Advanced Clinical Research Center

Division of Molecular Therapy
分子療法分野

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Our laboratory is primarily concerned with the development of novel therapeutic options against intractable hematological disorders including leukemia and lymphoma. In this year, our efforts based on molecular and cellular biology have produced the following achievements which are clinically oriented.

1. Hematopoietic Activity of Common Marmoset CD34 Cells Isolated by a Novel Monoclonal Antibody MA24

Izawa K, Tojo A, Asano S

We focused on a small New World monkey, the common marmoset (Callithrix jacchus) to establish a nonhuman primate model of the treatment of hematological disorders. In this study, we developed the first monoclonal antibodies (MAbs) against marmoset CD34 and tested the in vitro and in vivo hemopoietic activity of cell populations isolated using one of these MAbs. Marmoset cDNA encoding a human CD34 homologue was cloned from bone marrow (BM) -derived RNA using reverse transcription-polymerase chain reaction and rapid amplification of cDNA ends. The amino acid sequence of the marmoset CD34 had 81% homology with the human sequence. Five mouse MAbs were raised against marmoset CD34 transfectant. One representative MAb, MA24 (IgM), reacted with approximately 0.5-1% of BM mononuclear cells (MNCs), where the colony-forming unit-granulocyte/macrophage (CFU-GM) was enriched approximately 11 to 75 fold as compared with the whole BM MNCs. Multi-lineage differentiation of marmoset CD34+ cells in NOD/SCID mice was confirmed by flow cytometry 1 month after xenotransplantation. Conclusion. These results demonstrated that MA24 is useful for the analysis and enrichment of hematopoietic progenitor cells in the marmoset model for preclinical experiments.

2. Effective transduction and stable transgene expression in human blood cells by a third-generation lentiviral vector

Bai Y, Soda Y, Tojo A, Asano S

Difficulty in gene transduction of human blood cells, including hematopoietic stem cells, has hampered the development of gene therapy applications for hematological disorders, encouraging the development and use of new gene delivery systems. In this study, we used a third-

generation self-inactivating (SIN) lentiviral vector system based on human immunodeficiency virus type 1 (HIV-1) to improve transduction efficiency and prevent vector-related toxicity. The transduction efficiency of the HIV-1-based vector was compared directly with the Moloney murine leukemia virus (MLV) SIN vector in human leukemia cell lines. Initial transduction efficiencies were almost 100% for the HIV and less than 50% for the MLV vectors. Similar results were observed in 11 types of primary cells obtained from leukemia or myeloma patients. Transgene expression persisted for 8 weeks in cells transduced with the HIV vector, but declined with the MLV vector. In addition, resting peripheral blood lymphocytes and CD34(+) hematopoietic cells were transduced successfully with the HIV vector, but not with the MLV vector. Finally, we confirmed vector gene integration in almost all colony-forming cells transduced with the HIV vector, but not with the MLV vector. In conclusion, this lentiviral vector is an excellent gene transduction system for human blood cells because of its high gene transduction and host chromosome integration efficiency.

3. Lentiviral transduction of INK4A/ARF protein cooperates with molecular targeted agents to induce apoptosis of leukemia cells.

Bai Y, Soda Y, Tojo A, Asano S

Inactivation of the INK4A/ARF locus has been implicated in tumorigenesis, particularly in leukemogenesis. It has been found in 25-60% cases of acute leukemia and 20-50% cases of lymphoma. Although deletion of the INK4A/ARF locus is uncommon in AML, mRNA expression is likely to be disrupted, resulting from hypermethylation or mutation in the promoter region. There is some evidence that restoration of $p16^{INK4a}$ into p16-deficient leukemia cell lines suppresses their proliferation in suspension culture or their clonogenic growth in semisolid culture. However, previous studies were conducted by retrovirus or plasmid-mediated gene transfer under drug selection, suggesting low efficiency of transduction which appears disadvantageous to interpret the results of the growth inhibitory gene. Here we constructed a lentiviral vector expressing either $p16^{ink4a}$ or $p14^{arf}$, respectively and validated its effect on leukemia cells, especially in combination with molecular targeted agents including imatinib and ATRA. The p16, p14, hrGFP or mock lentiviral transfer vector was introduced into 293T cells along with packaging plasmids (pMDLg/p. RRE and pMD. G) and pRSV-Rev. The resulting VSV-G pseudotyped lentivirus supernatant was concentrated by ultracentrifugation for high-titer virus stock. K562, NB4 and IMS-M2 cells were infected with those prepared viruses at the multiplicity of infection (moi) of 20-50, which allowed almost 100% of infection efficiency based on hrGFP fluorescence. The former two cell lines were deficient in p16 and p14 transcripts and had mutated p53 but intact pRb, while the latter one expressed both transcripts and had mutated p53 but intact pRb. Overexpression of p16 induced leukemia cells to accumulate in the G1 phase, followed by partial (IMS-M2) or complete (K562 and NB4) inhibition of cell growth. In addition, overexpression of p14 resulted in partial (IMS-M2) or substantial (K562 and NB4) apoptotic cell death, suggesting the presence of p53-independent pathway of apoptosis through p14. On the other hand, clonal growth and differentiation of cord blood CD34-positive cells was not affected by enforced expression of either p16 or p14 at the same moi. Next, not only imatinib-induced apoptosis of K562 cells but also ATRA-induced growth arrest and apoptosis of NB4 cells was markedly enhanced by lentiviral transduction of either p16 or p14 at a low moi less than 2.5. These results suggest that INK4A/ARF protein-mimetic agents may be promising options for advanced Ph-positive leukemia as well as AML-M3 in combination with the present molecular targeted therapy since these diseases are relatively resistant to a single agent.

4. Prospective identification of putative telomerase-positive primary leukemia cells

Kobayashi S, Soda Y, Tojo A

There is considerable evidence that rare leukemic stem cells can self-renew and give rise to their progeny with limited life span. Replicative potential of normal as well as leukemic stem cells is primarily defined by their telomerase activity which closely correlates with expression of telomerase reverse transcriptase (hTERT) at the mRNA level. Here we developed a cell-based assay for transcriptional activity of the hTERT gene and applied it to prospective identification of putative telomerase-positive cells in AML. Self-inactivating lentiviral vector containing the expression cassette of the hrGFP gene driven by the 1.2kb hTERT promoter was constructed and transfected into 293T cells together with packaging plasmids (pMDLg/p. RRE and pMD. G) and pRSV-Rev. The resulting VSV-G pseudotyped hTERT-hrGFP lentivirus supernatant was concentrated for high-titer virus stock. K562 and NB4 cells were infected with this virus or mock

and then treated with imatinib and ATRA, respectively. Flow cytometric analysis of either cell line revealed that fluorescence intensity of hrGFP in virus-infected cells reduced in parallel with growth inhibition as well as decrease in telomerase activity of mock-infected cells, suggesting that the fluorescent profile of hrGFP may reflect intrinsic telomerase activity in this assay. Since primary AML cells exhibit much less telomerase activity than cell lines, to analyze patient samples hrGFP was substituted by a novel YFP-variant (Venus) with fast and efficient maturation (Nature Biotech. 20: 87, 2002). This novel reporter construct allowed us to detect hTERT promoter active leukemia cells, which appears telomerase-positive, in a living state. hTERT+ cell population occupied 3.3-62.4% of the total (n=6), and distributed in both CD34+ CD38- and CD34+CD38+ fraction. The fluorescence intensity of Venus showed an almost single peak, suggesting a successive hierarchy of hTERT promoter activity in leukemia cells. Some cases significantly increased in hTERT+ population in response to cytokine stimulation but others did not. A small but highly fluorescent cell population could be identified under microscopy, making it possible to trace its behavior in coculture with bone marrow stromal cells. This reporter assay is valuable for investigation of putative telomerase-positive cells in heterogenous cell populations.

5. Selection of chronic lymphocytic leukemia binding peptides.

Takahashi S

To provide cell-binding ligands for ex vivo gene therapy and chronic lymphocytic leukemia (CLL)-targeting ligands for in vivo drug and gene therapy, we selected 44 20-mer peptides from peptide-presenting phage libraries by panning against primary patient CLL cancer cells. Twenty-nine of the selected peptides were assayed for cell binding. Eight of the selected peptides bound CLL cells, B cells, T cells, and monocyte cells, 12 bound only CLL cells and B cells, and 1 peptide bound only B cells. However, eight of the selected peptides were CLL specific. When two of the peptides were tested out of the context of phage, the synthetic peptides were able to bind cells and functionally retarget adenovirus to increase ex vivo gene delivery to primary CLL cells. These data demonstrate the ability to identify lead cancer-targeting peptides by selection of phage libraries against primary human cancers cells.

6. *In vitro and in vivo* biological properties of novel tumor targeting peptides

Chen M, Soda Y, Tojo A, Asano S

Fight against refractory malignancies requires novel therapeutic approaches including cell and /or molecular targeting therapy. Although gene therapy is among those promising candidates, targeted delivery of therapeutic genes into tumor cells remains to be achieved. In case of current molecular targeted agents such as imatinib and gefitinib, related toxicities are frequently observed especially in high dose administration. Thus, tumor-specific delivery of molecular targeted agents seems more ideal. Cell type-specific peptides can be applied to delivery vehicles for small molecule compounds, nucleotides as well as diagnostic agents. Recently, several cell-specific targeting peptides have been identified by screening phage-display libraries. We also screened M13 phage-display libraries containing random 12-mer peptides by the subtraction method as follows. Daudi lymphoma cells were used to exclude non-specific and/or lymphoid-specific binding of peptide-presenting phages, and AML-M2 derived Kasumi-1 cells were used to isolate peptides preferentially targeting myeloid leukemia cells. After repeated screening of cell surface bound phages, individual clones were isolated and sequenced. As a result, we identified two kinds of phages presenting 12-mer peptides (K12#S1 and #S2). Specific binding of the purified phages to Kasumi-1 cells was confirmed by flow cytometry using anti-M13 monoclonal antibody. In addition, FITC-labeled peptides corresponding to the phage inserts were synthesized and subjected to a binding assay, indicating that these two peptides bound to Kasumi-1 cells as efficiently as the original phages. Unexpectedly, these phages not only bound to myeloid leukemia cells but also to lymphoid malignancies as well as a number of solid tumor cells such as Hela cells, but not to normal lymphocytes and bone marrow cells. Time-dependent internalization of these phages and peptides into Kasumi-1 and HeLa cells was confirmed by immunostaining and confocal laser microscopic analysis. Furthermore, NOD-SCID mice subcutaneously implanted with Hela cells were injected via tail vein with either of the two isolated phages and a control phage, and sacrificed for immunohistochemical analysis of its distribution 15 min later. Both K12#S1 and #S 2 phages but not a control phage specifically bound to Hela cells without evidence of distribution in normal tissues including spleen, bone marrow, kidney, heart, lung and liver, suggesting successful in vivo targeting. These peptides will be candidate vehicles for tumor cell target-

ing therapy.

