD26. While HIF-1α level also increased when cells were cultured in serum-depleted media, its expression was required but not sufficient for CD26 upregulation.

CD26 mRNA and protein levels increase in a confluence-dependent manner in colon carcinoma cell lines, with c-Myc acting as a repressor and Cdx2 acting as an enhancer of CD26 expression. The enhanced expression of CD26 in serum-depleted media and a requirement for HIF-1α suggest a role for nutrients or growth factors in the regulation of CD26 protein expression.

III. Therapeutically targetting transcription factors

Hirotoshi Tanaka, Noritada Yoshikawa (Rheumatology Clinic), Noriaki Shimizu, Takako Maruyama, Chikao Morimoto (Division of Clinical Immunology)

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. Our recent achievement is now been applied in clinical settings in the Research Hospital.

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 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. On the other hand, we have developed an efficient system to screen out the target genes of GR in glucocorticoid-responsive tissues, and are woking with clarification of tissue-specific effects of glucocorticoids.

a. 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 ascrived 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 the mineralocorticoid receptor (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.

b. 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.

c. Clarification of tissue-specific effects of glucocorticoids

We performed target gene identification and clarification of their biological significance in cardiac muscles and skeletal muscles.

The elucidation of tissue-specific target genes of GR action is difficult, since the GR overlaps functionally with MR at the level of ligand-binding specificity, and most metabolically active organs, including the heart, express substantial levels of both GR and MR. Endogenous glucocorticoid -namely, cortisol in humans and corticosterone (COR) in rodents -binds to both the GR and the MR with comparable affinity. In the absence of 11β-hydroxysteroid dehydrogenase 2, which converts the glucocorticoid to inactive metabolites, the intramyocardial concentration of glucocorticoid reflects the free concentration in plasma, which is 1,000-fold higher than that of the mineralocorticoid aldosterone (ALD). Therefore, it seems likely that glucocorticoid rather than mineralocorticoid occupies the MR and influences the proinflammatory response after myocardial infarction. Thus, it is crucial to clarify the GR-specific target genes independently of the functional redundancy with MR. Recently, we performed DNA microarray analysis to evaluate the changes in gene expression profiles in neonatal rat cardiomyocytes after stimulation with COR, the GR-selective agonist cortivazol (CVZ), or ALD. Unexpectedly, we found that the expression of genes that encode 2 key enzymes in a common pathway of prostaglandin biosynthesis were upregulated by glucocorticoids via the GR in cardiomyocytes: phospholipase A2 group IVA (Pla2g4a; encoding cytosolic calcium-depen-dent phospholipase A2 [cPLA2]), which belongs to the class of cPLA2s that preferentially cleave arachidonic acid from membrane phospholipids; and prostaglandin-endoperoxide synthase 2 (Ptgs2; encoding COX 2), which converts arachidonic acid into PGH2. Importantly, ALD did not have similar stimulatory effects on these genes. The induction of Pla 2g4a and Ptgs2 by GR is specific for cardiomyocytes, since GR has been shown to transrepress the activation of these proinflammatory genes in most cells. Therefore, we sought to investigate the major types of prostanoids produced in cardiomyocytes after exposure to glucocorticoids and to clarify the roles of these products in cardiac physiology. Among the genes for PGH2 isomerases, expression of Ptgds, which encodes lipocalin-type prostaglandin D synthase (L-PGDS), was selectively upregulated by a GR-specific ligand. Consistent with this result, PGD 2 was the most prominently induced prostaglandin by GR-spe-cific ligand stimulation of cultured cardiomyocytes and in vivo hearts. Using isolated Langen-dorff-perfused hearts and cultured cardiomyocytes, we demonstrate that the activation of L-PGDS-mediated production of PGD2 was crucial for the cardioprotection against ischemia / reperfusion conferred by glucocorticoid-GR signaling. Our results suggest what we believe to be a novel interaction between glucocorticoid-GR signaling and the arachidonic acid cascade-media-ted cardiomyocyte survival pathway.

