Advanced Clinical Research Center

Division of Molecular Therapy
分子療法分野

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

(1) Preclinical study of therapeutic gene transfer mediated by various viral vectors:
A main research project in this field is application of RNA interference (RNAi) technology to a human experimental model of hematological malignancies characterized by a leukemia specific fusion mRNA such as BCR-ABL.

(2) 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.

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

(4) Regeneration of tooth using the methods of tissue engineering: To accomplish this goal, we are focusing on the issues including a) identification and characterization of tissue stem cells from tooth germ, b) search for molecules to affect the growth and differentiation of stem cells, and c) search for suitable biomaterials as the scaffold to assemble these stem cells on.

1. The *in vitro* and *in vivo* effects of imatinib mesylate on $CD8^+$ T cell subsets defined by the level of CD26 expression.

Yokoyama K, Nagamura T, Ohno N, Uchimaru K, Takahashi S, Tojo A

Based on the expression level of CD26, $CD4^+$ and $CD8^+$ T cells can be divided into 3 (high/intermediate/low or negative) subsets. The significance of CD26 has been studied mainly on $CD4^+$ T cells, and $CD26^{high}CD4^+$ T cells are considered to represent effector memory T cells of a typical Th1 phenotype producing IL2 and IFNγ. The role of each subset of $CD8^+$ T cells has not yet been clarified. Multi-parameter flow cytometry analysis was performed to characterize $CD8^+$ T cells differentially expressing CD26 in combination with intracellular detection of cytokines including perforin (P) and granzyme B (Gr). According to the expression level of CD26, we could categorize $CD8^+$ T cells as follows: $CD26^{high}CD8^+$ T cells are defined as central memory T cells which has a phenotype of $CD45RO^+CD28^+CD27^+$ $IFN\gamma^+Gr^+P^{+/-}$, $CD26^{intermed}CD8^+$ T cells as naïve T cells of $CD45RO^-CD28^+CD27^+$ $IFN\gamma^-Gr^-P^-$, and $CD26^{low}CD8^+$ T cells as effector memory T cells of $CD45RO^{-/+}$ $CD28^-CD27^-$ $IFN\gamma^{++}$ $Gr^{++}P^{++}$, respectively. Peripheral blood mononuclear cells were primed with anti-CD3/CD28 MAb and subjected to the grading doses of imatinib mesylate (IM; ABL kinase inhibitor) for short term culture, followed by flow cytometory. CFSE labeling was used for monitoring cell proliferation. Intriguingly, we found that IM dose-dependently inhibits activation, cytokine production and proliferation of $CD26^{high}CD8^+$ central memory T cell subsets in a differentiation stage-specific manner. We compared the absolute number of peripheral blood $CD26^{high}CD8^+$ T cell subsets between 20 patients with CML in IM-induced CCR and 20 normal volunteers, clearly indicating a significant decrease of this subset in CML patients (22.30/μl vs 45.60/μl, $p<0.01$). The present study offers another evidence for immunomodulatory effects of imatinib or the critical role of Abl (-related) kinase in T cell development, and draws special attention to susceptibility to viral infection of CML patients under long-term IM therapy.

2. The marked perturbation of human myelopoiesis and B lymphopoiesis by forced expression of Pax5

Sekine R, Tojo A

We applied a co-culture system to the genetic manipulation of human B lymphoid and myeloid progenitor cells, using a murine bone marrow stromal cell support and investigated the effects of forced Pax5 expression in both cell types. Cytokine-stimulated cord blood $CD34^+$ cells could be transduced at 85% efficiency and 95% cell viability by a single 24 h infection with RD114-pseudotyped retroviral vectors, produced by a packaging cell line Plat-F and bicistronic vector plasmids pMXs-IG, pMYs-IG, or pMCs-IG, encoding EGFP. Infected $CD34^+$ cells were seeded onto HESS-5 cells in the presence of stem cell factor and granulocyte colony-stimulating factor, allowing extensive production of B progenitors and granulocytic cells. We examined cell number and CD34, CD33, CD19, and CD20 lambda and kappa expression by flow cytometry. Ectopic expression of Pax5 in $CD34^+$ cells resulted in small myeloid progenitors coexpressing CD33 and CD19 and inhibited myeloid differentiation. After 6 weeks, the number of Pax5-transduced $CD19^+$ cells was 40-fold lower than that of control cells. However, expression of CD20 and κ/λ chain on Pax5-transduced $CD19^+$ cells suggests that Pax5 transgene may not interfere with their differentiation. This report is the first description of the effects of forced Pax5 expression in human hematopoietic progenitors.

3. Efficient knockdown of $p190^{Bcr-Abl}$ delineates its downstream signaling pathway and implicates a novel combination of therapeutic agents for overcoming drug resistance in Ph+ALL.

Futami M, Tojo A

The 190kD (p190) and 210kD (p210) Bcr-Abl proteins are responsible for the development and progression of Philadelphia chromosome (Ph)-positive leukemia. We applied RNA interference (RNAi) technology to specific killing of $p190^+$ cells. After screening a series of small hairpin RNA (shRNA) targeting p190, we determined the optimal sequences for gene silencing in the BCR, junctional and ABL region of p190, respectively. Then, $p190^+$, $p210^+$ and both negative cell lines were infected with lentiviral vectors encoding these shRNAs, resulting in efficient killing of $p190^+$ cells, while $p210^+$ cells were also sensitive to the two other than junction-specific shRNA and both negative cells were resistant to all. In p190-transformed Ba/F3 cells, RNAi-mediated silencing of p190 specifically inhibited tyrosine phospohorylation of stat 5 prior to their death, but did not affect phosphorylation of Jak2, Akt, or MEK1/2. In contrast, down-regulation of p190 by their treatment with 17-allylamino-17- demetoxygeldana-

mycin (17-AAG) was associated with reduced protein levels of Jak2, Akt, and MEK1/2. shRNA targeting p190 collaborated additively with imatinib and 17-AAG in growth inhibition of Ba/F3-p190wt and BaF/3-p190Y253H cells. Collectively, RNAi-mediated silencing of p190 is a promising option both for delineating signal transduction and for therapeutic application in p 190-expressing leukemia.

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

Kobayashi S, Izawa K, Tojo A

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

5. Contribution of microenvironmental upregulation of NF-κB activity to survival, expansion and drug-resistance in Ph+ALL.

Tsai HJ, Kobayashi S, Izawa K, Tojo A

Constitutive activation of NF-κB was reported in primary blast cells and cell lines derived from Philadelphia chromosome (Ph)-positive acute lymphoblastic leukemia (Ph+ALL). However, microenvironmental regulation of NF-κB activity in Ph+ALL has not been clarified. We infected Ph+ALL cell lines (IMS-PhL1 and Sup-B15) with a lentiviral vector containing the NF-κB/Luc reporter gene, and the resulting cloned cells were cultured in the absence and presence of murine stromal cells (HESS5) and/or cytokines. NF-κB activity of Ph+ALL cells was analyzed by luminometer and *in vivo* imaging system (IVIS), and was not significantly activated by HESS5 alone but synergistically upregulated by the combination of HESS5 and TNFα. DHMEQ, a specific inhibitor of NF-κB nuclear translocation, induced apoptosis of Ph+ALL cells, suggesting the essential role of NF-κB in their growth and survival. However, DHMEQ-induced inhibition of NF-κB activity and apoptosis of Ph-ALL cells were hampered by the combination of HESS5 and TNFα. Next, we transplanted these NF-κB / Luc reporter-transduced Ph-ALL cells into NOD-SCID mice and periodically monitored in vivo regulation of their NF-κB activity. The mode and pattern of *in vivo* NF-κB activation was different between the two cell lines, but murine TNFα was significantly induced in the lesions where infiltrated Ph+ALL cells revealed high NF-κB activity. These results suggest that microenvironmental upregulation of NF-κB activity may contribute to survival, expansion and presumably drug-resistance of Ph+ALL cells. The present bioimaging model helps us to dissect the regulatory mechanism of NF-κB signal by cytokines and cellular interactions.

6. Feasibility and efficacy of bone tissue engineering using human bone marrow mesenchymal stromal cells cultivated in serum-free conditions

Agata H, Kagami H, Tojo A

Current standard techniques for bone tissue engineering utilize *ex vivo* expanded osteogenic cells. However, *ex vivo* expansion requires serum, which may hinder clinical applications. Here, we report the feasibility and efficacy of bone tissue engineering with human bone marrow stromal cells (BMSCs) expanded in serum-free conditions. Bone marrow was aspirated from 4 healthy donors and adherent cells were cultured in either serum-free medium or conventional serum-containing medium. Efficacy of expansion was greater in serum-free medium. Phenotypically, serum-free expanded BMSCs were smaller in cell-size and showed expression of $CD105^{++}$ and $CD146^{dim}$. After osteogenic induction, serum-free expanded BMSCs showed lower alkaline phosphatase activity. However, they showed higher responsiveness to induction. Ectopic bone-forming capability was also confirmed. In conclusion, bone tissue engineering with serum-free expanded BMSCs is feasible and as efficient as that obtained with BMSCs ex-

panded in conventional serum-containing medium.