7. A Versatile Targeted Drug Delivery System Using Streptavidin-tagged Pegylated Liposomes and Biotinylated Ligands

Chen M, Soda Y, Tojo A, Asano S

Recently several tumor-targeting strategies have been described to overcome clinical resistance to conventional chemotherapy without increasing toxicity to normal tissues. At the 44th ASH meeting, we reported that a CD19-targeted pegylated (PEG) liposome would be an effective delivery tool of imatinib for Ph-ALL cells. In the present study, we developed a versatile liposome-mediated drug delivery system based on the rigid interaction between biotin and streptavidin (SAv). Besides CD19, CD33 and CD 7 are representative surface antigens which are abundantly expressed in restricted cell populations and internalized rapidly and efficiently after binding of their ligands. Humanized anti(□) -CD33 monoclonal antibody (MoAb) was really conjugated with calicheamicin for AML treatment (Mylotarg™). G-CSF receptors (R) are alternate candidates for targeting myeloid cells because they are expressed exclusively by myeloid cells despite relatively low level expression. We exploited a SAv-conjugated PEG liposome (SAv-liposome) together with a biotinylated (b) version of G-CSF, □ CD33 or □ CD7 MoAb for targeted drug delivery. The introduction efficiency of calcein-encapsulated SAv-liposomes with bG-CSF into G-CSFR+ AML cells (Kasumi -1 and IMS-M2) was determined as almost 100% by flow cytometry. Similarly, those of SAv-liposomes with b□ CD33 or b□ CD7 MoAb into CD33+ cell lines (IMS-M2, NB4 and Meg-01) or CD7+ Jurkat cells were also 100%. Internalization of these immunoliposomes was confirmed by confocal laser microscopy. SAv-liposomes could not bind to target cells efficiently in the absence of G-CSF or MoAbs, and PEG-liposomes lacking SAv could not bind regardless of the presence of ligands. Internalization of SAv-liposomes with biotinylated ligands was competitively inhibited by unlabeled G-CSF, MoAbs or SA. Next, AraC-encapsulated SAv-liposomes were produced and tested for its ability to kill IMS-M2 cells. AraC-SAv-liposomes in combination with b□ CD33 MoAb killed these cells at significantly lower concentration than free AraC and AraC-PEG-liposomes. In conclusion, we successfully developed a liposome-mediated drug delivery system easily applicable to various cell types depending on the choice of ligands.

8. CD 19-targeting liposomes containing imatinib efficiently killed Philadelphia chromosome-positive acute lymphoblastic leukemia cells

Harata M, Soda Y, Ooi J

Patients with Philadelphia chromosome-positive acute lymphoblastic leukemia (Ph^+ ALL) have a poor prognosis, despite intensive treatments such as hematopoietic cell transplantation. Recently, imatinib (Glivec®, STI571), BCR-ABL tyrosine kinase inhibitor, has proven to be a useful agent for Ph^+ ALL, but almost all of them become resistant quickly to this agent. Although high-dose administration of imatinib would overcome this resistance, toxicities will not allow this approach. Therefore, new delivery system of imatinib for target cells alone is strongly desired. Since almost all Ph^+ ALL cells express CD19 on their surface, we have developed a new immunoliposome bound with anti-CD19 antibody (CD19-liposomes). The internalization efficiency of the CD19-liposomes was almost 100% in all CD19-expressing cell lines tested, including Ph^+ ALL cells, and was significantly higher than that of anti-CD19 antibody-unbound liposomes. In contrast, the internalization efficiency of CD19-liposomes to CD19-negative cells was very low. The cytocidal effect of imatinib-encapsulated CD19-liposomes (imatinib-CD19-liposomes) on Ph^+ ALL cell lines and primary leukemia cells from Ph^+ ALL cells was much greater than that of imatinib encapsulated in liposomes without anti-CD19 antibody or of free imatinib. In addition, those effects were maintained even when the target Ph^+ ALL cells harbor imatinib-resistant mutation in the *bcr-abl* gene. Importantly, the imatinib-CD19-liposomes did not affect the colony formation of CD34-positive hematopoietic cells, even when an inhibitory concentration of free imatinib was used. Taken together, it was clearly demonstrated that the imatinib-CD19-liposomes induced specific and efficient death of Ph^+ ALL cells. This new therapeutic approach might be useful for Ph^+ ALL with much less side effects than administration of free imatinib.

Publications

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Advanced Clinical Research Center

Division of Cellular Therapy
細胞療法分野

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Our major projects are (1) signal transduction of cytokine receptors, (2) identification and characterization of novel cytokines, cytokine receptors, soluble factors, and transcription factors, (3) the roles of small GTPases and GAPs, (4) molecular mechanism of leukemogenesis, (5) identification of self-renewal factor for embryonic stem cells, (6) ontogeny of hematopoiesis, (7) characterization of hematopoietic stem cells, (8) molecular mechanism regulating hematopoiesis.

1. Isolation and characterization of new genes by a novel signal sequence trap method SST-REX

Yuichi Ikeda, Wing-Chun Bao, Toshihiko Oki, Takuya Sugiyama, Hidetoshi Kumagai[1], Sizhou Feng[1], Kaori Tamitsu[1], Yoshihiro Morikawa[2], Hideaki Nakajima, Tetsuya Nosaka[3], and Toshio Kitamura: [1]Takada Research Labs, Chugai Pharmaceutical Co., Ltd., [2]Department of Neurobiology and Anatomy, Wakayama Medical School of Medicine, [3]Division of Hematopoietic Factors, The Institute of Medical Science, The University of Tokyo

Secreted and cell-surface proteins play essential roles in cell-cell interaction. We have recently established a novel and efficient signal sequence trap method (SST-REX), in which cDNA fragments fused to an extracellular deletion mutant of the constitutively active MPL were transduced into IL-3-dependent cells via retrovirus infection followed by the selection of factor-independent clones. Our method is quick and more accurate than the previously published methods. In addition, type II membrane proteins, which had never been isolated by the previous SST methods, were also obtained by our SST-REX.

Several interesting genes have been isolated by this method from various tissues including hemopoietic stem or progenitor cells, AGM (aorta-gonad-mesonephros) cells, mast cells, and cardiocytes, and their functions are currently being investigated.

2. Development of retrovirus vectors and packaging cell lines

Sumiyo Morita[3], Fumi Shibata, Dan Wang, Toshihiko Oki, Tetsuo Kojima[3], Yuko Koshino, Hideaki Nakajima, Tetsuya Nosaka[3], Carol Stocking[4], Wolfam Ostertag[4], Koichiro Tsuji and Toshio Kitamura: [4]Heinrich-Pette Institute, Hamburg University

We previously developed a MuLV-derived efficient retrovirual vector pMX that is suitable for library construction. Combination of transient retrovirus packaging cell lines such as Bosc23 and the pMX vector produced high titer (10^6-10^7 /ml) retroviruses that gave 100% infection efficiency in NIH3T3 cells, 10-100% infection efficiency in various hemopoietic cell lines, and 1-

20% in primary culture cells including T cells, monocytes, and mast cells. However, pMX did not work well in immature cells such as EC cells and ES cells. We have now developed pMY and pMZ vectors that utilize PCMV's LTR and primer binding site, and can express GFP in EC cells and ES cells.

Recently, usefulness of transient packaging cells has been recognized, however the titers of retroviruses are rather unstable during culture. In order to establish more stable packaging cell lines, we used the IRES sequence that allows simultaneous expression of both *gag-pol* or *env* gene and drug resistance gene from one transcript. We used the strongest promoter EF-1α in making packaging constructs. In addition, to avoid inclusion of retrovirus sequences as much as possible, we used only coding sequence of *gag-pol* and *env* genes for the packaging constructs, which will not allow the formation of replication-competent retroviruses by recombination in packaging cell lines. We established high-titer ecotropic (PLAT-E) and amphotropic (PLAT-A) packaging cell lines where the EF-1α-*gag-pol*-IRES-puro^r together with the corresponding EF-1α-*env*-IRES-bs^r were introduced into 293T cells. We have also established another new packaging cell line (PLAT-F) for efficient infection to human hematopoietic stem cells by using *env* gene of feline endogenous retrovirus RD114, and the efficiency of infection of the viruses produced by PLAT-F, into human CD34 positive cells, is being investigated by a long term reconstitution assay in SCID mice.

3. Analysis of the role of MgcRacGAP as a regulator of the small GTPase Rho family in differentiation and cytokinesis

Toshiyuki Kawashima[3], Yukinori Minoshima, Koichi Hirose[3], Yukio Tonozuka, Takaya Satoh[5], Yoshito Kajiro[5], Hideaki Nakajima, Tetsuya Nosaka[3], and Toshio Kitamura: [5]Faculty of Bioscience and Biotechnology, Tokyo Institute of Technology

In the search for key molecules that prevent murine M1 leukemic cells from undergoing IL-6-induced differentiation into macrophages, we isolated an antisense cDNA that encodes full-length mouse MgcRacGAP through functional cloning. In human HL-60 leukemic cells, overexpression of the human MgcRacGAP induced growth suppression and macrophage differentiation. Analysis using the mutants revealed that the GAP activity was dispensable, but the myosin-like domain and the cysteine-rich domain were indispensable for 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. Overexpression of an N-terminal deletion mutant resulted in the production of multinucleated cells in HeLa cells. This mutant lost the ability to localize in the mitotic spindle and midbody. MgcRacGAP was also found to bind α-, β-, and γ-tubulins through its N-terminal myosin-like domain. These findings indicate that MgcRacGAP dynamically moves during cell cycle progression probably through binding to tubulins and plays critical roles in cytokinesis. Furthermore, using a GAP-inactive mutant, we have disclosed that the GAP activity of MgcRacGAP is required for cytokinesis, suggesting that inactivation of Rho family GTPases may be required for normal progression of cytokinesis. We have recently found that MgcRacGAP is phosphorylated by some of the kinases that are known to work in the midbody.

4. Identification of a small molecule that inhibits leukemic cell growth caused by the internal tandem duplication mutations of *Flt-3*

Ken Murata[3], Hidetoshi Kumagai[1], Hideaki Nakajima, Toshiyuki Kawashima[3], Kaori Tamitsu[1], Mariko Irie[1], Shigetaka Asano[6], Tetsuya Nosaka[3], and Toshio Kitamura: [6]Division of Molecular Therapy, Advanced Clinical Research Center, Institute of Medical Science, The University of Tokyo

Internal tandem duplications of the juxtamembrane region of the *Flt-3* are found in about 20% of the human acute myeloid leukemia patients. In screening of the small compounds by the ability to selectively inhibit leukemic cell growth caused by such mutations of *Flt-3*, we have identified several small chemical compounds. These molecules show structural similarity to the tyrosine kinase inhibitor. One of the most effective molecules GTP14564 preferentially inhibited the growth of the Ba/F3 cells transformed by the mutant *Flt-3*, thereby suppressing the tyrosine phosphorylation of STAT5, but not very much in Ba/F3 cells driven by the Flt-3 ligand/wild type Flt-3. Forced expression of the dominant negative STAT5A, but not treatment with the MEK inhibitors suppressed the mutant Flt-3-driven cell growth. On the other hand, the proliferative signal through the wild type Flt-3 was dependent on the activation of MAP kinases. We also revealed that the N-terminal two tyrosine residues of the intracellular domain of the mutant Flt-3 were responsible for STAT5 activation and autonomous cell growth, but the corre-

sponding tyrosine residues of the intracellular domain of the wild type Flt-3 was dispensable for cell growth.