d. Restoration of Muscle atrophy

Muscle comprises ~40% of body mass and contributes not only to the structure and movement of the body but also to nutrient storage and supply. In adult mammals, skeletal muscle hypertrophy/atrophy is characterized by an increase/decrease in the size (as opposed to the number) of individual myofibers, respectively. The control of muscle mass is believed to be determined by a dynamic balance between anabolic and catabolic processes (Hoffman and Nader, 2004). Mammalian target of rapamycin (mTOR) is a crucial component of the anabolic machinery for protein synthesis. mTOR consists of two complexes: mTORC1, which includes Raptor, signals to S6K and 4E-BP1, controls protein synthesis, and is rapamycin sensitive; and mTORC2, which includes Rictor, signals to Akt, and is rapamycin insensitive. mTORC1 integrates four major signals: growth factors, energy status, oxygen, and amino acids, especially branched-chain amino acids (BCAA). Prototypically, insulin/IGF-1 activates mTOR via the PI3 K-Akt pathway. It is currently considered that mTORC1, and not mTORC2, is essential for the maintenance of muscle mass and function. Protein degradation in skeletal muscle cells is essentially mediated by the activity of two conserved pathways: the ubiquitin-proteasomal pathway and the autophagic/lysosomal pathway (Sandri, 2008). The ubiquitin-proteasomal pathway is responsible for the turnover of the majority of soluble and myofibrillar muscle proteins. The activity of this pathway is markedly increased in at-

rophying muscle due to the transcriptional activation of a set of E3 ligase-encoding genes, e.g., atrogin-1 and MuRF1 (Glass, 2003, Sandri et al., 2004). Autophagy also plays an important role in the degradation of skeletal muscle, and is indicated to be a consequence of an ordered transcriptional program involving a battery of genes, e.g., LC3 and Bnip3 (Mizushima et al., 2008). These positive and negative pathways are balanced in a highly coordinated manner for the determination of myofiber size and total muscle volume; however, distortion of this balance with a relative increase in degradation results in the generalized decrease of myofiber size and muscle atrophy (Hoffman and Nader, 2004). Pioneering studies demonstrated that muscle atrophy is a result of active processes that are transcriptionally controlled through the expression of a particular gene set; the forkhead box O (FoxO) transcription factors are common components of a number of atrophy models and act as critical liaison molecules for protein degradation and autophagy via the transcriptional regulation of, for example, atrogin-1, MuRF1, LC3, and Bnip3 (Mammucari et al., 2007; Sandri et al., 2004; Stitt et al., 2004; Zhao et al., 2007). In clear contrast, it is evident that each disease has proper signaling pathways to FoxOs and that other components of the cellular machinery often participate in the progression of atrophy (Moresi et al., 2010; Suzuki et al., 2007). Therefore, for the development of therapies against muscle atrophy, it should be addressed how the transcriptional program triggered by a particular atrophy pathway is orchestrated and how the balance of muscle protein synthesis and degradation is distorted in each disease.

Typically, glucocorticoid-induced muscle atrophy is characterized by fast-twitch type II glycolytic muscle fiber loss with reduced or no impact on type I fibers. The mechanism of such fiber specificity is yet unknown. Previous reports suggested that the glucocorticoid-GR system has anti-anabolic and catabolic effects and promotes degradation via the induction of a set of genes including atrogin-1, MuRF1, and myostatin (Menconi et al., 2007; Schakman et al., 2008). Although the involvement of FoxO transcription factors is reported in the gene regulation of atrogin-1 and MuRF1 under the presence of excess glucocorticoids (Sandri et al., 2004; Stitt et al., 2004), the biochemical role of GR in the transcriptional regulation of muscle tissue has not yet been determined. Therefore, we investigated how GR-mediated gene expression coordinately modulates anti-anabolic and catabolic actions to understand the functional coupling of metabolism and volume regulation in muscle.