Publications

Agata H, Watanabe N, Ishii Y, Kubo N, Ohshima S, Yamazaki M, Tojo A, Kagami H. Feasibility and efficacy of bone tissue engineering using human bone marrow mesenchymal stromal cells cultivated in serum-free conditions. Biochem Biophys Res Commun, 2009 Mar 9. [Epub ahead of print]

Ooi J, Takahashi S, Tomonari A, Tsukada N, Konuma T, Kato S, Kasahara S, Sato A, Monma F, Nagamura F, Iseki T, Tojo A, Asano S. Unrelated cord blood transplantation after myeloablative conditioning in adults with ALL. Bone Marrow Transplant. 2008 Oct 27. [Epub ahead of print]

Kiryu S, Inoue Y, Watanabe M, Izawa K, Shimada M, Tojo A, Yoshikawa K, Ohtomo K. Evaluation of Gd-EOB-DTPA as a contrast agent for mouse liver imaging: comparison with Gd-BOPTA. Magnetic Resonance Imaging, 2008 Jul 2. [Epub ahead of print]

Inoue Y, Izawa K, Kiryu S, Kobayashi S, Tojo A, Ohtomo K. Bioluminescent evaluation of the therapeutic effects of total body irradiation in a murine hematological malignancy model. Exp Hematol, 36: 1634-41, 2008

Takahashi N, Sato N, Fukushima T, Takahashi S, Tojo A. Gene expression profiles of peripheral blood mononuclear cell subpopulations in acute graft-versus-host disease following cord blood transplantation. Exp Hematol. 36: 1760-1770, 2008

Nagamura-Inoue T, Kai S, Azuma H, Takanashi M, Isoyama K, Kato K, Takahashi S, Taniguchi S, Miyamura K, Aoki1 K, Hidaka1 M, Nagamura F, Tojo A, Fang1 XM, Kato S for Japan Cord Blood Bank Network. Unrelated cord blood transplantation in chronic myelogenous leukaemia: Japan Cord Blood Bank Network analysis. Bone Marrow Transplant. 42: 241-51, 2008

Futami M, Hatano T, Soda S, Kobayashi S, Miyagishi M, Tojo A. RNAi-mediated silencing of $p190^{Bcr-Abl}$ inactivates Stat5 and cooperates with imatinib mesylate and 17-allylamino-17-demetoxygeldanamycin in selective killing of $p190^{Bcr-Abl}$-expressing leukemia cells. Leukemia. 22: 1131-8, 2008

Sekine R, Kitamura T, Tsuji T, Tojo A. Efficient Retroviral Transduction of Human B Lymphoid and Myeloid Progenitors: Marked Inhibition of Their Growth by the *Pax5* Transgene. Int J Hematol. 87: 351-62, 2008

Inoue Y, Izawa K, Kiryu S, Tojo A, Ohtomo K. Diet and abdominal autofluorescence detected by in vivo fluorescence imaging of living mice. Molecular Imaging, 7: 21-7, 2008

Watanabe N, Takahashi S, Ishige M, Ishii Y, Ooi J, Tomonari A, Tsukada N, Konuma T, Kato S, Sato A, Tojo A, Nakauchi H. Recipient-derived cells after cord blood transplantation: dynamics elucidated by multicolor FACS, reflecting graft failure and relapse. Biol Blood Marrow Transplant. 14: 693-701, 2008

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Our major projects are (1) Analysis of co-ordinate control of cell division and differentiation by a crosstalk between JAK/STAT and small GTPases, (2) Development of 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, Yukinori Minoshima, Ying Chun Bao, Tomonori Hatori, Yasushi Nomura, Takaya Satoh[1], Yoshito Kaziro[2], Hideaki Nakajima[3], Tetsuya Nosaka, David Williams[4] and Toshio Kitamura: [1]Kobe University, [2]Biochemistry and Cell Biology Unit, HMRO, Kyoto University Graduate School of Medicine, [3]Project of mesenchymal stem cells, The 21st century center of excellence program, Institute of Medical Science, The University of Tokyo, [4]Cincinnati Children's Hospital Medical Center, USA

In a search for key molecules that prevent murine M1 leukemic cells from undergoing IL-6-induced differentiation into macrophages, we isolated an antisense cDNA that encodes full-length mouse MgcRacGAP through functional cloning. In human HL-60 leukemic cells, overexpression of human MgcRacGAP induced growth suppression and macrophage differentiation. Interestingly, MgcRacGAP localized to the nucleus in interphase, accumulated to the mitotic spindle in metaphase, and was condensed in the midbody during cytokinesis. These findings indicate that MgcRacGAP dynamically moves during cell cycle progression and plays critical roles in cytokinesis. Moreover, the experiment using a GAP-inactive mutant showed that the GAP activity of MgcRacGAP was required for completion of cytokinesis. We also found that MgcRacGAP is phosphorylated by Aurora B at the midbody. Intriguingly, this phosphorylation induced the Rho-GAP activity of MgcRacGAP, which was critical for completion of cytokinesis. We identified S387 as a phosphorylation site responsible for the acquirement of Rho-GAP activity during cytokinesis at the midbody. On the other hand, MgcRacGAP mainly localizes in the nucleus in the interphase. In the interphase, MgcRacGAP directly binds transcription factors STAT3 and STAT5, and enhances transcriptional activation of STAT proteins as a Rac GAP. Recently, we present evidence showing that MgcRacGAP harbors functional NLS and works as a nuclear chaperon together with Rac1. In summary, our results suggest that MgcRacGAP plays distinct roles depending on the cell cycle

thereby co-ordinating cell division and cell differentiation/proliferation.

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

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

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

STAT3 is frequently activated in many cancers and leukemias, and is required for transformation of NIH3T3 cells. Therefore, we have started searching for STAT3 inhibitors. We already established an efficient screening protocol for identification of STAT3 inhibitors, and identified several compounds that inhibit STAT3 activation. 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 companies to modify these compounds for optimization, thus eventually to develop anti-cancer drugs.

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

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

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

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

Toshihiko Oki, Jiro Kitaura, Koji Eto[6], Yang Lu, Yoshinori Yamanishi, Hideaki Nakajima[3], Hidetoshi Kumagai, and Toshio Kitamura: [6]Laboratory of Stem Cell Therapy, Institute of Medical Science, The University of Tokyo

Integrin αIIb, a well-known marker of megakaryocyte-platelet lineage, has recently been 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 αIIbβ3-dependent manner. In addition, the binding of mast cells to fibrinogen enhanced proliferation, cytokine production and migration and induced the uptake of soluble fibrinogen, implicating integrin αIIbβ3 in a variety of mast cell functions. Our goal is to delineate the biological significance of integrin αIIbβ3 on mast cells by in vivo allergy and inflammation models using integrin αIIb knockout mice.

5. Identification and characterization of a

new pair of immunoglobulin-like receptors, leukocyte mono-Ig-like receptors (LMIRs).

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

We originally identified and characterized two mouse cDNAs from a mouse bone marrow-derived mast cell cDNA library. They encoded type I transmembrane proteins including a single variable immunoglobulin (Ig) motif in the extracellular domain with about 90% identity of amino acids. LMIR1 contains immunoreceptor tyrosine-based inhibition motif (ITIM) in the intracellular domain, while LMIR2 harbors a short cytoplasmic tail associating with immunoreceptor tyrosine-based activation motif (ITAM)-bearing molecules such as DAP12. In addition to LMIR1/2, related genes were identified by homology search in the close proximity on the same chromosome 11: LMIR3 is an inhibitory type receptor like LMIR1, and LMIR4, 5, 6 are activation type receptors like LMIR2 (LMIR4 is missing in humans). LMIRs are also called CLMs or MAIRs. Those receptors are mainly expressed in cells involved in innate immunity including mast cells, granulocytes, monocytes, dendritic cells. In addition, some of the receptors are also expressed in some cells in colon, trachea, and lung, indicating that these receptors play important roles in innate immunity. Investigation to elucidate the biological roles of LMIRs on immune cells is now underway; we attempt to identify the ligands and analyzing LMIR-deficient mice.