5. STAT5 induces macrophage differentiation of M1 leukemia cells through activation of IL-6 production mediated by NF-κB p65

Toshiyuki Kawashima[3], Ken Murata[3], Yukio Tonozuka, Yukinori Minoshima, Tetsuya Nosaka[3], and Toshio Kitamura

Using a constitutively active STAT5A (STAT5 A1*6), we have shown that STAT5 induces macrophage differentiation of mouse leukemic M1 cells through a distinct mechanism, autocrine production of IL-6. The supernatant of STAT5A1*6-transduced cells contained sufficient concentrations of IL-6 to induce macrophage differentiation of parental M1 cells, and STAT3 was phosphorylated on their tyrosine residues in these cells. Treatment of the cells with anti-IL -6 blocking antibodies profoundly inhibited the differentiation. We have also found that the STAT5A1*6 transactivated the IL-6 promoter, which was mediated by the enhanced binding of NF-κB p65 (RelA) to the promoter region of IL-6. These findings indicate that STAT5A cooperates with Rel/NF-κB to induce production of IL-6, thereby inducing macrophage differentiation of M1 cells in an autocrine manner. In summary, we have shown a novel mechanism by which STAT5 induces its pleiotropic functions.

6. A novel secreted form of immune suppressor factor with high homology to vacuolar ATPases identified by a forward genetic approach of functional screening based on cell proliferation

Edgardo E Tulin[3], Hideaki Nakajima, N Onoda[7], M Maeda[7], M Hasegawa[7], Atsushi Urano, Fumi Shibata, Tetsuya Nosaka[3], H Nomura[7], Shigetata Asano[6], and Toshio Kitamura: [7]Chugai Research Institute for Molecular Medicine

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, which we termed ShIF, 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, a membrane protein harboring six transmembrane domains, was reported to have immunosuppressive functions. The coding region of ShIF started from the third transmembrane domain of ISF. Biochemical analysis demonstrated that ShIF was expressed in both the secreted and membrane-bound forms of 27-kDa protein, which was supposed to have an internal ATG present in the third transmembrane domain of ISF as a start codon. In addition to the full-length form of ISF, a major protein with a molecular size of 27 kDa was also expressed through the proteolytic process of ISF. ShIF resembles this naturally occurring short form of ISF (sISF). Deletion analysis of the major domains of ISF cDNA revealed that ShIF is an active functional domain of ISF with a capability to support proliferation of S21 cells. Enforced expression of ShIF in MS10 cells, bone marrow stroma cells that do not express endogenous ShIF or ISF, conferred on the cells an ability to support the growth of S21 cells as well as bone marrow cells. Interestingly, ShIF shows a high sequence homology to the C-terminal part of a 95-kDa yeast vacuolar H (+)–ATPase subunit, Vph1p (39%), and a 116-kDa proton pump (VPP 1) (54%) of the rat and bovine synaptic vesicle. Therefore, it is possible that ShIF also acts as a proton pump and somehow prevents the cells from undergoing apoptosis. We are currently examining the effects of ISF and ShIF on the growth of hematopoietic progenitor cells from bone marrow, and also trying to identify the molecules that interact with these factors.

7. Molecular mechanism of stem cell self renewal on bone marrow stroma

Hideaki Nakajima, Yuko Koshino, Fumi Shibata, Yumi Fukuchi and Toshio Kitamura

Hematopoietic stem cells (HSC) keep self-renewing in the bone marrow in order to support continuous blood cell production. These processes are thought to occur in the bone marrow niche, a special microenvironment created by stromal cells. HSC-stromal cell interaction is thought to provide unknown signals to keep HSC in immature state and makes them undergo extensive self-renewal. However, molecular mechanism of these processes is poorly understood. We are trying to address this question by following approaches. 1) Identify cell surface molecules that are expressed on stromal cells and important for HSC self-renewal by utilizing a variety of technologies (i.e. signal sequence trap, mRNA subtraction) and analyze their function in vitro and in vivo. 2) Identify genes that are induced in HSC by contacting with stromal cells. These genes are strong candidates that are

involved in the self-renew processes evoked by stromal cell contact. We are now characterizing two novel molecules that are speculated to be important for these processes.

8. Role of co-repressors in STAT5-dependent transcription

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STAT5 is a latent transcription factor activated by a variety of cytokines including IL-3, GM-CSF and erythropoietin. To gain more insight into the molecular mechanism how STAT5 regulates the variety of cytokine responses, we set out to explore the proteins that interact with STAT5 *in vivo*. We employed yeast two-hybrid screening to look for STAT5-interacting molecules, and identified silencing mediator for retinoid and thyroid hormone receptor (SMRT) as a potential binding partner. SMRT interacted with both STAT5A and 5B, and the association was detected both *in vitro* and *in vivo*. Interestingly, SMRT strongly repressed STAT5-dependent transcription both on heterologous and native promoters in reporter assays. To further clarify the physiological role of this interaction, we created a stable cell line overexpressing SMRT. In clear contrast to parental cell line, expression of STAT5 target genes in this cell line was not sustained and was quickly suppressed within 2 hours after normal initial phase of induction. Conversely, histone deacetylase inhibitor, Trichostatin A effectively enhanced and prolonged induction of STAT5-target genes in a parental cell line. Extensive mutational and binding analysis revealed that this interaction was mediated through N-terminal coiled-coil region of STAT5. Surprisingly, previously identified point mutation in the coiled-coil region that makes STAT5 hyperactive, disrupted Stat5-SMRT interaction, suggesting overall transcriptional activity of STAT5 is determined by the balance of coactivators and corepressors bound to it. In addition, above data predicts that naturally occurring dominant negative mutant of STAT5 lacking carboxyl-transactivation domain interacts only with SMRT, but not with CBP, suggesting that it acts as dominant negative by actively repressing target genes through SMRT. Together, this study illuminates the potential role of SMRT in negative regulation of STAT5-dependent transcription, and reveals a novel crosstalk between nuclear receptor and JAK-STAT signaling pathways.

9. The role of CCAAT/enhancer-binding protein ε and α in normal hematopoiesis and leukemogenesis

Hideaki Nakajima, Yumi Fukuchi, Naohide Watanabe[9], Makoto Handa[9], Yasuo Ikeda[10], James N Ihle[8] Scott Kogan[11], Grant McArthur[12] and Toshio Kitamura: [10]Division of Hematology, Department of Internal Medicine, Keio University School of Medicine, [11]Comprehensive Cancer Center, University of California, San Francisco, [12]Division of Hematology/ Medical Oncology, Peter MacCallum Cancer Institute

Granulocyte colony-stimulating factor (G-CSF) is a major cytokine that regulates proliferation and differentiation of myeloid cells, although the underlying mechanisms by which G-CSF controls myeloid differentiation are largely unknown. Differentiation of hematopoietic cells is regulated by lineage-specific transcription factors, and gene-targeting studies previously revealed the critical roles of CCAAT/enhancer-binding protein C/EBPα and C/EBPε, respectively, in the early and mid-late stages of granulocyte differentiation. The expression of C/EBPε in 32Dcl3 cells and FDCP1 cells expressing mutant G-CSF receptors was examined and it was found that G-CSF up-regulates C/EBPε. The signal for this expression required the region containing the first tyrosine residue of G-CSF receptor. Dominant-negative STAT3 blocked G-CSF-induced granulocytic differentiation in 32D cells but did not block induction of C/EBPε, indicating that these proteins work in different pathways. It was also found that overexpression of C/EBPε greatly facilitated granulocytic differentiation by G-CSF and, surprisingly, that expression of C/EBPε alone was sufficient to make cells differentiate into morphologically and functionally mature granulocytes. Overexpression of c-myc inhibits differentiation of hematopoietic cells, but the molecular mechanisms of this inhibition are not fully understood. In 32Dcl3 cells overexpressing c-myc that do not differentiate by means of G-CSF, induction of C/EBPε is completely abrogated. Ectopic expression of C/EBPε in these cells induced features of differentiation, including changes in nuclear morphologic characteristics and the appearance of granules. The data show that C/EBPε constitutes a rate-limiting step in G-CSF-regulated granulocyte differentiation and that c-myc antagonizes G-CSF-induced myeloid differentiation, at least partly by suppressing induction of C/EBPε.

Acute promyelocytic leukemia is characterized by the balanced taranslocation t (15;17), which

generates PML-RARα fusion protein. This fusion protein is thought to affect key differentiation pathway of normal myeloid development, one of which is C/EBPε. We employed PML-RARα transgenic mouse model to show that restoration of C/EBPε expression can revert leukemic phenotype of these mice. These observations reveal that C/EBPε is a critical target of PML-RARα and suggest that targeted modulation of C/EBP activities could provide a new approach to therapy of AML

10. Identification of factor(s) supporting self-renewal of primate embryonic stem cells

Takuya Sugiyama[3], Atsushi Urano, Tetsuya Nosaka[3], Hideaki Nakajima and Toshio Kitamura

Dissection of molecular nature of embryonic stem (ES) cells may promote our understanding of cellular pluripotency and inner cell mass (ICM) development, and also can assist ES-based tissue engineering. Both mouse and human ES cells require feeder layer cells to retain the undifferentiated state. Whereas mouse ES cells were reported to remain undifferentiated without feeder cells in the presence of leukemia inhibitory factor (LIF), primate ES cells without feeder cells do differentiate even in the presence of LIF. Our goal is to identify the feeder-derived factor(s) supporting undifferentiated state and growth of the primate ES cells, through cDNA expression cloning. This project is in collaboration with Drs. Suemori and Nakatsuji at Kyoto University.

11. Whole embryo culture (WEC) analysis on hematopoietic cell development

Daisuke Sugiyama, Feng Ma, Kohichiro Tsuji

Although precursors with the potential to generate definitive hematopoietic stem cells (HSC) appear independently in yolk sac (YS) and intraembryonic paraaortic splanchnopleura (P-Sp) as mentioned above, it remains unanswered whether both early YS and P-Sp contribute to definitive hematopoiesis in their circumstances *in vivo*. To address this issue, we developed an embryo-grafting system using WEC. In this system, whole embryos from 8.25 days postcoitum (dpc), a time before the formation of omphalomesenteric artery which connect between YS and embryo proper, could be cultured to 11.0 dpc after its formation. We first investigated whether hematopoietic cell development of embryos in WEC can compare with normal one. We isolated and dispased AGM region from the embryos after WEC, and performed fetal thymus organ culture (FTOC) assay and co-culture with OP9 stromal cells to detect T and B lymphoid and hematopoietic potentials. After 2.75 days of WEC, lymphohematopoietic progenitors existed in AGM region of the cultured embryo as well as normal 11.0 dpc embryo. We then made YS-YS chimera embryos at 8.25 dpc. When the chimera embryos were cultured in WEC, vigorous blood flow was formed within the YS graft. The developed system may provide a useful tool for analysis of hematopoieitc cell development, especially its origin.