We identified REDD1 and KLF15 genes as direct targets of GR. REDD1 is known to be induced by various stressors, including glucocorticoid, and to inhibit mTOR activity via the sequestration of 14-3-3 and the increase of TSC1/2 activity (Wang, et al., 2006; DeYoung et al., 2008). We clearly identified the functional GRE via the promoter analysis of REDD1 gene. On the other hand, KLF15 is a recently discovered transcription factor that is involved in several metabolic processes in skeletal muscle; e.g., KLF 15 transcriptionally upregulates the gene expression of branched-chain aminotransferase 2 (BCAT2), a mitochondrial enzyme catalyzing the first reaction in the catabolism of BCAA to accelerate BCAA degradation and alanine production in skeletal muscle (Gray et al., 2007). Moreover, phenotypic analysis of cardiac-specific KLF 15 knockout mice revealed marked left ventricular hypertrophy, indicating the negative regulatory role of KLF15 on muscle mass (Fisch et al., 2007). We here demonstrated that KLF15 participates in muscle catabolism via the transcriptional regulation of atrogin-1 and MuRF1. Moreover, KLF15 affects mTOR through BCAA degradation and negatively modulates myofiber size. mTOR activation inhibits GR-mediated transcription by suppressing GR recruitment onto target genes, strongly suggesting a mutually exclusive crosstalk between mTOR and GR. Pharmacological activation of mTOR with BCAA attenuated GR-mediated gene expression, leading to the substantial restoration of muscle in glucocorticoid-treated rats. We, therefore, indicate the critical importance of the interaction of GR and mTOR in the regulation of metabolism-volume coupling in skeletal muscle.

IV. Cancer Stem Cells

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

a. Regulation of stem cell properties by CD9 in human B-acute lymphoblastic leukemia

Although the prognosis of acute lymphoblastic leukemia (ALL) has improved considerably in recent years, some of the cases relapse and exhibit therapy-resistant. We have previously reported that CD9 is expressed heterogeneously in B-ALL cell lines and only CD9+ cells proliferated by an asymmetric cell division-like manner. CD9+ cells also exhibited greater tumorigenic potential than CD9- cells and were serially transplantable in immunodeficient mice, indicating that CD9+ cell possess self-renewal capacity. In the current study, we performed more detailed analysis of CD9 function for the stem

cell properties in B-ALL. In patient sample analysis, CD9 is expressed in most cases of B-ALL cells with significant correlation of CD34- expression. Gene expression analysis revealed that Wnt family proteins, leukemogenic fusion proteins, and Src family proteins were significantly regulated in the CD9+ population of both cell lines and corresponding patient samples. Moreover, CD9+ cells exhibited drug-resistance for both anti-cancer drugs and Src inhibitors, but proliferation of bulk cells of the cell line was inhibited by anti-CD9 monoclonal antibody. Knockdown of CD9 by shRNA remarkably reduced the leukemogenic potential, resulting in longer survival of the transplanted mice. Furthermore, gene ablation of CD9 affected the expression and tyrosine-phosphorylation of Src family proteins and reduced the expression of USP22, a known cancer stem cell marker and deubiquitination enzyme of histone H2B. Taken together, our results suggest that CD9 links to several signaling pathways and epigenetic modification for regulating the stem cell properties of B-ALL.

b. Identification of cancer stem cell markers in human malignant mesothelioma cells.

Malignant mesothelioma (MM) is an aggressive and therapy-resistant neoplasm arising from the pleural mesothelial cells and usually associated with long-term asbestos exposure. Recent studies suggest that tumors contain cancer stem cells (CSCs) and their stem cell characteristics are thought to confer therapy-resistance. However, whether MM cell has any stem cell characteristics is not known. To understand the molecular basis of MM, we first performed serial transplantation of surgical samples into NOD/SCID mice and established new cell lines. Next, we performed marker analysis of the MM cell lines and found that many of them contain SP cells and expressed several putative CSC markers such as CD9, CD24, and CD26. Interestingly, expression of CD26 closely correlated with that of CD24 in some cases. Sorting and culture assay revealed that SP and CD24(+) cells proliferated by asymmetric cell division-like manner. In addition, CD9(+) and CD24(+) cells have higher potential to generate spheroid colony than negative cells in the stem cell medium. Moreover, these marker-positive cells have clear tendency to generate larger tumors in mouse transplantation assay. Taken together, our data suggest that SP, CD9, CD24, and CD26 are CSC markers of MM and could be used as novel therapeutic targets.