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

Naoko Watanabe, Yukiko Komeno, Naoko Kato, Toshihiko Oki, Koichiro Yuji, Yuka Harada[7], Hironori Harada[7], Toshiya Inaba[8], Hideaki Nakajima[3], Tetsuya Nosaka, Jiro Kitaura, and Toshio Kitamura: [7]Department of Hematology/Oncology and [8]Department of Molecular Oncology, Research Institute for Radiation Biology and Medicine, Hiroshima University

To elucidate the molecular mechanisms of leukemia, MDS, and MPD, we established mouse model using bone marrow transplant (BMT); we transduced mouse bone marrow cells with genes of leukemogenic mutations such as MLL-fusions or AML-1 using retroviruses. In the result, we are now able to reproduce acute leukemia, MPD and MDS-like symptoms in mice, and are now in the process of characterizing these mouse models. We also establish bone marrow-derived immature cell lines transduced with MLL fusions and AML-1 with mutations. While the differentiation of these cell lines are blocked probably through the dominant negative effects of MLL-fusions and the mutated AML-1, they still remain dependent on cytokines including SCF, IL-3, and Flt-3 ligand. In the mouse BMT model, we are beginning to understand that leukemogenesis (acute leukemia as well as MDS overt leukemia) require multiple mutations; mutations that block differentiation, and mutations that block apoptosis or induce factor-independent proliferation. Based on the mouse BMT model, we assume that there are the second hit mutations in addition to mutations such as MLL-fusions and AML-1 in patients' leukemic cells. To identify such mutations, we use retrovirus-mediated expression cloning method. To this end, we make cDNA libraries of patients' leukemic cells, and will isolate cDNAs that give rise to the autonomous growth of the cytokine-dependent cell lines established as stated above. In this way, we isolated ITD-Flt3 (constitutively activated Flt3 mutant found in 30% of patients with acute myeloid leukemia) and RasGRP4 that activate Ras pathways. We are now testing the effects of RasGRP4 in mouse BMT models.

Publications

Kawashima, T., Bao, Y.C., Minoshima, Y., Nomura, Y., Hatori, T., Hori, T., Fukagawa, T., Takahashi, N., Nosaka, T., Inoue, M., Sato, T., Kukimoto-Niino, M., Shirouzu, M., Yokoyama, S., and Kitamura, T. A Rac GTPase activating protein MgcRacGAP is an NLS-containing nuclear chaperone in the activation of STAT transcription factors. Mol Cell Biol., 29: 1796-1813, 2009.

Islam, S.M., Shinmyo, Y., Okafuji, T., Su, Y., Naser, I.B., Ahmed, G., Zhang, S., Chen, S., Ohta, K., Kiyonari, H., Abe, T., Tanaka, S., Nishinakamura, R., Terashima, T., Kitamura, T., and Tanaka, H. Draxin, a novel repulsive guidance protein for spinal cord and forebrain commissures. Science 323: 388-393, 2009.

Sugano, Y., Takeuchi, M., Hirata, A., Atsushita, H.M., Kitamura, T., Tanaka, M., and Miya-

jima, A. Junctional adhesion molecule-A, JAM-A, is a novel cell-surface marker for long-term repopulating hematopoietic stem cells. Blood 111: 1167-1172, 2008.

Watanabe-Okochi, N, Kitaura, J., Ono, R., Harada, H., Harada, Y., Nakajima, H., Nosaka, T., Inaba, T., and Kitamura, T. AML1 mutations induced MDS and MDS/AML in a mouse BMT model. Blood 111: 4297-4308, 2008.

Yamanishi, Y., Kitaura, J., Izawa, K., Matsuoka, T., Oki, T., Lu, Y., Shibata, F., Yamazaki, S., Kumagai. H., Nakajima, H,, Maeda-Yamamoto, M., Tybulewicz, V.L.J., Takai, T., and Kitamura, T. Analysis of mouse LMIR5/CLM7 as an activating receptor: differential regulation of LMIR5/CLM7 between mouse and human. Blood 111: 688-698, 2008.

Kawashima, T., and Kitamura, T. Rac and Nuclear Translocation of STAT Transcription Factors. Methods in Enzymology 431: 171-180, 2008.

Sekine, R., Kitamura, T., Tsuji, T. and Tojo, A. Efficient retroviral transduction of human B-lymphoid and myeloid progenitors: mark inhibition of their growth by the Pax5 transgene. Int. J. Hemal. 87: 351-362, 2008.

Komori, T., Gyobu, H., Ueno, H., Kitamura, T., Senba, E. and Morikawa Y. Expression of kin of irregular chiasm-like 3/mKirre in proprioceptive neurons of the dorsal root ganglia and its interaction with nephrin in muscle spindles. J. Comp. Neurol. 511: 92-108, 2008.

Ikeya, M., Nosaka, T., Fukushima K., Kawada, M., Furuta, Y., Kitamura, T., and Sasai, Y. *Twisted gastrulation* mutation suppresses skeletal defect phenotypes in Crossveinless 2 mutant mice. Mech. Dev. 125: 832-842, 2008.

Ma F, Ebihara Y, Umeda K, Sakai H, Hanada S, Zhang H, Zaike Y, Tsuchida E, Nakahata T, Nakauchi H, Tsuji K. Generation of functional erythrocytes from human embryonic stem cell-derived definitive hematopoiesis. Proc Natl Acad Sci USA 105: 13087-13092, 2008.

Ma F, Kambe N, Wang D, Shinoda G, Fujino H, Umeda K, Fujisawa A, Ma L, Suemori H, Nakatsuji N, Miyachi Y, Torii R, Tsuji K, Heike T, Nakahata T. Direct development of functionally mature tryptase/ chymase double positive tissue-type mast cells from primate ES cells. Stem Cells 26: 705-714, 2008.

Mizutani T, Tsuji K, Ebihara Y, Ohba Y, Taniguchi T, and Kenya Honda K. Homeostatic erythropoiesis by the transcription factor IRF2 through attenuation of type I interferon signaling. Exp Hematol 36: 255-264, 2008.

Konuma T, Ooi J, Takahashi S, Tomonari A, Tsukada N, Kobayashi T, Sato A, Kato S, Kasahara S, Ebihara Y, Nagamura-Inoue T, Tsuji K, Tojo A, Asano S: Cardiovascular toxicity of cryopreserved cord blood cell infusion. Bone Marrrow Transplant 41: 861-865, 2008.

Fukuchi, Y., Ito, M., Shibata, F., Kitamura, T., and Nakajima, H. Activation of C/EBPa or PU.1 in hematopoietic stem cells leads to their reduced self-renewal and proliferation. Stem cells, 26: 3172-3188, 2008.

Hiwatari, M., Ono, R., Taki, T., Hishiya, A., Ishii, E., Kitamura, T., Hayashi, Y., and Nosaka, T. Novel gain-of-function mutation in the extracellular domain of the PDGFRA gene in infant acute lymphoblastic leukemia with t (4;11) (q21;q23). Leukemia 22, 2279-2280, 2008.

Shibata, F., Goto-Koshino, Y., Morikawa, Y., Komori, T., Ito, M., Fukuchi, Y., Hauchins, J.P., Tsang, M., Kitamura, T., and Nakajima, H. Robo4 is expressed on hematopoietic stem cells and potentially involved in the niche-mediated regulation of the side population phenotype. Stem cells, 27: 183-190, 2009.

Nakajima, H., Tamura, T., Ito, M., Shibata, F., Kuroda, K., Fukuchi, Y., Watanabe, N., Kitamura, T., Ikeda, Y., and Handa M. SHD1 is a novel cytokine-inducible, negative feedback regulator of STAT5-dependent transcription. Blood, 113: 1027-1036, 2009.

Watanabe-Okochi, N., Oki, T., Komeno, Y., Kato, N., Yuji, K., Ono, R., Harada, Y., Harada, H., Hayashi, Y., Nakajima, H., Nosaka, T., Kitaura, J., and Kitamura, T. Possible involvement of RaoGRP4 in leukemogenesis. Int. J. Hematol. in press.

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Division of Infectious Diseases
感染症分野

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The long-term goal of our division is medical sciences on infectious diseases in two directions, from clinic to bench and from bench to clinic. Our current main subject is immune-pathogenesis of HIV-1 infection. We wish to clarify how cellular immune responses can control HIV-1 infection in some patients but not in others. We work together with the staffs in the Department of Infectious Diseases and Applied Immunology in the IMSUT hospital and apply the research results to the people living with HIV-1/AIDS.

1. Highly restricted TCR repertoire in the CD 8-positive T cell response against an HIV-1 epitope with a stereotypic amino acid substitution

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HIV-specific CD8+ T cells play an important role in controlling HIV-1 infection. However, despite the presence of HIV-specific CTLs, the majority of HIV-1-infected patients eventually progress to AIDS, probably because HIV-1 escapes from the CTLs by mutating and changing the amino acid sequences in the epitopes for CTL recognition. Investigating the mechanisms of CTL failure to control the virus is critical to understanding the pathogenesis of HIV-1 infection. In this study we wanted to elucidate the difference of T cell receptor (TCR) usage between the CD8+ T cells recognizing the wild type virus and those recognizing a common escape mutant.

Nef138-10 (RYLPTFGWCF) was known as an immunodominant CTL epitope restricted by HLA-A*2402, the most common HLA class I allele among the Japanese. Our previous study revealed that 80% of HIV-1 recovered from A24+ Japanese patients had a stereotypic amino acid substitution in this epitope, i.e., phenylalanine (F) for tyrosine (Y) at the 2nd position (designated as Nef138-10(2F)). We made two tetramers labeled with different fluorochrome; one presented the wild type Nef138-10, while the other presented Nef138-10(2F) in the context of HLA-A24. PBMCs from HIV-1-infected patients were stimulated with Nef138-10 peptide and stained by these tetramers. The CD8+ population was sorted into different fractions: positive only to the wild-type tetramer (wt-positive), positive only to the mutant tetramer (2F-positive), and positive to both wt- and mutant-tetramers (dual-positive). TCR repertoires of sorted epitope-specific CD8+ populations were determined by sequencing.