12. Erythropoiesis from acetylated low-density protein (Ac-LDL)-incorporating endothelial cells into circulation at pre-liver stage.

Daisuke Sugiyama, Kohichiro Tsuji

Erythropoiesis is characterized by two distinct waves of production during mouse embryogenesis: a primitive one originating from YS and a definitive one produced from both the YS and the embryo proper. How this last wave is generated remains unclear. We put forward the hypothesis that erythroid cells could be generated by endothelial cells (ECs). To investigate this problem, we have designed a method to label ECs at 10 dpc. This label associates two techniques: an intracardiac inoculation that allows molecules to be delivered into the blood stream, followed by a WEC period. DiI-conjugated acetylated low-density lipoprotein (Ac-LDL-DiI) was used to specifically tag ECs from the inside. One hour after inoculation, DiI staining was found along the entire endothelial tree. Flow cytometric analysis revealed that DiI^+ cells were $CD31^+$, $CD34^+$ and $CD45^-$, an antigen make-up characteristic for the endothelial lineage. Twelve hours after inoculation, 43% of DiI^+ circulating cells belonged to the erythroid lineage. These cells expressed Ter 119 and displayed an adult globin chain arrangement, thus belonged to the definitive lineage as confirmed in erythroid colony formation. The rest of the cells likely represent committed white blood cells or multipotent progenitors as revealed by a mix-colony formation. Beyond the 29-somite stage, the proportion of the DiI^+ erythroid cells gradually decreased. These results demonstrate, for the first time in the mouse embryo, the generation of hematopoietic cells from an endothelial intermediate, using an *in vitro* tracing. We thus provide evidence for a release of these cells are able to colonize the fetal liver and generate definitive erythrocytes *in vivo*.

13. Reconstitution of human hematopoiesis in NOD/SCID mice by clonal cells expanded from single $CD34^+CD38^-$ cells expressing Flk2/Flt3

Yasuhiro Ebihara, Atsushi Manabe, Mika Wada, Kohichiro Tsuji,

In the present study, we examined the expression of Flk2/Flk3, a tyrosine kinase receptor, on human CB $CD34^+$ hematopoietic progenitor/stem cells. In flow cytometric analysis, Flk2/Flt3 was expressed on four fifths of $CD34^+$ cells and their immature subpopulations, $CD34^+CD33^-$ and $CD34^+CD38^-$ cells. Methycellulose clonal culture of sorted Lin^- $CD34^+Flk2/Flt3^+$ and Lin^- $CD34^+Flk2/Flt3^-$ cells showed that most of myelocytic progenitors expressed Flk2/Flt3, but erythroid and hematopoietic multipotential progenitors were shared by both fractions. When 1×10^4 $Lin^-CD34^+Flk2/Flt3^-$ cells were transplanted into four NOD/SCID mice, no recipients possessed human $CD45^+$ cells in bone marrow 11 to 12 weeks after the transplantation. By contrast, all of four recipients transplanted with 1×10^4 $Lin^-CD34^+Flk2/Flt3^+$ cells showed a successful engraftment. Furthermore, clonal cells expanded from single $Lin^-CD34^+CD38^-Flk2/Flt3^+$ cells in the culture with Flk2/Flt3 ligand (FL), stem cell factor (SCF), thrombopoietin (TPO), and a complex of IL-6/soluble IL-6 receptor (IL-6/sIL-6R) were individually transplanted into NOD/SCID mice. Twenty to 21 weeks after the transplantation, 3 of 10 clones harvested at day 7 of culture, and 3 of 6 clones at day 14 could reconstitute human hematopoiesis in recipient marrow. These results demonstrated that Flk2/Flt3 was expressed on a wide variety of human hematopoietic cells including long term-repopulating hematopoietic stem cells.

14. TEK expression and hematopoietic and angiogenic potentials in cord blood CD34 + cells.

Mika Wada, Yasuhiro Ebihara, Feng Ma, Miyuki Ito, Kohichiro Tsuji

Tunica interna endothelial cell kinase (TEK) is expressed on commonly expressed on both hematopoietic and endothelial cells, and plays some crucial roles in mouse development. In human, however, little has been known about the hematopoietic and angiogenic ability of TEK-expressing cells in CB cells, which originate from human fetus period. We then compared the hematopoietic and angiogenic ability between CB $CD34^+TEK^+$ and $CD34^+TEK^-$ cells, using clonogenic assay and xenotransplantation into NOD/SCID mice. The result showed that colony-forming cells and cells capable of repopulating in NOD/SCID mice were present in both $CD34^+TEK^+$ and $CD34^+TEK^-$ cells, and their hematopoietic activities were similar. By contrast, the potential to differentiate into endothelial cells *in vivo* was greater in the former. All the seven NOD/SCID mice engrafted with CD 34^+TEK^+ cells had human CD31 and VE-cadherin -expressing endothelial cells in vessels of ischemic musles and/or human CD31, KDR and ecNOS-expressing endothelial cells in liver sinusoidal cells, while such endothelial cells were detected in only three of the seven recipients engrafted with $CD34^+TEK^-$ cells. The present result has important implications in cellular therapy for hematopoietic disorders and vascular diseases using CB cells.

15. Impaired neutrophil maturation in the truncated mouse granulocyte colony-stimulating factor (G-CSF) receptor-transgenic mice.

Tetsuo Mitsui, Sumiko Watanabe[13], Sachiyo Hanada, Yasuhiro Ebihara, Kohichiro Tsuji: [13]Division of Molecular and Developmental Biology, IMSUT

Severe congenital neutropenia (SCN) is a hematopoietic disorder characterized by neutropenia in peripheral blood and maturation arrest of neutrophil precursors in bone marrow. Patients with SCN may evolve to have myelodysplastic syndrome or acute myelocytic leukemia. In approximately 20% of SCN cases, a truncation mutation is found in the cytoplasmic region of the G-CSF receptor (G-CSFR). We then generated mice carrying murine wild type G-CSFR and its mutants equivalent to truncations at amino acids 718 and 731 in human G-CSFR, those were reported to be related to leukemic transformation of SCN. Although numbers of peripheral white blood cells, red blood cells and platelets had no difference among mutant and wild type G-CSFR transgenic (Tg) mice, both of the mutant receptor Tg mice had one third of peripheral neutrophil cell counts compared to wild type receptor Tg mice. The mutant receptor Tg mice also showed impaired resistance to the infection with *Staphylococcus aureus*. Moreover, bone marrow of these Tg mice had an increased percentage of immature myeloid cells, a feature of SCN. This maturation arrest was also observed in *in vitro* cultures of bone marrow cells of truncated G-CSFR Tg mice under G-CSF stimulation. In addition, clonal culture of bone marrow cells of the truncated G-CSFR Tg mice showed the hypersensitivity to G-CSF in

myeloid progenitors. Our Tg mice may be useful in the analysis of the role of truncated G-CSFR in SCN pathobiology.

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Main subjects of the Division of Infectious Diseases are human immunodeficiency virus (HIV) infection and related disorders.

1. Immunogene therapy for AIDS by presenting a CTL-epitope peptide with Sendai virus vectors.

Ai Kawana-Tachikawa et al.

We study a Sendai virus (SeV) vector system for expression of major histocompatibility complex (MHC) class I/peptide complexes, which may be an eligible therapeutic molecules in immunogene therapy. We cloned the extracellular domain of a human MHC class I heavy chain, HLA-A*2402, and human beta-2 microglobulin (beta2m) fused with HLA-A*2402-restricted human immunodeficiency virus type 1 (HIV-1) cytotoxic T-lymphocyte (CTL) epitopes (e-beta2m) in separate SeV vectors. When we coinfected nonhuman mammalian cells with the SeVs, naturally folded human MHC class I/peptide complexes were secreted in the culture supernatants. Biotin binding peptide sequences on the C terminus of the heavy chain were used to tetramerize the complexes. These tetramers made in the SeV system recognized specific CD8-positive T cells in peripheral blood mononuclear cells of HIV-1-positive patients with a specificity and sensitivity similar to those of MHC class I tetramers made in an Escherichia coli system. Solo infection of e-beta2m/SeV produced soluble e-beta2m in the culture supernatant, and cells pulsed with the soluble protein were recognized by specific CTLs. Furthermore, when cells were infected with e-beta2m/SeV, these cells were recognized by the specific CTLs more efficiently than the protein pulse per se. SeV is nonpathogenic for humans, can transduce foreign genes into nondividing cells, and may be useful for immunotherapy to enhance antigen-specific immune responses. Our system can be used not only to detect but also to stimulate antigen-specific cellular immune responses.

2. Dynamics of HIV variations in HIV-infected Japanese patients

Tae Furutuki, Ai Kawana-Tachikawa, Mariko Tomizawa, and Noriaki Hosoya et al.

Deciphering the dynamics of HIV variation will bring deeper understanding of the mechanisms that generate viral diversity. Because HLA-A24 is the most common HLA genotype among Japanese, we are investigating HLA-A24-restriction in cytotoxic T-cell (CTL) response

with this aim. From clinical isolates we are cloning and sequencing part of *nef* regions that encodes CTL epitope -Nef138-10 (RYPLTFGWCF)-restricted by HLA-A24. We found three dominant mutations that resulted in amino acid substitutions in this region: Nef138-10 (2F) (RFL PTFGWCF), Nef138-10 (5C) (RYLPCFGWCF), and Nef138-10(2F5C) (RFLPCFGWCF).

To confirm that these viruses were mutants escaping from CTLs, we first investigated binding affinity of these epitope peptides by binding assay. The "2F" peptide bound to T2-A*2402 cells, which expresses HLA-A24 molecules, as efficiently as the wild-type. In contrast, the "5C" and "2F5C" peptides bound less efficiently than the wild-type. Next we examined CTL response of five HIV-infected HLA-A24 patients by enzyme-linked immunospot assay with their peripheral blood mononuclear cells: we counted the number of cells producing interferon-gamma on stimulation by each epitope peptides. We found tendency that "5C" and "2F5C" required significantly more peptides than "2F" and the wild-type for counting a half of the maximum number of interferon-producing cells. Taking altogether into account, we concluded that "5C" and "2F5C" but not "2F" substitutions resulted in generating CTL epitope peptides that bind poorly to the HLA-A24 molecule, and thus those mutants escaped from CTLs. At the moment, the reason why "2F" replicated dominantly in the HLA-A24 population is unknown.

3. Immunogenetherapy for AIDS

To present HIV-derived antigenic peptides on dendritic cells (DCs) is an important way for enforcement of cytotoxic T-cell response against HIV in HIV-infected patients., we designed adenoviral and Sendai-viral vectors (AdV and SeV, respectively) for expression of green fluorescent protein (GFP). We compared cytotoxity and efficiency of AdV with those of SeV for introduction of HIV genes into DCs. DCs were obtained by culturing healthy volunteers' peripheral monocytes for seven days in the presence of IL-4 and GM-CSF. SeV, either version with or without the Fusion (F) protein, expressed GFP in DCs in an optimal condition of 24 hour culture after infection at an M.O.I of 2, but with considerable cytotoxicity. In contast, AdV expressed GFP in a bit less amount in an optimal condition of 48 hour culture after infection at an M.O. I of 1000, with much less cytotoxicity. Either vector seems promising for expression of exogenous genes in DCs with improving some disadvantages we found in this study.