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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 cancers

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To clarify the molecular mechanisms of colorectal cancer and discover target molecules for therapy, we previously compared expression profiles of colorectal cancer tissues with the corresponding non-cancerous colon tissues using cDNA microarray. As a result, we found that MRGBP (MRG-binding protein; also known as C 20orf20) was up-regulated in the majority of colorectal tumors and the enhanced expression was associated with cell proliferation. We furthermore investigated its role in colorectal carcinogenesis and searched for genes regulated by MRGBP. Immunohistochemical staining of 22 adenomas and 47 carcinomas in the colon and rectum showed that high levels of MRGBP expression were observed more frequently in carcinomas (45%) than adenomas (5%), linking its role to malignant properties of colorectal tumors. Copy number analysis revealed that gene amplification is involved in the elevated expression. Using colorectal cancer cells expressing abundant MRGBP, we suppressed its expression using MRGBP siRNA. Genome-wide expression analysis of the cells treated with or without MRGBP siRNA identified a total of 41 genes up-regulated by MRGBP. These genes were implicated in biological processes including DNA replication, minichromosome maintenance, and cell division, suggesting that MRGBP contributes to colorectal carcinogenesis through rendering advantages in cell proliferation and/or division of cancer cells. 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 carcinomas, and breast cancer

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

To clarify the physiological role of SMYD3, a molecule over-expressed in colorectal cancer, hepatocellular carcinoma, and breast cancer, we investigated its role in development using zebrafish. Two forms of *smyd3* were identified in a zebrafish database. Semi-quantitative RT-PCR

analysis disclosed that both forms were expressed from early developmental stages. We found that knockdown of Smyd3 with morpholino oligonucleotides led to pericardiac edema and curved trunk suggesting that Smyd3 plays a crucial role in the development of heart and skeletal muscle. We further investigated the expression of cardiac and skeletal muscle markers in the zebrafish treated with the morpholinos (morphants). *In situ* hybridization revealed that expression of anterior lateral plate mesoderm markers including *gata4, scl, nkx2.5*, and *hand2*, was normal in the morphants. However, expression of *cmlc2, amhc*, and *vmhc* was deregulated in the morphants. Expression of myogenic regulatory factors including *myod* and *myog* was high in both the morphants and controls at early developmental stages. Although control zebrafish decreased their expression at late stages, Smyd3 morphants persisted high levels of their expression. To investigate which variants play a role in the development of cardiac and skeletal muscle, we co-injectioned variant1 or variant2 mRNA into the eggs together with the Smyd3 morpholinos. Consequently, co-injection of variant2 decreased the heart and trunk defects, but co-injection with variant1 did not complement the defects. These data suggest that Smyd3 variant2 may play a major role in the heart and trunk development through the regulation of cardiac chamber-specific and myogenic regulatory factors.

3. Establishment and investigation of novel mouse models of gastrointestinal, pancreas, and liver cancer

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Genetically engineered mice (GEM) are useful tools for studying human diseases, including cancer. We have tried to establish mouse models of gastrointestinal, pancreas, and liver cancers using GEM with tissue-specific activation and/or inactivation of genes involved in various intracellular signaling pathways.

In this project, we have already succeeded in the establishment of a mouse model of pancreatic ductal adenocarcinoma (PDAC) using pancreas-specific deletion of *TGF β type II receptor* and *K-ras* activation. In addition, we recently succeeded in the development of a novel mouse model displaying aggressive intrahepatic cholangiocarcinoma (ICC). This model was generated by the liver-specific deletion of *Pten* and *K-ras* activation, because it has been reported that PI3K/AKT and RAS signaling pathways are frequently involved in human ICC. Now we are characterizing the phenotypes of these mouse models. Intense investigation of these models should provide better understanding of PDAC and ICC and facilitate the development of new therapies to these tumors.

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. 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 colorectal cancer was determined. For genetic diagnosis, we analyzed the three responsible genes by PCR-direct sequencing and Multiplex Ligation-dependent Probe Amplification. 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. The analysis identified alterations not only in exons but also in introns. We have developed a minigene assay system to examine genetic alterations associated with disrupted splicing, and proved that this system is useful for the characterization of variants in introns as well as exons. Using this system, two genetic alterations were diagnosed as pathogenic mutations because both alterations resulted in either exon-skipping or activation of cryptic-splicing. We have been analyzing cases that do not harbor any pathogenic mutations in *MLH1*, *MSH2*, *or MSH6*, three responsible genes, but contain large deletions in the *TACSTD (Ep-CAM)* gene. Challenges of increasing the sensitivity of diagnosis and finding people at a genetically high-risk to this disease are ongoing.

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