A 2F-positive population was rarely observed under our culture and staining conditions. We analyzed TCR repertoire of the wt-positive and dual-positive cells. The wt-positive CD8+ populations had a diverse TCR repertoire, but the

TCR repertoires in dual-positive CD8+ populations were highly restricted. In the dual-positive CD8+ populations most clonotypes used the TRBV4-1 and TRBJ2-7 gene segments for the TCR β-chain and the TRAV8-3 and TRAJ40-1 for the TCR α-chain. The CDR3 region of the TCR β-chain showed little variation. These results provide an example of restricted TCR repertoire in a specific CTL response against the escaping epitope. We speculate that impairment of antigen presentation in the escaping virus may underlie the restricted repertoire.

2. Contribution of Gag-specific CD8+ T cells to HIV control in a population lacking HLA-B57/27 that are protective against HIV-1 infection

Ai Kawana-Tachikawa, Kaori Nakayama, Michiko Koga, Takashi Odawara, Takeshi Fujii, Aikichi Iwamoto.

HIV-specific CD8+ T cells play an important role in controlling HIV infection. Especially, Gag-specific CD8+ T cells are considered to be crucial for HIV control. Some HLA-alleles, such as HLA-B27 and B57 that are known to be protective alleles, confer CTL-epitopes on Gag, and Gag-specific CD8+ response appears to have impact on viral setpoint and disease progression. However, as HLA distribution in Japanese cohort is quite different from any reported cohorts, we don't know whether HIV-specific CD8+ T cells contribute to HIV control among Japanese patients. To determine the impact of HIV-specific CD8+ T cells on HIV control, the association between HIV-specific CD8+ T cell responses and viral loads was analyzed.

Freshly prepared PBMCs from 51 Japanese individuals in chronic HIV infections were used for Interferon gamma (IFN-γ) ELISPOT assay. No individual among them had HLA-B57 or 27. Overlapping peptides that covered whole Gag, Pol, Env, and Nef proteins (576 OLPs) were used as antigen. We examined the association between viral loads and the magnitude or proportion of IFN-γ producing cells against each protein.

Although there was no correlation between viral loads and magnitudes of total response (sum of Gag, Pol, Env, Nef responses), only Gag-specific responses had inverse correlation with viral loads ($p=0.024$). Furthermore, we found that there was strong inverse correlation between the proportion of Gag-specific response (% of magnitude of Gag-specific response to magnitude of total response) and viral load ($p<0.0001$). These data suggested that Gag-specific CD8+ T cell responses had impact on viral set point in the studied Japanese cohort, even though there was no HLA allele known to be protective.

3. The impacts of HLA class I alleles on HIV-1 plasma virus loads in a unique Asian population with a narrow spectrum of HLA, and the change at the population level over time

Michiko Koga, Ai Kawana-Tachikawa, Takashi Odawara, Toshiyuki Miura, Aikichi Iwamoto

The expression of HLA class I types are associated with the level of plasma virus loads (pVLs) and disease outcome of HIV-1 infection, probably being mediated by CTL responses. We previously reported that CTL escape mutations can accumulate in Japanese population (as described above), suggesting that some HLA alleles might be losing the protective effects as the epidemic continues. We speculated that the Japanese population is genetically less diverse and can provide us a unique opportunity to observe the loss of viremia control by some class I alleles at the population level over time. Thus, we determined HLA class I genotypes from the 141 untreated HIV-1-infected Japanese individuals diagnosed from 1999 to 2007, and analyzed the associations between the expression of HLA alleles and the pVLs during the asymptomatic phase of infection.

By investigating the published data, we confirmed that the general Japanese population has a much narrower spectrum of HLA class I alleles (total 28 alleles with >1% frequency) compared to those in Caucasians in the U.S. (total: 46 alleles with >1% frequency); and noticed that they are intriguingly lacking HLA-B27/B57 that are the most protective against HIV-1 infection in the Caucasians. However, several protective alleles in the Caucasians (A11, A26, B51 and Cw14) including the 3rd protective HLA-B51, were seen at high frequency in the Japanese population. Although our data set in the present study was smaller and limited to the HIV-1-infected individuals, it had a similar HLA distribution to the general population.

The cross-sectional analyses revealed no significant associations between any individual alleles and pVLs. Nevertheless, we observed a clear trend that the B51+Japanese diagnosed before 2001 had lower pVLs than those diagnosed after 2005 ($p=0.08$), whereas no differences were observed for the other allelels protective in the Caucasians between the periods. These results indicate that HLA alleles that are protective against HIV-1 infection in a given population, are not necessarily beneficial in the

distinct population; and of more importance, some alleles like HLA-B51, might be losing their protective effects at a population level as the epidemic continues.

4. DNA microarray analysis on the host genes differently expressed between the patients with high viral loads and low viral loads during chronic HIV-1 infection

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In the natural course of HIV-1 infection, a stable level of plasma viral load (pVL) called "set point" is attained after the initial steep viremia. The set point is different from patient to patient and most importantly correlates with the prognosis of HIV-1 infection; the lower the set point the better the natural course. It has been believed that cellular immune response plays a crucial role on the level of set point. However, it is not known what kind of host reactions exactly determines the viral set point.

In this study, we examined gene expression profile in PBMCs from chronically HIV-1-infected patients. The patients were divided into two groups, i.e, low pVL group (plasma viral load $<$5000copies/ml) and high pVL group (plasma viral load $>$30000copies/ml). Freshly prepared PBMCs were stimulated with Gag overlap peptides (OLPs) or mock-stimulated for 18 hours, and cellular RNAs were collected and reverse transcribed. Cy5- and Cy3-labeled antisense RNAs were produced from Gag-stimulated aliquots and mock controls, respectively. Competitive hybridization was performed on a 9000-gene-loaded microarray "human immunity chip" (Toray).

447 and 492 genes were up-regulated by Gag-OLP-stimulation in high pVL and low pVL groups, respectively, whereas 211 and 228 genes were down-regulated in respective groups. Only 77 genes (21%) were commonly down-regulated in both groups, while 346 genes (58%) were commonly up-regulated. There was no gene whose expression changed in the opposite direction between high and low pVL groups, but significant difference ($P<0.005$) of expression change between the two groups was noticed for 71 genes. Of these 71 genes, 44 genes (62%) were classified as "more up-regulated in low pVL". In contrast, the expression of only one gene was more down-regulated in low pVL group. The other 26 genes, of which 11 genes were up-regulated and 15 were down-regulated, showed more changes in high pVL group. Further analysis on these genes is in progress.

Publications

1. Miyazaki E, Kawana-Tachikawa A, Tomizawa M, Nunoya J, Odawara T, Fujii T, Shi Y, Gao FG, Iwamoto A. Highly restricted TCR repertoire in the CD8+ T-cell response against an HIV-1 epitope with a stereotypic amino acid substitution. AIDS. In press.
2. Hou W, Aoki C, Yu L, Wen X, Xue Y, Gao B, Liu W, Gao GF, Iwamoto A, Kitamura Y. A recombinant replication-competent hepatitis C virus expressing Azami-Green, a bright green-emitting fluorescent protein, suitable for visualization of infected cells. Biochem Biophys Res Commun. 377: 7-11, 2008.
3. Zong L, Chen Y, Peng H, Gao F, Iwamoto A, Gao GF. Rhesus macaque: A tight homodimeric CD8alphaalpha. Proteins. 75: 241-244, 2008.
4. Nagai H, Iwasaki N, Odawara T, Okada S. Actual status of AIDS-related lymphoma management in Japan. Int J Hematol. 87: 442-443, 2008.
5. Maeda T, Yamada H, Akao N, Iga M, Endo T, Koibuchi T, Nakamura T, Odawara T, Iwamoto A, Fujii T. Unusual radiological findings of Fasciola hepatica infection with huge cystic and multilocular lesions. Intern Med. 47: 449-52, 2008.
6. Hosoya N, Miura T, Kawana-Tachikawa A, Koibuchi T, Shioda T, Odawara T, Nakamura T, Kitamura Y, Kano M, Kato A, Hasegawa M, Nagai Y, Iwamoto A. Comparison between Sendai virus and adenovirus vectors to transduce HIV-1 genes into human dendritic cells. J Med Virol. 80: 373-82, 2008.