4. Polymorphisms and haplotypes of the human gene and their association with the clinical outcome of HIV-positive Japanese patients.

Dayong Zhu and Hitomi Taguchi-Nakamura et al.

Patients infected with human immunodeficiency virus type 1 (HIV) show variable response to antiretroviral therapy with HIV protease inhibitors (PIs) such as nelfinavir (NFV), which are substrate of the P-glycoprotein (P-gp), the product of the multidrug resistance-1 (*MDR 1*) gene. In exons of *MDR1* are present three single-nucleotide polymorphisms (SNPs) at *MDR1* 1236, *MDR1* 2677, and *MDR1* 3435. First, we readily genotyped these SNPs by the SNaPshot method (n=79), and found incomplete linkage disequilibrium between these SNPs. Because the SNP at *MDR1* 3435 has been found to be associated with P-gp expression, we evaluated the effect of that SNP on export of NFV from HIV-positive patients' lymphoblastoid cell lines by measuring time-dependent decrease in the amount of intracellular NFV by HPLC. We found that the amount of intracellular NFV decreased in a distinct biphasic fashion: the fast Phase I followed by the slow Phase II. The half-life period of Phase II in LCLs with the homozygous T/T genotype at *MDR1* 3435, were three times longer than that with C/C genotype (612 min. vs. 204 min.). This difference was statistically significant ($p=0.0286$, Mann-Whitney U-test.). Then evaluating in a retrospective study of HIV-positive patients (n=31) the effect of the SNPs on the response to antiretroviral treatment with PIs, we found a statistically significant association of the SNP at *MDR1* 1236 but not at *MDR1* 2677 or *MDR1* 3435. As *in vitro* results were not consistent with the clinical evaluation, clinical importance of *MDR1* genotype monitoring for antiretroviral therapy remains to be investigated in a larger, case-controlled study.

6. HIV vectors

Ryuta Sakuma et al.

To examine whether vectors based on human immunodeficiency virus type 1 (HIV) with viral central polypurine tract (cPPT) express transgenes more efficiently than those without cPPT, we constructed a series of vectors carrying a Neo^r gene as a stable marker, which is driven by an internal synthetic thymidine kinase promoter. Insertion of cPPT, either 282bp or 178bp long, resulted in about 3-8-fold increase of the amount of produced vector particles. Insertion of the longer cPPT resulted in decrease in transduction

efficiency in about eight-fold, whereas insertion of the shorter resulted in increase in transduction efficiency in about two-fold independently of the orientation of the insert. Moreover, in a transient expression system using a *Renilla* luciferase as a reporter, we found that insertion of cPPT/CTS upstream of heterologous promoters increased the expression of a downstream luciferase in about two-fold. However, additional insertion of an LTR sequence upstream of these cPPT/CTS in turn decreased luciferase activity in about five-fold. This function of cPPT/CTS may partly account for the observed decrease in transduction efficiency with our vectors.

7. Variation of Kaposi's sarcoma-associated herpesvirus in HIV-infected Japanese patients

Tokiomi Endo et al.

Kaposi's sarcoma (KS) -associated herpesvirus, or human herpesvirus type 8 (HHV8) can be classified into distinct subtypes by the sequence polymorphisms in several open reading frames (ORFs). We analyzed the subtypes of HHV8 in 59 HIV-infected Japanese patients using polymorphism of ORF26, and found that over two thirds of HHV8 fell into major subtype A. We also found that single-nucleotide polymorphisms (SNPs) at nucleotide positions 1032 (C to A substitution) and 1055 (G to T substitution) in ORF26 were correlated with increased susceptibility to KS as compared to HHV8 with the wild type nucleotides at these positions (p=0.0106). This observation suggests that molecular heterogeneity of HHV8 genome affects the biological properties of HHV8, resulting in different clinical features of HHV8 infection. Since sensitive PCR of ORF26 allows us to analyze the SNPs by using peripheral blood of HHV8-infected patients, ORF26 SNPs seem a potent tool to investigate the pathogenesis of HHV8 infection.

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Our department has two major goals in basic research; 1) Development of innovative cancer therapy using immunologic approaches and gene therapy strategies, and 2) Mechanistic study on transplantation immunology to further develop clinical transplantation.

1. Development of innovative cancer therapy

a. Induction of anti-tumor immunity using intra-tumor administration of adenoviral vector expressing biologically active IL-18

Takuya Takayama, Tetsuya Saitoh, Hideaki Tahara

We have reported that interleukin (IL)-18 has potent antitumor effects mediated by CD4+T cells and NK cells, but in IFN-gamma- and IL-12 -independent pathways. In order to develop IL-18 cancer gene therapy, we have investigated the in vivo antitumor effects of intratumoral administration of an adenoviral vector expressing biologically active murine IL-18. Substantial antitumor effects were observed when established MCA205 fibrosarcoma was treated in syngeneic immunocompetent mice with intratumoral injection of Ad.PTH.IL-18. This study suggests that intratumoral administration of an adenoviral vector expressing biologically active murine IL-18 could be a new strategy of gene therapy in the clinical setting to treat patients with cancer.

b. In vivo electroporation of human FLT3-Ligand plasmid DNA induce effectively mobilize and activate dendritic cells in situ

Takuya Takayama, Tetsuya Saitoh, Shigenori Nagai, Hideaki Tahara

We have established the genetically modified DC to regulate the immune response. We have also focused on Flt3-Ligand, a recently reported cytokine, is a stimulator for proliferation and differentiation of DC not only *in vitro* but in vivo. In this study, we evaluated the effects of Flt3-Ligand on DC mobilization, proliferation, maturation and immune response using in vivo electroporation (IVE). After Flt3-Ligand trasfection using IVE, significantly high level of Flt3-Ligand was detected in the serum during 10 days after IVE. The frequency of DC both in spleen and bone marrow significantly was increased after Flt3-Ligand IVE when compared with those of control group. In mouse tumor model, Flt3-Ligand IVE induced the migration of dendritic cells to local tumor site that was associated with proliferation and mobilization of DC. These results implied that Flt3-Ligand gene transfer using IVE could utilize to the clinical application for cancer gene therapy.

c. Dendritic cells stimulated with a bacterial product, OK-432, efficiently induce cytotoxic T lymphocytes specific to tumor rejection peptide

Saori Nakahara, Takuya Tsunoda, Toshiyuki Baba, Hideaki Tahara.

Dendritic cells (DC) are potent antigen presenting cells which has recently been used for cancer immunotherapy using epitope peptides derived from tumor rejection antigens. Accumulating results of the clinical trial of such strategy suggest that maturation of the DCs applied is one of the key factors which influence the outcome of the vaccination. It has been suggested that DCs need to have "mature" phenotype which is capable of inducing cytotoxic T cells (CTL) efficiently. The characteristics of the mature DCs (mDCs) include high expression of MHC and co-stimulatory molecules and the production of IL-12. In this study, we examined the effects of penicillin-killed Streptococcus pyogenes (OK-432, clinical grade in Japan) on DC maturation. Furthermore, we also examined the potency of OK-432 stimulated DCs on the induction of CTLs specific to the epitope peptide. Peripheral blood mononuclear cells (PBMC) were obtained from healthy donors, selected by the adherence, and cultured in AIM-V medium supplemented with 1000U/ml of GM-CSF and 1000U/ml of IL-4 for 5-7 days. Phenotypic analysis on them showed that more than 90% of prepared cells showed the immunophenotype consistent with immature DC (iDC). These iDC were divided into 4 groups and cultured further in AIM-V containing following agents; A. AIM-V alone, B. TNF-α (100 ng/ml), C. LPS (100 ng/ml), D. OK-432 (10 μg/ml) (OK-DC). After 72 hours, cells were harvested and surface phenotypes and cytokine production using FACS and ELISA respectively. DCs in groups B, C, and D showed significantly higher CD83 expression (B, 85.9%; C, 84.7%; D, 61.0%) when compared with control, (A,3.82%). Furthermore, DCs in group D showed significantly higher production of IL-12 (40.7±3.1ng/ml) and IFN-γ (1976.8±272.6pg/ml) when compared with those of other groups. These results indicate that OK-432 could promote the maturation of iDC to produce significant amount of Th1 type cytokines. To examine the influence of the OK-432 on the induction of peptide specific CTLs, CE3 (HLA-A*2402 restricted 9 mer peptide derived from Carcinoembryonic antigen, TYACFVSNL) was used for inducing peptide specific CTLs. The ^{51}chromium-releasing assay and the tetramer assay of the CD 8+T cells showed that highest cytotoxic activity and highest CTL frequency were induced with OK-DC stimulation. Furthermore, we investigated the signaling pathway of OK-432 using the TLR indicator cell lines and the blocking antibodies. These results showed that OK-432 does not use either TLR2 or TLR4, but the β2 integrin for the stimulation. These results strongly suggest that OK-432 could be a useful agent for peptide-based cancer vaccine using DCs.

d. Generation of mature dendritic cells fully capable of T helper type 1 polarization using OK-432 combined with prostaglandin E2

Marimo Sato, Takuya Takayama, Hiroaki Tanaka, Juichiro Konishi, Toshihiro Suzuki, Teruo Kaiga, Hideaki Tahara

Dendritic cell (DC) administration appears to be a very promising approach as the immunotherapy of cancer. The results of clinical studies have been suggested that the nature and the magnitude of antitumor immune responses are critically affected by DC functions including production of Th1-inducing cytokines, activation of the T cell subsets and NK cells, and migration from peripheral tissues to T cell area of the draining lymph nodes. Administration of immature DCs could fail to fully stimulate antigen-specific immune responses and might induce tolerance in some conditions. In this study, we developed the measures to obtain fully mature DCs and compared in detail with the effects of maturation stimulus termed MCM-mimic, which is a mixture of recombinant cytokines and PGE2 mimicking the content of monocyte-conditioned medium. Using DCs derived from monocytes of advanced cancer patients in this study, we have shown DCs stimulated with OK-432 alone showed phenotypes similar to those of mature DCs induced using MCM-mimic with better secretion of IL-6 and IL-12. However, these DCs were found to have poor migratory capacity associated with the marginal expression of CCR7. When OK-432 was combined with PGE2, CCR7 expression and migratory capacity of DCs were significantly improved without impairing other immuno-stimulatory functions. These results suggest that OK-432 stimulation combined with PGE2 could be applicable as an alternative to MCM-mimic in clinical trials, which require fully matured DCs to induce Th1-type immune responses against tumor cells even in the patients with advanced cancer.