Advanced Clinical Research Center

Division of Bioengineering
臓器細胞工学分野

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

I. The role of molecules involved in apoptotic cell phagocytosis in compromising antitumor immunity and promoting tumorigenesis

a. The role of milk-fat globule EGF8 (MFG-E8) in melanoma progression

Masahisa Jinushi, Hideaki Tahara

Milk-fat globule-EGF8 (MFG-E8) critically contributes to form immunosuppressive environments through the expansion of $Foxp3^+$ regulatory T cells and inhibition of antigen-specific CTL activities (Jinushi M, et al. J. Clin. Invest., 2007). We identified milk-fat globule-EGF8 (MFG-E8) as a major regulator of tumor progression in murine and human melanomas. Immunohistochemical examination of human melanomas showed that MFG-E8 is highly expressed in tumor infiltrating macrophages and/or tumor cells in aggressive melanomas with vertical growth phase, which may be associated with disease progression. The detailed evaluation using animal models reconstituted with bone marrow expressing MFG-E8 as well as the melanoma cells engineered to secrete MFG-E8 unveiled the critical role of this factor in tumor cell growth, invasion, and metastasis. MFG-E8 promoted resistance to apoptosis, an epithelial-mesenchymal transition, and angiogenesis through the activation of the PI-3 kinase/Akt and twist-1 pathways. Furthermore, MFG-E8 compromised tumor immunity through the expansion of $FoxP3^+$ Tregs in the tumor microenvironment. The inhibition of MFG-E8 in human melanoma cells through siRNA mediated gene silencing enhanced tumor cell apoptosis, particularly in combination with various kinase inhibitors. Collectively, these studies demonstrate that MFG-E8 produced in the tumor microenvironment promotes tumor progression through coordinated effects on tumor cells, vasculature, and immune elements and thereby identify MFG-E8 as an attractive target for cancer therapy.

b. The identification of novel immunological targets from patients with advanced cancers who received experimental immunotherapies.

Masahisa Jinushi, Hideaki Tahara

The identification of novel the identification of cancer antigens that contribute to transformation and are linked with immune-mediated tumor destruction is an important goal for immunotherapy. Towards this end, we screened a murine renal cell carcinoma cDNA expression library with sera from mice vaccinated with irradiated tumor cells engineered to secrete GM-CSF. Multiple non-mutated, over-expressed proteins that function in tumor cell migration, protein/nucleic acid homeostasis, metabolism, and stress responses were detected. Among these, the most frequently recognized clone was NKG2 D ligand MICA, protein disulfide isomerase (PDI) and multiple angiogenic factors (VEGF, angiopoietin, and MIF). High titer antibodies to these targets were similarly induced in patients with melanoma, ovarian carcinoma, non-small cell lung carcinoma and acute myelogenous leukemia, who achieved durable clinical response following vaccination with GVAX and /or anti-CTLA 4 antibodies (Ipilumumab). Moreover, these targets also evoked potent humoral and antigen-specific cellular reactions in diverse malignancy patients. Together, these findings reveal the potent immunogenic target triggered by GVAX and ipilumumab combination treatments, and raise the possibility that these gene products might serve as targets for further broadening therapeutic options.

II. Development of innovate cancer therapy using immunologic approaches

a. Th1 and Th17 in IL-23 induced anti-tumor immune responses

Marimo Sato, Hideaki Tahara

Interleukin-23 (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 preferentially act on Th1 effector/memory CD4+ T-cells and to induce their proliferation and IFN-γ production. The IL-23 is also reported to act on Th17-CD4+ T-cells which are involved in inducing tissue injury. In this study, we examined the anti-tumor effects associated with systemic administration of IL-23 and their mechanisms using mouse tumor system. Methods: Systemic administration of IL-23 was achieved using in vivo electroporation of IL-23 plasmid DNA of C57BL/6 mice. Results: The IL-23 treatment was associated with significant suppression of the growth of pre-existing MCA205 fibrosarcoma and prolongation of the survival of treated mice without significant toxicity. Although the therapeutic outcomes were similar to those with the IL-12 treatment, the IL-23 treatment induced characteristic immune responses distinctive to those of IL-12 treatment. The IL-23 administration even at the therapeutic levels did not induce detectable IFN-γ concentration in the serum. In vivo depletion of CD4+ T-cells, CD8+ T cells, or NK cells significantly inhibited the anti-tumor effects of IL-23. Furthermore, the CD4+ T-cells in the lymph nodes in the IL-23-treated mice showed significant IFN-γ and IL-17 response upon anti-CD3 Ab stimulation in vitro. Conclusions: These results and the ones in the IFN-γ or IL-12 gene knockout mice suggest that potent anti-tumor effects of IL-23 treatment could be achieved when the Th1-type response is fully promoted in the presence of endogenously expressed IL-12.

b. Cancer Immunotherapy using dendritic cells

Marimo Sato, 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 and the genetic modification of DCs to have better function in situ. In order to obtain DCs suitable for the vaccination with class I-restricted epitope peptides derived from tumor-related antigens, we have been using monocyte-derived DCs stimulated with OK-432 and prostaglandin E2 (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 capacity to migrate. Based on these preclinical results, we initiated phase I clinical protocol to treat stage IV melanoma patients 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 administered DCs was confirmed with 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 in all patients. These results warrant further development of our vaccination strategy using OK-P-DCs.

c. Combined mobilization and stimulation of tumor-infiltrating dendritic cells and natural killer cells with Flt3 ligand and IL-18 in vivo induces systemic antitumor immunity.

Tetsuya Saito, Marimo Sato, Hideaki Tahara

It was hypothesized that if dendritic cells (DC) could be efficiently manipulated in vivo, this might enable functional maturation and retention of their potent functions and might represent a more promising approach in DC immunotherapy. The present study focused on the modulation of DC in tumor microenvironment using Fms-like thyrosine kinase 3 ligand (Flt3L) combined with interferon-gamma-inducing factor (IL-18). Tumor-inoculated mice were treated with in vivo electroporation (IVE) of expression plasmids carrying complementary DNA of Flt3 L. As a combination therapy, mice in the other group were treated with intra-tumoral injection of adenoviral vector carrying IL-18 gene (Ad.IL-18). Significant antitumor effect was observed in mice treated with Ad.IL-18 alone when compared with that of control ($P < 0.01$). Complete eradication was observed more frequently (100 %versus 33%: $P < 0.05$) in the mice treated with Flt3L and Ad.IL-18 when compared with the mice treated with Ad.IL-18 alone. In uninjected distant tumor, significant antitumor responses were observed only in the mice treated with combination therapy. Lymphoid cells in lymph nodes of mice treated with combination therapy showed significant cytolytic activity against inoculated tumor cells and YAC-1 cells when compared with the lymphoid cells in other groups. In the tumor microenvironment, combination therapy resulted in the recruitment of mobilized DC into the tumor bed, although Flt3L-IVE alone had an effect in the peri-tumoral area. Tumor-infiltrating DC in mice treated with combination therapy showed higher CD86 expression and more potent allogeneic T-cell stimulatory capacity. These results may suggest that local expression of IL-18 combined with in vivo DC mobilization with Flt3L is clinically applicable as a new strategy of DC immunotherapy.

III. Immunological monitoring for Phase I Clinical trial of cancer vaccination using HLA-Class I restricted epitope-peptides derived from endothelial growth factor Receptor 2.

Marimo Sato, Hideaki Tahara

We performed Phase I clinical study using class I-restricted epitope peptides derived from tumor-related antigens including Vascular Endothelial Growth Factor Receptor 2 (VEGFR2). Peptide vaccination was administered to the patients with HLA-A*0201 and A*2402 as the suspension with incomplete fluid adjuvant (IFA). In this study, we have evaluated peptide-specific immunological responses in the enrolled patients using the methods established for the analysis of PBMCs. Patients, such as esophageal cancer, colon cancer, gastric cancer, gastrointestinal stromal cancer (GIST), pancreatic cancer and breast cancer, were enrolled to a phase I studies, and received intra-dermal administration of the epitope peptides for four times with one week interval. During the vaccination, bloods (PBMCs) collections were performed regulatory. Immunological responses using PBMCs were examined after the short time culture with peptides and low dose recombinant human IL-2 (20 U/ml). These samples were analyzed for the frequency of IFN-γ-producing cells using ELISPOT assay and the frequency of peptide specific CD 8^+ T cells using MHC-Dextramer assay. All the patients enrolled have well tolerated the treatment with no serious adverse events related to the treatment. These results suggest that the peptides used can be safety administrated to the cancer patients. Significant immune responses were detected as early as 2 weeks after the 1^{st} injection in some patients. These results warrant further development of our peptide vaccination protocol and immunological assays for detecting specific immune responses in the patients. This type of assay would become even more useful, when the analysis would be required on the patients treated with multiple peptides.

IV. Development of innovative cancer therapy using gene therapy strategies. Development of targeted HSV vector: A single-chain antibody against the EGF receptor is superior to EGF as a targeting ligand.

Kenji Nakano and Hideaki Tahara

We are pursuing on the development for immuno-therapy by bioengineering using vaccines, viral vectors and immune cells. Upon applying the viral biomaterials for clinical use, tropism-based targeting is one of important issues to resolve. We have previously shown that HSV can use the EGF receptor (EGFR) for entry into cells when provided with an adapter protein comprising the nectin-1 V-domain fused to a single chain antibody (scFv) against EGFR [Mol Ther 2005; 11: 617-26]. The current study examines if and how replacement of the scFv portion of the adapter with the natural ligand for the receptor, EGF, affects the mechanism and efficiency of adapter-mediated HSV entry. Immunocytochemistry, flow cytometry, and transmission electronmicroscopy (EM) were used to compare the binding activities of the scFv and EGF adapters to EGFR and HSV glycoprotein D

(gD) and to examine the early stages of virus entry. EGFR internalization induced by adapter-coated virus was evaluated by receptor protection from protease degradation. Adapter-mediated HSV entry was determined in EGFR-overexpressing cells by measurement of reporter gene expression. Adapter-mediated entry pathways were examined by entry inhibition assays using various endosome-modulating agents and EM. While the binding affinity for EGFR was similar between the two adapters, the affinity for gD expressed on CHO cells was higher for the scFv adapter than for the EGF adapter. EM revealed tighter cell attachment of viral particles incubated with the scFv adapter than with the EGF adapter. The efficiency of scFv adapter-mediated HSV infection was significantly higher than that mediated by the EGF adapter. Infection mediated by the scFv adapter was partly inhibited by ammonium chloride, bafilomycin A 1, sucrose and amiloride, suggesting clathrin-dependent and -independent endocytic virus uptake. Differences were observed between the two adapters in EGFR internalization and intracellular adapter persistence. In conclusion, EGF is a less efficient targeting ligand for adapter-mediated HSV infection through the EGFR than the scFv used in this study.

Publications

Schoenfeld J, Jinushi M, Wiener D, Nakazaki Y, Park J, Soiffer R, Neuberg, D, Mihm M, Hodi, FS & Dranoff G. Immunotherapy Induced Antibodies Target Tumor Angiogenesis. *Proc. Natl. Acad. Sci. U.S.A*. (in press).

Fonseca C, Soiffer R, Ho V, Vanneman M, Jinushi M, Ritz J, Neuberg D, Stone R, Deangelo D & Dranoff G. Protein disulfide isomerases are antibody targets during immune-mediated tumor destruction. *Blood*, (in press).

Jinushi M, Nakazaki Y, Carrasco DR, Draganov D, Souders N, Johnson M, Mihm MC, Dranoff G. Milk fat globule EGF-8 promotes melanoma progression through coordinated Akt and twist signaling in the tumor microenvironment. *Cancer Res*. 68, 8889-8898, 2008.

Jinushi M, Hodi FS, & Dranoff G. Improving antitumor efficacy of GM-CSF secreting tumor cell vaccines. *Immunol Rev*., 222, 287-298, 2008.

Jinushi M, Vanneman M, Munshi NC, Tai Y-T, Prabhala RH, Ritz J, Neuberg D, Anderson KC, Carrasco DR & Dranoff G. MHC class I chain-related protein A antibodies and shedding are involved in theprogression of multiple myeloma. *Proc. Natl. Acad. Sci. U.S.A*., 105, 1285-1290, 2008.

Saito T, Takayama T, Osaki T, Nagai S, Suzuki T, Sato M, Kuwano H, Tahara H. Combined mobilization and stimulation of tumor-infiltrating dendritic cells and natural killer cells with Flt3 ligand and IL-18 in vivo induces systemic antitumor immunity. Cancer Sci. 99: 2028-36, 2008

Takahashi H, Ishizaki H, Tahara H, Tamaki Y, Yanagi Y. Suppression of choroidal neovascularization by vaccination with epitope peptide derived from human VEGF receptor 2 in an animal model. Invest Ophthalmol Vis Sci. 49: 2143-7, 2008

Suzuki T, Ogawa S, Tanabe K, Tahara H, Abe R, Kishimoto H. Induction of antitumor immune response by homeostatic proliferation and CD 28 signaling. J Immunol. 180: 4596-605, 2008

Advanced Clinical Research Center

Division of Clinical Immunology
免疫病態分野

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

I. β1 integrins and Cas-L/NEDD9

Satoshi Iwata, Yutaka Hashizume, Koji Yo, Shunsuke Kondo, Sayaka Nomura, Tomonori Katayose, Satoshi Murakami, Akiko Souta-Kuribara, Osamu Hosono, Hiroshi Kawasaki, Hirotoshi Tanaka, and Chikao Morimoto.

β1 integrins play crucial roles in a variety of cell processes such as adhesion, migration, proliferation, and differentiation of lymphocytes. Previously we showed that co-immobilized anti-β1 integrin mAbs or its ligand with a submitogenic dose of anti-CD3 mAb induced a marked increase of IL-2 secretion and proliferative response of T cells, indicating that β1 integrins are costimulatory molecules of T cells. Pp105 was first described in our laboratory as a protein predominantly tyrosine phosphorylated by the ligation of β1 integrins in H9 T cells. By cDNA cloning, we demonstrated that pp105 was a homologue of p130Cas (Crk-associated substrate)/BCAR1 (Breast Cancer Antiestrogen Resistance 1), and designated as Cas-L (Cas lymphocyte type). It has been shown that Cas-L, HEF1 (human enhancer of filamentation), and Nedd9 (neural precursor cell expressed, developmentally down-regulated 9) are identical gene products. We found that transfection of Cas-L cDNA into Jurkat T cells restored β1 integrin-mediated costimulation and cell migration, indicating that Cas-L plays a key role in the β1 integrin-mediated T cell functions.

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

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

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

b. SHP-2 regulates beta-1 integrin signaling by dephosphorylation of Cas-L.

The Src homology 2 (SH2) domain-containing protein tyrosine phosphatase, SHP-2, plays an important role in cell migration, by interacting with various proteins. In this report, we identified Cas-L/Nedd9, a member of Cas family as a novel substrate for SHP-2, and found that SHP-2 negatively regulates migration of A549 lung adenocarcinoma cells induced by fibronectin. Cas-L is a docking protein with diverse functional properties, including regulation of cell division, proliferation, migration and invasion, and it is a key molecule in integrin-dependent signaling at focal adhesions. We showed that SHP-2 co-localizes with Cas-L at focal adhesions and that exogenous expression of SHP-2 abrogated cell migration mediated by Cas-L. SHP-2 dephosphorylates Cas-L in vitro, and associates with Cas-L to form an enzyme-substrate complex in a tyrosine phosphorylation-dependent manner. Finally, immunoprecipitation experiments with deletion mutants revealed that the substrate domain of Cas-L, and c-terminal SH2 and PTP domain of SHP-2 are necessary for the association. These results suggest that SHP-2 is a negative regulator of integrin-dependent cell migration mediated by Cas-L.

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

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

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

We evaluated the potential role of Cas-L/Nedd9 in the phosphorylation and downstream signaling of EGFR in NSCLC. Subsequently, we found that EGFR stimulation promote tyrosine phosphorylation of Cas-L in human non small cell lung cancer cell lines (PC-9 and A549), which was abrogated by inactivation of EGFR using Gefitinib. Introduction of siRNA for Cas-L reduced the migratory activity of PC-9 and A 549 cells. The extent of reduction was significantly higher in the case with siRNA for Cas-L compared to that of p130Cas. These results indicate that the crosstalk between EGF and integrin signaling pathways might be occurred at the level of Cas-L, and that Cas-L may be a therapeutic target for the treatment of malignancies

such as lung cancer.

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

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

CD26 is a 110-kDa cell surface glycoprotein that posseses dipeptidyl peptidase IV(DPPIV) (EC. 3.4.14.5) activity in its extracellular domain and a primary marker of activated T cells. In the resting state, CD26 is preferentially expressed on a subset of CD4 memory T cells where they account for the majority of IL-2 secretory capabilities and help for B cell Ig production and are the primary responders to recall antigen such as tetanus toxoid. CD26 is also capable of providing a potent costimulatory or "second" signal which can augment other activation pathways leading to proliferation, cytokine production and effector functions. The mechanism of costimulation remains unclear since the cytoplasmic domain consists of only 6 amino acid and lacks a phosphorylation site, leading to the conclusion that CD26 interacts with other cell surface molecules. We have already shown that CD26 may interact with CD45RO which modulates TcR/CD3 activity through its intracellular tyrosine phosphatase domain. Recently, we have detected another CD26 binding protein, the mannose-6-phosphate/insulin-like growth factor II receptor (M6P/IGFIIR) as being critical for this interaction for CD26 mediated T cell costimulation in addition to adenosine deaminase (ADA). More recently, we have shown that CD26 localizes into lipid rafts, and targeting of CD26 to rafts is necessary for signaling events through CD26. Importantly, aggregation of CD 26 by anti-CD26 mAb crosslinking also causes coaggregation of CD45 into rafts. In addition, we have demonstrated that recombinant soluble CD26 (sCD26) has an enhancing effect on T cell proliferation in the presence of the recall antigen, tetanus toxoid. This enhancement resulted in an increase in the surface expression of the costimulatory molecule CD86 on monocytes following sCD26 binding to Caveolin-1 expressed on monocytes.