e. Development of novel chemoimmunotherapy using S-1 and Lentinan

Hiroyuki Mushiake, Takuya Tsunoda, Hideaki

Tahara

Cancer chemotherapy has limitations that it is difficult to obtain survival benefit, even if the tumor regression was accomplished temporarily. Combine usage of biological response modifier with anti-cancer drug, so called "chemoimmunotherapy", has been paid attention to these effects and benefits. However, little is known about the mechanisms. This investigation was conducted to clarify the mechanisms of synergistic effect of β-glucan, Lentinan, and a novel oral anti-cancer drug, S-1, in cancer cachexic mouse model. On the hypothesis that a β-glucan, Lentinan will be able to enhance the phagocyte efficiency of dendritic cells, we have been trying to break the peripheral T cell tolerance toward tumor self antigen, CEA, expressed by MC-38 stably transduced with CEA in C57BL /6J mice transgenic for CEA.

f. Optimal condition for RNA transfection of dendritic cells using electroporation

Yoshikazu Kato, Yuichi Ando, Hideaki Tahara

Dendritic cells are professional antigen presenting cells that can efficiently activate antigen-specific T cells. Various strategies have been investigated to load antigens on the dendritic cell for efficient CTL induction. Recent reports have shown that strong immune responses can be induced by dendritic cells transfected with RNA coding tumor-associated antigens using electroporation. However, the optimal condition of RNA transfection with electroporation is still controversial. We examined various conditions of RNA electroporation for dendritic cells to determine the optimal conditions for expression. We used EGFP RNA transcribed in vitro from pTNT/EGFP and pGEM4Z/EGFP/A64, and lacZ RNA transcribed in vitro from pTNT/lacZ and pGEM4Z/lacZ/A64. In vitro transcribed RNA was transfected into day-7 bone marrow derived dendritic cells of C57BL/6 mouse with electroporation. The 5x10e6 cells in 200 microl Opti-MEM (Invitrogen) were electroporated with RNA in 0.2-cm gapped cuvette. Multiple conditions of voltage, pulse length, number of pulse and RNA amount were examined ranging between 200-1000 V, 150-3000 microsec, 1-5 pulses and 0-50 microg, respectively. Effects of OK-432 or LPS on EGFP expression in transfected dendritic cell were also examined. Flow cytometry and X-gal staining showed the expression of EGFP and β-galactosidase in dendritic cells electroporated with EGFP and lacZ RNA transcribed in vitro, respectively. The EGFP expression was better with capped RNA than with RNA including β globin leader sequence or with uncapped RNA. The best efficiency with minor cell damage of electroporation was obtained under conditions of 300 V, 500 microsec and one pulse. The EGFP expression reached a plateau in DC transfected with 25 microg of capped of EGFP RNA. The EGFP expression was detected at 12 hr after electroporation, and was at its peak at 24 hr after electroporation. Stimulation with LPS or OK-432 was associated with increased EGFP expression. We demonstrated that RNA of interest could be efficiently transfected and expressed with electroporation only with well-examined specific conditions.

2. Mechanistic study on transplantation immunology

a. Analysis of tolerance induction following intra-thymic administration of allopeptide in HLA.TGM heterotopic heart transplant model.

Sumihito Tamura, Yoshifumi Beck, Hideaki Tahara

T cells of C3H.B51 recognize HLA-B*3501 molecules expressed on C3H.B35 as allo-MHC class I antigens and rejects vascularized C3H.B 35 grafts by cellular mechanism. (Ando and Beck et al., Transplantation 68, 904-908, 1999.) HLA-B*3501 derived peptide (CDLGPDGRLLR-GHDQSAYDGKDYIA designated hereafter as Peptide C) induces long-term heart graft survival in C3H.B35 into C3H.B51 cardiac transplantation model by intra-thymic injection. (Transplant proceedings, 30, 3890-3891, 1998). We considered that elucidating the mechanism underlying the allo-graft acceptance in this model may lead to a novel modality of allograft specific tolerance induction applicable to human clinical transplantation. Last year, the state known as "split tolerance" has been confirmed in the HLA.TGM model. In brief, T cell obtained from tolerant recipients in HLA.TGM heart transplantation model showed both proliferation and CTL generation following stimulation by C3H.B35 derived APC. The finding suggested the denial of deletion hypothesis based on MHC-allo-peptide molecular mimicry theory playing a primary role in graft acceptance in the model. On the other hand, preliminary adoptive transfer experiment with bulk splenocytes obtained from C3H.B51 donors receiving intrathymic administration of allo-peptide resulted in prolongation of graft survival, suggesting that cells with regulatory capability may be involved. In order to clarify the regulatory cell

population, adoptive transfer experiments were carried out. The study revealed CD4CD25 cells obtained from C3H.B51 mouse receiving intra-thymic inoculation of peptide C to be the most potent in inducing long term acceptance of HLA.B3501 bearing C3H.B35 heart graft in C3H. B35 into C3H.B51 heterotopic heart transplant model. CD4CD25 cells from naive C3H.B51 mouse or CD4CD25 cells from C3H.B51 mouse receiving intra-thymic inoculation of peptide E (a peptide derived from HLA.B3501 as peptide C but not tolerogenic) did not induce the acceptance of HLA.B3501 bearing C3H.B35 heart graft. Further more, adoptive transfer of CD4CD 25 cells obtained from C3H.B51 mouse receiving intra-thymic inoculation of peptide C to naive C 3H.B51 recipients resulted in acceptance of HLA.B3501 bearing C3H.B35 heart grafts but not major mismatch (A/J) third party allo-grafts. Similar results have been observed in preliminary studies with minor mismatch (CBA/N) graft. According to these findings we have come to believe that CD4CD25 regulatory T cell is the key player. Next, we proceeded to study the mechanism of allo-specific suppression by CD4 CD25. Addition of either CD4CD25 cells obtained from naive or C3H.B51 mouse receiving intra-thymic inoculation of allo-HLA.B3501 derived peptide (peptide C) reduced the proliferation of C3H.B35 skin graft primed C3H.B51 stimulated with C3H.B35 splenic DC. No difference in the level of suppression between the two, however, was found in this *in vitro* assay system. Since the suppressive effect following TCR specific activation of CD4CD25 has been reported as non-specific in vitro, our results were somewhat acceptable despite the in vivo observation. In order to explain the allo-specific suppression by CD4CD25 cells in vivo in our model, we moved on to investigate the so called "Civil Service Hypothesis", an idea that CD4CD 25 regulatory cells suppress the cellular effector's mechanism in close vicinity to target antigens. Graft infiltrating lymphocytes (GIL) were obtained and studied. Since CD25 is a component of IL2R, an element that can be expressed in any activated lymphocytes including CD8 CTL and activated B cells, we have used Foxp3 as a surrogated marker of regulatory CD4CD25 cells among GIL. Foxp3 is a gene encoding Scurfin, a Forkhead-winged-helix transcription factor. The current consensus is that the mRNA expression is predominantly restricted to CD4 CD25 with regulatory capacity. Infiltrating lymphocytes obtained on day 14 post transplantation from C3H.B35 graft heart engrafted into C3 H.B51 recipients receiving intrathymic inoculation of peptide C showed higher normalized Foxp3 mRNA levels by real time PCR compared to that obtained from C3H.B35 graft heart engrafted into naive C3H.B51 recipients. Based on the result we hypothesize that CD4CD25 regulatory cells generated by intra-thymic inoculation of allo-peptide exists in the vicinity to allo-heart graft, possibly playing a important role in allograft specific tolerance *in vivo*. Our future plan is to confirm the above hypothesis. Currently, the following studies are in progress. Evaluation of Foxp3 levels on day 28 post transplantation in GIL obtained from heart grafts engrafted into recipients receiving intra-thymic inoculation of HLA.B3501 allo-peptide C and evaluation of Foxp3 expression in GIL obtained from heart grafts engrafted into recipients receiving adoptive transfer of CD4CD25.

b. Association between gene-based single nucleotide polymorphisms and the side effects of immunosuppressive drugs in patients after renal transplantation

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Although immmunosuppressive drugs are necessary for the organ transplantation, a lot of severe side effects including fatal infectious diseases and cardiovascular disorders are caused by their use. Prograf (FK-506) and Cyclosporin (CYA) are essential immunosuppressive drugs and their doses are determined by measurement of blood concentrations. However, we often experience that one patient was suffering from side effects and the other was not, although their blood concentrations were the same level. It was considered that these differences might be caused by distinctions of each genetic background. To clarify this issue, we performed a genome-wide analysis of gene-based single nucleotide polymorphisms (SNPs) in 75 patients after renal transplantation in our hospital. SNPs of the genes encoding some signal transducing factors were strongly associated with cardiovascular disorders caused by immunosuppressive drugs. Subsequently, the functional analysis of these genes will be performed.

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免疫病態分野

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

I. β1 integrins and tetraspanin projects

Satoshi Iwata, Takahiro Sasaki, Akiko Souta-Kuribara, Hiroshi Kobayashi, Sayaka Nomura, Yasuyo Urasaki, Osamu Hosono, Hiroshi Kawasaki, and Chikao Morimoto

β1 integrins and their associating molecules, tetraspanins play crucial roles in a variety of cell processes such as adhesion, migration, proliferation, and differentiation of lymphocytes. Previously, we identified some of our established mAbs as tetraspanins, CD9 and CD82 by expression cDNA cloning. We showed that co-immobilized anti-β1 integrin mAbs or anti-tetraspanin mAbs (CD82 and CD9) with a sub-mitogenic dose of anti-CD3 mAb induced a marked increase of IL-2 secretion and proliferative response of T cells, indicating the role of β1 integrins and tetraspanins in T cell activation. Furthermore, we showed that the ligation of β1 integrins, CD9, or CD82 induces protein tyrosine phosphorylation of pp125FAK (focal adhesion kinase), paxillin, and pp105 in H9 cells as well as peripheral T cells. Pp105 was first described in our laboratory as a protein that is predominantly tyrosine phosphorylated by the ligation of β1 integrins in H9 cells. Recently, we have demonstrated that pp105 is a hematopoietic variant of p130Cas (Crk-associated substrate) by cDNA cloning, and thus designated Cas-L (Cas lymphocyte type). Cas-L is not only expressed in immune system, but also in neurons at their developmental stages. The expression of Cas-L in neurons is developmentally down-regulated, however, it re-appears in the neurons under pathological conditions such as brain ischemia. By two-hybrid screening, we have screened cDNA library of a HTLV-I-transformed T cell line (SLB-I) and fetal brain, and identified some of the Cas-L binding proteins, which turned out to be HTLV-I (human T-lymphotropic virus type I) Tax, Smad7, SMN (survival of motor neuron) and so on.

Our present projects aim at investigating the biological significance of interaction between

Cas-L and its binding proteins in vitro, and evaluating the clinical relevance of those protein -protein interactions in a variety of disorders, such as inflammatory diseases, malignancies, and neurological diseases, and characterizing immunological significance of CD9.

a. Cas-L associates with HTLV-I Tax and regulates its transactivation of NF-κB

Cas-L is a docking protein that is heavily tyrosine phosphorylated by the engagement of β1 integrins in T cells. In the present study, we attempted to evaluate the role of Cas-L in the pathophysiology of adult T cell leukemia (ATL).

Examination of clinical samples from ATL patients as well as HTLV-I-infected T cells lines demonstrated an elevation in protein expression and tyrosine phosphorylation of Cas-L in these cells. To investigate the role of Cas-L in the pathophysiology of ATL, we evaluated cell migration activity and protein tyrosine phosphorylation. We showed that Cas-L expression was markedly elevated through the induction of HTLV-I Tax in JPX-9 cells, with these cells showing marked motile behavior on FN-coated Transwell™ insert. To elucidate the biological roles of Cas-L in HTLV-I infected T cells, we next performed Yeast Two-Hybrid screening of cDNA library from an HTLV-I transformed T cell line, which resulted in the identification of Tax as a putative binding partner for Cas-L. Co-precipitation experiments revealed that the serine-rich region of Cas-L might serve as the binding site with the highest affinity for Tax, although several other domains also demonstrated binding capability. Meanwhile, co-localization study showed that Tax and Cas-L partly merged in the cytoplasm. Finally, we showed that exogenous Cas-L inhibited Tax-mediated transactivation of NF-κB, while that of TRE remained intact, hence indicating that Cas-L might specifically regulate Tax-NF-κB pathway.

Taken together, our results strongly suggest a key role for Cas-L in the pathophysiology of ATL.

b. Role of Cas-L in the pathophysiology of Rheumatoid Arthritis in *tax* transgenic mice and in humans

We investigate the role of Cas-L, a downstream signaling molecule of β1 integrins, in the pathophysiology of rheumatoid arthritis (RA). We analyzed human T lymphotropic virus type I (HTLV-I) *tax* transgenic mice as well as samples from human RA patients. Splenocytes from *tax* transgenic mice were cultured on mouse endothelial cell-covered Transwell inserts, and cells migrating through the endothelial monolayer were counted. Biochemical studies were performed to analyze the protein expression and tyrosine phosphorylation of Cas-L. Immunohistochemical analysis was performed to detect Cas -L positive cells that had infiltrated into the joints. Migratory activity of splenocytes from *tax* transgenic mice with arthritis (ATg) was much higher than that of *tax* transgenic mice without arthritis (NTg) and littermate control mice. The expression of Cas-L protein and its tyrosine phosphorylation were increased in ATg mice compared with NTg and control mice, and this was accompanied by enhanced autophosphorylation of Fyn and Lck. Immunohistochemical analysis demonstrated a large number of Cas-L-positive lymphocytes migrating into the affected joints. Furthermore, in human RA, Cas-L-positive lymphocytes were shown to infiltrate to the inflammatory lesions. These results strongly suggest that Cas-L plays an important role in the pathophysiology of RA.

c. Nedd9 protein, a Cas-L homologue, is upregulated and may remodel neurons after transient global ischemia.

Identification of an endogenous protein involved in self-repair after stroke in adult brain can potentially widen the therapeutic time window. Some of these proteins are primarily expressed during embryonic development and strongly downregulated during the early postnatal phase. Neuronal precursor cell-expressed, developmentally down-regulated gene (Nedd) 9 was recognized to be identical to Cas-L, a docking protein that associates with a variety of signaling molecules such as FAK, proline-rich tyrosine kinase 2, and Crk. We investigated the expression and function of Nedd9 protein in the pathogenesis of transient global ischemia in rats. We found that Nedd9 was a splicing variant of Cas-L and selectively induced in neurons of cerebral cortex and hippocampus 3 to 14 days after the ischemia. Induced Nedd9 protein was tyrosine phosphorylated and was bound to FAK in dendrite and soma of neurons after the ischemia. In physiological process, Nedd9 was transiently expressed in neurites and cell body of developing neurons in cerebral cortex and hippocampus during development. Nedd9 promoted neurite outgrowth of PC-12 cells in an NGF-independent manner. These results suggest that delayed upregulation of Nedd9 in neurons plays an important role in neuron remodeling after global ischemia in rats.

d. Preferential expression of CD9 on human $CD4^+CD45RA^+$ naive T cell population

Human $CD4^+$ T cells can be divided into reciprocal memory and naive T cell subsets based on their expression of CD45 isoforms and CD29 /integrin β1 subunit. To identify unique cell surface molecules on human T cells, a novel monoclonal antibody termed anti-5H9 has been established. Binding of anti-5H9 triggers a costimulatory response in human peripheral blood T cells. Retrovirus-mediated expression cloning has revealed that the antigen recognized by anti-5H9 is identical to CD9. Here we show that human CD9 is preferentially expressed on the $CD4^+CD45RA^+$ naive T cell subset, and that $CD9^+CD45RA^+$ T cells preferentially respond to the autoantigen β2-glycoprotein I, as compared to $CD9^-CD45RA^+$ T cells. Furthermore, anti-5H9 inhibits the response of T cells to the autoantigen β2-glycoprotein I but not to the recall antigen tetanus toxoid. These results suggest that CD9 may be involved in β2-glycoprotein I induced T cell proliferation.

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

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

Currently we are focusing on the molecular and structural basis for CD26 mediated T cell activation signaling and enhancement of memory T cell response by sCD26 and searching for the putative ligand which is closely involving CD26-mediated costimulation. Furthermore, we are focusing on the translational research of utilization of anti-CD26 mAb as well as recombinant soluble CD26 for malignant tumors, immune-mediated disorders and immune deficiency diseases.

a. Association of CD26 with CD45RA outside lipid rafts attenuates cord blood T-cell activation.

CD26 is a T-cell activation antigen that contains dipeptidyl peptidase IV activity and binds adenosine deaminase. Recent work showed that specialized membrane microdomains, also known as lipid rafts, play a key role in T-cell signaling. In this study, we investigate the role of CD26 in cord blood T-cell activation and signal transduction. We demonstrated that different expression levels of CD26 were observed between cord blood T cells (CBTCs) and peripheral T cells (PBTCs), and that CD26+CD45RA+ CBTCs were different as compared with CD26+ CD45RA+ PBTCs. Moreover, the comitogenic effect of CD26 was not as pronounced in CBTCs as in PBTCs. We also showed that CD26 crosslinking induced less phosphorylation of T-cell receptor signaling molecules Lck, ZAP-70, TCR-zeta, and LAT in CBTCs than in PBTCs. Furthermore, CD26 molecules associated with CD45RA molecules outside lipid rafts in CBTCs. Our results suggest that strong physical linkage of CD26 with CD45RA outside lipid rafts may be responsible for the attenuation of T-cell activation signaling through CD26, which may be responsible for immature immune response and the low incidence of severe graft-versus-host disease in cord blood transplantation.

b. CD26/dipeptidyl peptidase IV enhances expression of topoisomerase II alpha and

sensitivity to apoptosis induced by topoisomerase II inhibitors.

CD26/dipeptidyl peptidase IV (DPPIV) is a cell surface-bound ectopeptidase with important roles in T-cell activation and tumour biology. We show that CD26/DPPIV enhances sensitivity to apoptosis induced by the antineoplastic agents doxorubicin and etoposide. In particular, CD26/DPPIV presence is associated with increased susceptibility to the mitochondrial pathway of apoptosis, documented by enhanced cleavage of poly (ADP ribose) polymerase (PARP), caspase-3 and caspase-9, Bcl-xl, and Apaf-1, as well as increased expression of death receptor 5 (DR5). We also show that the caspase -9-specific inhibitor z-LEHD-fmk inhibits drug-mediated apoptosis, leading to decreased PARP and caspase-3 cleavage, and reduced DR5 expression. Importantly, through detailed studies that demonstrate the association between topoisomerase II alpha expression and DPPIV activity, our data provide further evidence of the key role played by CD26 in biological processes.

c. T-large granular lymphocyte lymphoproliferative disorder: expression of CD26 as a marker of clinically aggressive disease and characterization of marrow inhibition.

T-large granular lymphocyte lymphoproliferative disorder (T-LGL LPD) is an indolent disease characterized by prolonged cytopenia and the presence of circulating large granular lymphocytes in the patient's peripheral blood. Although the disease is commonly thought of as indolent, most patients eventually require therapy because of recurrent infections secondary to neutropenia as well as a need for frequent blood product transfusions. CD26 is a 110-kDa surface glycoprotein with an essential role in T-cell function, including being a marker of T-cell activation and a mediator of T-cell activating signals. We evaluated CD26 expression in T-LGL patients and correlate CD26 expression with clinical behaviour. In addition, we examined the potential mechanism of cytopenia that is associated with this disorder. Our findings suggest that CD26 is a marker of aggressive T-LGL LPD and that CD26-related signalling may be aberrant in T-LGL LPD. Furthermore, inhibition of granulocyte-macrophage colony-forming units may be mediated by CD8+ cells of T-LGL LPD patients and is major histocompatibility complex class I-restricted.

d. Effect of CD26/dipeptidyl peptidase IV on Jurkat sensitivity to G2/M arrest induced by topoisomerase II inhibitors.

CD26/dipeptidyl peptidase IV (DPPIV) is a surface antigen with multiple functions, including a role in T-cell activation and the development of certain human cancers. We previously demonstrated that CD26/DPPIV enhanced sensitivity of Jurkat cells to doxorubicin. We show that expression of CD26/DPPIV enhanced sensitivity of CD26 Jurkat transfectants to G(2)-M arrest mediated by the antineoplastic agent etoposide. The increased sensitivity to etoposide -induced G(2)-M arrest was associated with disruption of cell cycle-related events, including hyperphosphorylation of p34 (cdc2) kinase, change in cdc25C expression and phosphorylation, and alteration in cyclin B1 expression. CD 26/DPPIV-associated enhancement of doxorubicin and etoposide-induced G(2)-M arrest was also observed in serum-free media, suggesting an effect of CD26 on cell-derived processes rather than serum-derived factors. Importantly, our work elucidated a potential mechanism for the enhanced susceptibility of CD26-expressing Jurkat cells to the topoisomerase II inhibitors by demonstrating that CD26/DPPIV surface expression was associated with increased topoisomerase II alpha levels and enhanced enzyme activity. Besides being the first to show a functional association between the multifaceted molecule CD26 and the key cellular protein topoisomerase II alpha, our studies provide additional evidence of a potential role for CD26 in the treatment of selected malignancies.

III. Studies in regulation of gene expression and therapeutic targeting transcription machineries

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We are interested in the mechanism of eukaryotic gene expression and development of novel therapy and/or drug 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-1α. Moreover, we have been also working with various modulators of transcriptional regulation. In this line, studies in a nuclear protein HEXIM1 have provided a novel aspect of modulation of transcription.

a. Glucocorticoid receptor project

Glucocorticoid hormones are effective in controlling inflammation, but the mechanisms that confer this action are largely unknown. It has been shown that both positive and negative regulation of gene expression are necessary for this process. The genes whose activity is negatively modulated in the anti-inflammatory process code for several cytokines, adhesion molecules. Most of them do not carry a classical binding site for regulation by the glucocorticoid receptor (GR), but have instead regulatory sequences for transcription factors such as AP-1 or NF-κB. Considering various severe side effects of glucocorticoids, it may be pharmacologically important to dissociate these negative regulatory function of the GR from induction of metabolic enzymes, gene expression of which has 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 the strategy for identification of novel therapeutic strategy.

(i) Development of Dissociating Ligand for the Glucocorticoid Receptor

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

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.