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 as well as recombinant soluble CD26 for treatment of malignant tumors, immune-mediated disorders and immune deficiency diseases. Hopefully we will perform phase I/II clinical trial utilizing humanized CD26 antibody for the treatment of the above diseases, such as Malignant mesothelioma and other CD26 positive malignant Tumors soon.

a. 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 alloSCT (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/LtSz-scid or NOD/Cg-Prkdcscidil2 rgtm1Sug/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 CD 3+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.

b. Molecular analysis and therapeutic targeting of CD26-depedent cancer cell growth

It has been reported previously that CD26 is upregulated during enterocytic differentiation of the colon cancer cell lines Caco-2 and HT-29. We also have found that the colon cancer cell lines HCT-116 and HCT-15 exhibit a confluence -dependent increase in CD26 expression. Moreover, we have shown that c-Myc protein expression decreases in a confluence-dependent manner in both cell lines. Our studies involving ectopic c-Myc and c-Myc siRNA expression suggested a role for c-Myc as a repressor of CD26 expression. In addition, our experiments involving Cdx2 siRNA treatment suggested that Cdx2 significantly contributes to the confluence-dependent increase in CD26 expression. Importantly, we have found that cell culture conditions involving serum-depleted culture media, but not hypoxic or acidic culture conditions, significantly contribute to the increase in CD26 levels, hence suggesting a putative role for growth factors in the regulation of CD26 protein expression. Under serum-depleted culture conditions, treatment of HCT-116 cells with the anti-CD26 mAb 1F7 resulted in elevated protein levels of p 27kip1 as compared with those seen following treatment with the isotype-matched control mAb. These findings may result in the expansion of the use of CD26-targetd therapy in the clinical setting for selected cancers, by regulating the expression of CD26 itself.

c. Molecular mechanisms of CD26-mediated cancer cell invasion

CD26 is a multifunctional membrane-bound glycoprotein that regulates tumor growth partly by modulating the activity of biopeptides and binding extracellular matrix constituents. SDF-1 α/CXCL12 and its receptor CXCR4 play a critical role in tumorigenesis besides their role in mediating migration and activation of leukocytes during immune and inflammatory responses. To assess CD26 involvement in cell invasion, we performed in vitro invasion assays with human T cell lines expressing different levels of CD26. These included the parental CD26-positive T- lymphoblast cell line HSB-2 and clones infected with a retrovirus expressing siRNA vectors that either targeted CD26 or encoded a mis-sense siRNA, and the parental CD 26-negative T-leukemia cell line Jurkat and clones expressing CD26. The role of SDF-1α in cell invasion was evaluated, as well as the phosphorylation status of key signaling proteins in cell invasion. Our results showed that CD26 enhances SDF-1α-mediated invasion of T-cell lines. This process is regulated by the PI-3K and MEK 1 pathways, as indicated by the involvement of the key signaling molecules Akt and p44/42 MAP kinase. Activation of these kinases leads, in turn, to induction and secretion of MMP-9. In addition, our data suggest that CD26-associated enhancement of SDF-1α-induced invasion may be mediated by CD45.

III. Therapeutically targetting transcription factors

Hirotoshi Tanaka, Noritada Yoshikawa, Noriaki Shimizu, Chikao Morimoto.

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

a. Glucocorticoid receptor (GR) project

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

(i) Redox Regulation of the GR

Redox regulation is currently considered as a

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

(ii) Development of Dissociating Ligand for the GR

The GR function could be differencially regulated by ligands. We have recently shown that not only synthetic glucocorticoids but also certain bile acids could differentially modulate GR function. Moreover, the effects of those compounds are indicated to be 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 MR have differential role in homeostatic regulation in non-classical corticosteroid target tissues including the heart. Notably, collaboration with Professor Miyano's laboratory greatly contributed to development of this program.

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

Expression of HEXIM1 is induced by treatment of vascular smooth muscle cells with a differentiation inducer hexamethylane bisacetamide. It is shown that HEXIM1 binds 7SK snRNA and inhibits P-TEFb-mediated transcriptional elongation process. On the other hand, we have found that HEXIM1 directly associates with the GR in the absence of 7SK and represses GR-mediated transcription. We are currently working on regulation of HEXIM1 expression, physiological role of HEXIM1 in GR action. Indeed, HEXIM1 has differential roles in gene regulation in a context and gene specific fashion. We have recently characterized that HEXIM1 may play an important role in tissue-specific regulation of glucocorticoid-mediated gene expression. Physiological significance of HEXIM1 is being studied using newly generated transgenic mice.

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

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

IV. Cancer Stem Cells

Hiroto Yamazaki, Hiroko Nishida, Ghani Farhana Ishrat, and Chikao Morimoto

a. Identification of cancer stem cells in human B-ALL

Cancer stem cell (CSC) theory suggests that

only a small subpopulation of cells having stem cell-like potentials can initiate tumor development. While recent data on acute lymphoblastic leukemia (ALL) are conflicting, some studies have demonstrated the existence of such cells following CD34-targeted isolation of primary samples. Although CD34 is a useful marker for the isolation of CSCs in leukemias, the identification of other specific markers besides CD34 has been relatively unsuccessful. To identify new markers, we first performed extensive analysis of surface markers on several B-ALL cell lines. Our data demonstrated that every B-ALL cell line tested did not express CD34 but certain lines contained cell populations with marked heterogeneity in marker expression. Moreover, the CD9+ cell population possessed stem cell characteristics within the clone, as demonstrated by in vitro and transplantation experiments. More importantly, our examination of primary pre-B-ALL samples showed significant correlation between CD9 and CD34 expression, with the CD9+ cells having greater engraftment potential than the CD9- cells. Microarray analysis also revealed that expression of several Src family proteins was differentially regulated in the CD9+ cell population. These results hence suggest that CD9 is a useful positive-selection marker for the identification of CSCs in B-ALL.

b. Identification of cancer stem cells in human T-ALL

Although cancer stem cells (CSCs) have been recently identified in myeloid leukemia, published data on lymphoid malignancy have been sparse. T-acute lymphoblastic leukemia (T-ALL) is characterized by the abnormal proliferation of T-cell precursors and is generally aggressive. As CD34 is the only positive-selection marker for CSCs in T-ALL, we performed extensive analysis of CD markers in T-ALL cell lines. We found that some of the tested lines consisted of heterogeneous populations of cells with various levels of surface marker expression. In particular, a small subpopulation of CD90 (Thy-1) and CD 110 (c-Mpl) double-positive cells were shown to possess stem cell characters both in vitro and in transplantation experiments. As these markers are expressed on hematopoietic stem cells, stem-like cells were supposed to be enriched in CD 90+/CD110+ fraction. We also examined pediatric T-ALL patient samples and found that 3 of 8 cases contained abundant CD90+/CD110+ cells with significant correlation with CD34 expression, showing higher engraftment potential in immunodeficient mice. In addition, gene expression analysis revealed that the Src family proteins Lyn, Blk, and Fgr were down-regulated in CD90+/CD110+ cells. These results suggest that CD90 and CD110 are useful positive markers for the isolation of CSCs in some cases of T-ALL.

IV. Immunomodulatory effects of RXM and its analogs

Noriko Otsuki, Emi kumagai, Chikao Morimoto

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

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

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

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

Publications

Nishida H, Yamada T, Iwata S, Dang NH, Inukai T, Sugita K, Ikeda Y, Morimoto C. CD9 correlates with cancer stem cell potentials in human B-acute lymphoblasticleukemia cells. Biochem. Biophys. Res. Commun. In press (2009).

Iwata S, Morimoto C. BCAR1 Targeted Proteins Database 1. In press (2009).

Harima A, Nakaseko C, Yokota A, Kitagawa M, Morimoto C, Harigaya K, Saito Y. Fibronectin promotes cell proliferation of human pre-B cell line via its interactions with VLA-4 and VLA-5. Hematology. 2008; 13: 236-43.

Iwata S, Nori M, Hashizume Y, You K, Morimoto C. Effect of H-1-receptor antagonists on proliferative response, cytokine production, and cellular migration of human T cells and macrophages. Clinical and Experimental Allergy Reviews 2008; 8: 21-29.

Ohnuma K, Hosono O, Kawasaki H, Yoshikawa N, Katayose T, Oyaizu N, Tanaka H, Morimoto C. An adult case of Henoch-Schönlein purpura complicating common peroneal nerve mononeuropathy. Mod Rheumatol. 2009; 19: 73-9.

Fujimaki W, Takahashi N, Ohnuma K, Nagatsu M, Kurosawa H, Yoshida S, Dang NH, Uchiyama T, and Morimoto C. Comparative study of regulatory T cell function of human CD25 +CD4+ T cells from thymocytes, cord blood, and adult peripheral blood. Clin Dev Immunol. Clin Dev Immunol. 2008; 2008: 305859.

Ohnuma K, Dang NH, Morimoto C. Revisiting an old acquaintance: CD26 and its molecular mechanisms in T cell function. Trends Immunol. 2008; 29: 295-301.

Yamochi T, Ohnuma K, Hosno O, Tanaka H, Kanai Y, Morimoto C. SSA/Ro52 autoantigen interacts with Dcp2 to enhance its decapping activity. Biochem Biophys Res Commun. 2008; 370: 195-199.