(ii) Molecular biology of a novel protein HEXIM1

We have recently cloned the cDNA encoding a novel protein HEXIM1, expression of which is induced by treatment of vascular smooth muscle cells with a differentiation inducer hexamethylane bisacetamide. We showed that HEXIM1 is a nuclear protein and represses NF-κB-dependent transcription. Since NF-κB plays a pathological role in smooth muscle cell proliferation, our study will not only unveil pathogenesis of but also contribute to therapy of atherosclerotic vascular disorders. Moreover, we have also shown that HEXIM1 interacts with not only NF-κB but also other nuclear proteins including GR and PSF/p54nrb. Since HEXIM1 locks the GR within the nucleus into transcriptionally silencing complexes, clarification of underlying mechanism would provide a novel milieau for regulation of glucocorticoid hormone action.

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

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

Angiogenesis is regulated by a combination of variety factors including transcription factors. Recently, we have isolated cDNA encoding the novel protein IPAS which can squelch HIF-1α. Its tissue-specific expression argues the physiological role of transcriptional network for orchestrated regulation of angiogenesis. We are currently studying the molecular mechanism of the interaction between HIF-1α and IPAS. This negative regulator may also therapeutically applicable for treating a number of angiogenic disorders including cancer, diabetic retinopathy, and rheumatoid arthritis. Moreover, we have recently shown that IPAS is a splice variant of HIF-3α, and its mRNA expression is enhanced under hypoxic conditions. This conditional regulation of splicing is our current interest.

On the other hand, we have recently identified that HIF-1α function is regulated in a various fashion in certain physiological settings, which may be important of homeostatic control of tissue function. Especially, we have shown that in human peripheral T lymphocytes HIF-1α protein level is determined under hypoxic conditions via not only inhibition of degradation but also synthesis. Furthermore, HIF-1α protein synthesis requires ERK- and mTOR-dependent pathways. We are now identifying the molecular mechanism for such regulation of HIF-1α.

IV. Immunobiology and clinical applications of chemokines and their receptors

Hiroshi Kawasaki, and Chikao Morimoto (in collaboration with Katsuaki Sato, Takami Matsuyama, and Kouichi Hirai)

We have been pursuing the structure and

functional analysis of human chemokine · chemokine receptor system in order to clearly address their roles in innate and acquired immune system. Since the discovery of chemokine receptors as the HIV co-receptors, this area of immune mediators have drawn tremendous attention. We are in the process of therapeutically apply antibodies to chemokine receptors in a fully humanized fashion. Major target molecules at present are CCR-1, CCR-3 and IL-12.

a. An abortive ligand-induced activation of CCR 1-mediated downstream signaling event and a deficiency of CCR5 expression are associated with the hyporesponsiveness of human naive CD4+ T cells to CCL3 and CCL5.

Human memory CD4(+) T cells respond better to inflammatory CCLs/CC chemokines, CCL 3 and CCL5, than naive CD4(+) T cells. We analyzed the regulatory mechanism underlying this difference. Memory and naive CD4(+) T cells expressed similarly high levels of CCR1; however, CCR5 was only expressed in memory CD4(+) T cells at low levels. Experiments using mAbs to block chemokine receptors revealed that CCR1 functioned as a major receptor for the binding of CCL5 in memory and naive CD4(+) T cells as well as the ligand-induced chemotaxis in memory CD4(+) T cells. Stimulation of memory CD4(+) T cells with CCL5 activated protein tyrosine kinase-dependent cascades, which were significantly blocked by anti-CCR1 mAb, whereas this stimulation failed to induce these events in naive CD4(+) T cells. Intracellular expressions of regulator of G protein signaling 3 and 4 were only detected in naive CD4(+) T cells. Pretreatment of cell membrane fractions from memory and naive CD4(+) T cells with GTP-gamma S inhibited CCL5 binding, indicating the involvement of G proteins in the interaction of CCL5 and its receptor(s). In contrast, CCL5 enhanced the GTP binding to G(i alpha) and G(q alpha) in memory CD4(+) T cells, but not in naive CD4(+) T cells. Thus, a failure of the ligand-induced activation of CCR1-mediated downstream signaling event as well as a deficiency of CCR5 expression may be involved in the hyporesponsiveness of naive CD4(+) T cells to CCL3 and CCL5.

b. CC-chemokine receptor 3: a possible target in treatment of allergy-related corneal ulcer.

To determine the suppressive effects of antibodies (Abs) against CC-chemokine receptor (CCR)-1 and CCR-3 on eosinophil chemotaxis induced by culture supernatant from corneal keratocytes and by tears from severely allergic patients with corneal ulcer. Primary cultures of human corneal keratocytes were incubated with interleukin (IL)-4 (33.3 ng/mL) and tumor necrosis factor (TNF)-alpha (33.3 ng/mL) for 48 hours. In tear samples collected from five severely allergic patients and three nonallergic control subjects , eosinophils were immunostained for CCR. Next, eosinophils purified from peripheral blood were preincubated with or without anti-CCR-1 and anti-CCR-3 Abs before a Boyden chamber assay was conducted. Recombinant human (rh) eotaxin, rh-regulated on activation normal T-cell expressed and secreted (rh-RANTES), culture supernatant from human corneal keratocytes, and tear samples were used as chemoattractants. Eosinophils in tears from allergic patients expressed CCR-1 and -3 on their surfaces. Anti-CCR-1 and -3 Abs each inhibited eosinophil chemotaxis induced by rh-RANTES. Anti-CCR-3 Ab (but not anti-CCR-1 Ab) also inhibited eosinophil chemotaxis induced by rh-eotaxin. Anti-CCR-1 and -3 Abs, respectively, inhibited up to 75.2% and 94.6% of eosinophil chemotaxis induced by culture supernatant, as well as 27.8% and 74.5% of chemotaxis induced by tear samples. CONCLUSIONS: Anti-CCR-1 and -3 Abs inhibited eosinophil chemotaxis induced by culture supernatant from corneal keratocytes and tear samples from severely allergic patients. Anti-CCR-3 Ab was more effective than anti-CCR-1 Ab. Inhibition of CCR-3 on eosinophils may be a treatment for corneal ulcer in patients with ocular allergy.

c. Langerhans Cell-mediated Transferred Antigen-Loaded Dendritic Cells Initiate T Cell Activation

Evidence obtained from experiments on animal suggests that epidermal Langerhans cells (LCs) and dermal dendritic cells (DCs) play a crucial role in epicutaneous immune responses. However, the mechanism underlying the initiation of the epicutaneous immune response in humans remains obscure. To clarify the mechanism responsible for the initiation of an Ag-specific immune response in epidermis in the human system, we examined the role of the interplay between DCs and LCs, both derived from human peripheral blood (PB) monocytes, in vitro. DCs exhibited more potent expressions of the MHC product and costimulatory molecules than LCs. LCs were less effective for the internalization of exogenous Ag, and the activation of allogeneic and autologous Ag-specific T cells than DCs. DCs and LCs expressed different in chemokine receptor repertoire and respon-

siveness. LCs can transfer unprocessed Ag to DCs via cell to cell contact, and these trans-Ag-loaded DCs induced an Ag-specific T cell response. Thus, cross-priming between DCs and LCs is crucial for the initiation of epicutaneous immune responses.

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Advanced Clinical Research Center

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

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The purpose of the Division of Medical Data Processing Network System for the Research Hospital is to research and develop advanced system engineering methodology and computer technology suitable for the 21-th century type research hospital. The system is called "Infrastructure for Translational Research of Genome Based Medicine", which is expected to strongly support bi-directional translation between genome based life-science and clinical medicine. Our main research objectives are as follows;
-Construction of clinical knowledge database system for evidenced based medicine,
-Computer technologies suitable for genome based medicine,
-Data processing methodologies of advanced medical instrument, and
-Pattern recognition methodologies and control technologies of living human cells.

1. Research and development of information network system for Translational Research under the concept of Evidence Based Medicine

National cost for Japanese health now amounts up to 3000 billion yen per year. Medical accidents and insufficient or surplus medical cares cause serious social problems. These facts will strongly require translational research which means application of genome science to clinical medicine. For promotion of translational research, EBM (Evidence Based Medicine) is the most necessary method for assurance of clinical protocol. For realizing true EBM, it is necessary to research and develop the most appropriated genome science based clinical databases and their processing system.

Basic clinical database should be consist of the set of correct descriptions of clinical actions. An clinical action means 5w1h (when, where, who, whom, why, and how) and its quantitative result from the logical atomism point of view. These clinical databases, which are processed by the appropriate statistical method including data mining technology, reveal the most appropriate clinical protocol.

The research hospital has introduced clinical order entry system. New clinical information systems are to be introduced and EBM-oriented sever computer system is going to operate in the background.

Because the Research Hospital has many experiences in blood and immunity disease, our EBM database will be expected to give us much useful information about the mechanism of these diseases. We are constructing pathological knowledge database including genome, protein, and their relation, i.e. dynamic pathway database for pathological application of genome science, named "e-pathfinder", which include protocol management utilities for translational re-

search of genome based medicine.

2. Research and development of experimental system for cell therapy

Pattern recognition and control technologies of human cell and microscopic cell organs are very important for genomic sciences and clinical applications. New clinical protocol should be tested in human cell experimental system before its clinical application. We are researching to develop automatic cDNA (RNA, or protein) mechanical injection machine to human cell nucleus. This machine is expected also to be useful to inject variable drugs in human cells.

At first the computational pattern recognition method of human cell organs must be developed. We are successful to recognize the fluorescence pattern in cell organ over about 90% efficiency. This result will be applied to the automatic cDNA injection machine combined with flow metric equipment, which will process over 100000 human cells in a day and will be applied to cell therapy for many kinds of blood or immunity disease.

We are also constructing a cell observation system, which will enables us to monitor living human cells and track them continuously. The instrument will be expected to give us much pathological knowledge concerning living cell dynamism.

3. Research and development of bioinformatics simulation system to explain blood system differentiation and immunity mechanism

Many research projects started to study genomic disease in Japan. For the clinical application of these results, systematic studies are necessary how to integrate the genomic database and clinical data as well as new computer simulation algorithm. We are going to simulate blood differentiation and immunity mechanism.

At first, genomic database for blood differentiation pathway should be arranged. The next step is to collect concerning protein pathway database. At last, the differentiation process and immunity mechanism will be simulated by bioinformatics technology under development in this Institute. The results will be verified by automatic human cell processing system to be applied to the clinical protocol. In this integrated system consisting of mutually interconnected in- vivo, in- vitro, and in- silico simulation, many kind of blood and immunity disease will be explained. This infrastructure will be hoped to contribute much to national health programs of Japan.

We started hematological bioinformatics simulation of erythrocyte maturation. Using appropriate genome and protein interactive pathway data including many interactions between nucleus and mitochondria, we obtained good simulation result which will be expected to give us explanation of the pathological processes concerning hemoglobin accumulation in the erythrocyte. This system is gradually expanded to the cell society simulation, which enables interactions of many kinds of cell into consideration, and it is expected to explain the mechanism of blood and immunity diseases.

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