Yamashita T, Ohneda O, Nagano M, Iemitsu M, Makino Y, Tanaka H, Miyauchi T, Goto K, Ohneda K, Fujii-Kuriyama Y, Poellinger L, Yamamoto M. Abnormal heart development and lung remodeling in mice lacking the hypoxia-inducible factor-related basic helix-loop-helix PAS protein NEPAS. Mol Cell Biol. 2008; 28: 1285-1297.

Kagawa T, Watanabe N, Mochizuki K, Numari A, Ikeno Y, Itoh J, Tanaka H, Arias IM, Mine T. Phenotypic differences in PFIC2 and BRIC2 correlate with protein stability of mutant Bsep and impaired taurocholate secretion in MDCK II cells. Am J Physiol Gastrointest Liver Physiol. 2008; 294: G58-67

Yoshikawa N, Shimizu N, Sano M, Ohnuma K, Iwata S, Hosono O, Fukuda K, Morimoto C, Tanaka H. Role of the hinge region of glucocorticoid receptor for HEXIM1-mediated transcriptional repression. Biochem Biophys Res Commun. 2008; 371: 44-49

Shimizu N, Yoshikawa N, Wada T, Handa H, Sano M, Fukuda K, Suematsu M, Sawai T, Morimoto C, Tanaka H. Tissue- and context-dependent modulation of hormonal sensitivity of glucocorticoid-responsive genes by hexamethylene bisacetamide-inducible protein 1. Mol Endocrinol. 2008; 22: 2609-2623.

Shibata S, Nagase M, Yoshida S, Kawarazaki W, Kurihara H, Tanaka H, Miyoshi J, Takai Y, Fujita T. Modification of mineralocorticoid receptor function by Rac1 GTPase: implication in proteinuric kidney disease. Nat Med. 2008; 14: 1370-1376.

Hozawa S, Nakamura T, Nakano M, Adachi M, Tanaka H, Takahashi Y, Tetsuya M, Miyata N, Soma H, Hibi T. Induction of matrix metalloproteinase-1 gene transcription by tumour necrosis factor alpha via the p50/p50 homodimer of nuclear factor-kappa B inactivated human hepatic stellate cells. Liver Int. 2008; 28: 1418-1425.

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We are now working on the following three projects, 1) The identification of novel diagnostic and therapeutic strategies of human cancer, 2) the application of genome-data forpersonalized medicine, and 3) genetic diagnosis and care of patients with hereditary cancer (HNPCC). 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

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

To identify novel target molecules for diagnosis and treatment of human cancers arising in colorectal cancers (CRCs), we have been studying their global gene expression profiles using genome-wide cDNA array. Among the genes overexpressed in the tumors, we selected MRG-binding protein (*MRGBP*) or *C20orf20*, a subunit of TRRAP/TIP60-containing histone acetyltransferase complex, as a candidate gene for clinical application. Quantitative PCR confirmed that expression of *MRGBP* was elevated in the great majority of the CRCs examined. Immunohistochemical staining with anti-MRGBP antibody further corroborated its accumulation in 20 of 27 CRC tissues (74%). Importantly, suppression of *MRGBP* by short interfering RNA (siRNA) resulted in growth suppression of CRCs. We also found that MRGBP associates with bromodomain containing 8 (BRD8) by yeast two-hybrid screening. This interaction changed subcellular localization of BRD8 from cytoplasm to nucleus, and led to its accumulation in the nucleus in a post-transcriptional manner. Since *BRD8* siRNA also suppressed proliferation of CRC, BRD8 may be an important down-stream target of MRGBP for the promoting effect of cell proliferation. 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, HCC, and breast cancer

Fabio Pittella Silva, Masaki Kunizaki, Tomoaki Fujii, Kiyoshi Yamaguchi, Yusuke Nakamura[1], Yoichi Furukawa: [1]Laboratory of Molecular Medicine, Human Genome Center, IMSUT

We earlier showed that SMYD3, a histone H3-Lysine 4-specific methyltransferase, is frequently up-regulated in human colorectal, liver, and breast cancer cells compared to their matched non-cancerous cells, and that its activity is associated with the growth of these tumors. To clarify the function of SMYD3, we are working on genes regulated by SMYD3. In the study, we found that human cancer cells express both the

full-length and a cleaved form of SMYD3 protein. Amino acid sequence analysis uncovered that the cleaved form lacks the 34 amino acids in the N-terminal region of the full-length protein. Interestingly the cleaved protein and mutant protein containing substitutions at glycines 15 and 17, two highly conserved amino acids in the N-terminal region, revealed a higher histone methyltransferase (HMTase) activity compared to the full-length protein. Furthermore, the N-terminal region is responsible for the association with heat shock protein 90alpha (HSP90α). These data indicate that the N-terminal region plays an important role for the regulation of its methyltransferase activity and suggest that a structural change of the protein through the cleavage of the region or interaction with HSP90 α may be involved in the modulation. These findings may help for a better understanding of the mechanisms that modulate the histone methyltransferase activity of SMYD3, and contribute to the development of novel anticancer drugs targeting SMYD3 methyltransferase activity.

Additionally our finding may facilitate the development of strategies that may inhibit the progression of cancer cells.

3. Prediction of sensitivity of Gefitinib to lung cancer, and that of Imatinib to CML.

Yoichi Furukawa, Yusuke Nakamura[1,2], Toyomasa Katagiri[1], Yataro Daigo[2], Naoyuki Takahashi[3], Tsuyoshi Fujii[4]: [1]Laboratory of Genome Technology, Human Genome Center, IMSUT, [2]Laboratory of Molecular Medicine, Human Genome Center, IMSUT, [3]Department of Applied Genomics, Research Hospital, IMSUT, [4]Department of Infectious Diseases and Applied Immunology, Research Hospital, IMSUT

In the second project, we are working on a prospective study of the prediction of sensitivity to gefitinib (Iressa) in patients with lung adenocarcinoma, in collaboration with Human Genome Center, and Department of Applied Genomics and Department of Infectious Diseases and Applied Immunology in Research Hospital, IMSUT. In our earlier study, we investigated expression profiles of lung adenocarcinomas that were treated with gefitinib, which identified 12 genes that can discriminate tumors with sensitivity to the drug from those without sensitivity. Two other groups reported that genetic alteration of epidermal growth fartor receptor (*EGFR)*, the target of gefitinib, was associated with the efficacy. Since these studies and ours analyzed a limited number of clinical samples, sensitivity and reliability of the two prediction methods remain unresolved. Therefore, we started a prospective study to analyze both expression profile and *EGFR* mutation of tumor tissues prior to treatment with gefitinib. An outpatient clinic for consultation of the applicants was opened in Research Hospital, IMSUT in September 2004, and Department of Respiratory Medicine, Kawasaki Medical University joined to this project in 2005.

4. Genetic diagnosis of HNPCC

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

Hereditary non-polyposis colorectal cancer (HNPCC) is an autosomal dominant hereditary disease accompanied by tumors arising mainly in the colon and other associated organs, such as stomach, renal pelvis, and endometrium. The frequency of HNPCC in Caucasian patients with colorectal cancer is estimated between two and five percent. However, the frequency in Japanese patients with colorectal cancer remains undetermined. Therefore, Japanese Study Group for Colorectal Cancer started a collaborative project of registration of Japanese HNPCC patients and genetic analysis of mutations in *MSH2*, *MLH1*, and *MSH6*, the responsible genes for HNPCC. All patients with colorectal cancer and those who were diagnosed as HNPCC by Amsterdam's II criteria in the collaborative hospitals have been registered, and the frequency of HNPCC in registered patients with colon cancer has been determined. Collaborating to this project, we have analyzed genetic alteration in a total of 131 patients using PCR-direct sequencing and Multiplex Ligation-dependent Probe Amplification. Among the 131 cases, 69 cases harbored pathogenic mutation in one of the three responsible genes. We have clarified that gastric cancer is frequently observed in HNPCC pedigrees, and that it should be considered as an HNPCC-related tumor in Japanese. In addition, we generated an algorithm to predict patients with a pathogenic mutation. These data will provide valuable information for the understanding of the frequency, penetrance and phenotypes of Japanese HNPCC. The results will be helpful for the identification and diagnosis of Japanese HNPCC patients.

Publications

1. Obama K, Satoh S, Hamamoto R, Sakai Y, Nakamura Y, Furukawa Y. Enhanced expression of RAD51AP1 is involved in the growth of intrahepatic choloangiocarcinoma cells. Clin. Cancer Res 14: 1333-1339, 2008.
2. Silva FP, Hamamoto R, Kunizaki M, Tsuge M, Nakamura Y, Furukawa Y. Enhanced methyltransferase activity of SMYD3 by the cleavage of its N-terminal region in human cancer cells. Oncogene 27, 2686-2692, 2008.
3. Kidokoro T, Tanikawa C, Furukawa Y, Katagiri T, Nakamura Y, Matsuda K. CDC20, a potential cancer therapeutic target, is negatively regulated by p53. Oncogene 27: 1562-1571, 2008.