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. A novel maxizyme vector targeting a bcr-abl fusion gene induced specific cell death in Philadelphia chromosome-positive acute lymphoblastic leukemia.

Soda Y, Tani K, Bai Y, Tojo A, Asano S.

Patients with Philadelphia chromosome-positive (Ph) acute lymphoblastic leukemia (ALL) generally have a poor prognosis and would benefit from the development of new therapeutic approaches. We previously demonstrated that an allosterically controllable ribozyme, maxizyme (Mz), can induce apoptosis in chronic myelogenous leukemia (CML) cells. Ph-ALL cells harbor a bcrabl fusion gene (e1a2) encoding a 190-kDa fusion protein (p190) involved in disease pathogenesis. In this study, we have designed a Mz that specifically cleaves e1a2 mRNA and transduced this e1a2Mz into Ph-ALL cells using a third-generation lentiviral vector system. In 3 of 5 Ph-ALL cell lines, e1a2 Mz transduction resulted in a significant decrease in viability and increased cell apoptosis. We observed a decrease in e1a2 mRNA in all Ph -ALL cells transduced with e1a2Mz, and the e1a 2 mRNA level was higher in e1a2Mz-resistant cells than in e1a2Mz-sensitive cells. All samples of primary Ph-ALL cells tested showed e1a2Mz-induced growth inhibition and apoptosis. Importantly, e1a2Mz did not influence the colony formation of normal $CD34^+$ cord blood cells. These results indicate that e1a2Mz kills Ph-ALL cells specifically, suggesting that it may be used as a novel gene therapy strategy for Ph-ALL.

2. CD19-targeting liposomes containing imatinib efficiently kill Philadelphia chromosome-positive acute lymphoblastic leukemia cells.

Harata M, Soda Y, Tani K, Ooi J, Tojo A, Asano S.

Patients with Philadelphia chromosome-positive acute lymphoblastic leukemia (Ph-ALL) have poor prognosis despite intensive therapeutic intervention. Recently, imatinib, a BCR-ABL tyrosine kinase inhibitor, has been proven to be an effective treatment for Ph-ALL, but nearly all patients rapidly acquire resistance. High-dose imatinib administration might overcome this resistance; however, systemic toxicities would likely limit this approach. Therefore, a new de-

livery system allowing for the specific targeting of imatinib is urgently needed. Because almost all Ph-ALL cells express CD19 on their surface, we have developed an immunoliposome carrying anti-CD19 antibody (CD19-liposomes). The internalization efficiency of the CD19-liposomes approached 100% in all Ph-ALL cells but was very low in $CD19^-$ cells. The cytocidal effect of imatinib-encapsulated CD19-liposomes (imatinib -CD19-liposomes) on Ph-ALL cell lines and primary leukemia cells from patients with Ph-ALL was much greater than that of imatinib with or without control liposomes. Importantly, the imatinib-CD19-liposomes did not affect the colony formation of $CD34^+$ hematopoietic cells, even at inhibitory concentration of free imatinib. Taken together, these data clearly demonstrate that the imatinib-CD19-liposomes induced specific and efficient death of Ph-ALL cells. This new therapeutic approach might be a useful treatment for Ph-ALL with fewer side effects than free imatinib.

3. Leptomycin B overcomes imatinib resistance mediated by stromal cells and mutant BCR-ABL in Philadelphia chromosome-positive acute lymphoblastic leukemia cells.

Izawa K, Tojo A.

Philadelphia chromosome-positive acute lymphoblastic leukemia (Ph-ALL) readily acquires resistance to chemotherapeutic drugs including imatinib mesylate. We hypothesize that the adhesive interaction of Ph-ALL cells with bone marrow stromal cells might cause their escape from drug-induced apoptosis and subsequent minimal residual disease, resulting in the generation of a chemoresistant clone. To gain insight into this possibility and a novel strategy against imatinib resistance, we used two Ph-ALL cell lines designated as IMS-PhL1 (L1) and IMS-PhL2 (L2). L1 cells had wild type BCR-ABL, whereas L2 cells had Y253H mutant and revealed 10-fold or more resistance to imatinib, as compared with L1 cells. Both cell lines adhered to and migrated beneath the HESS-5 cell layer, resulting in the formation of cobblestone areas (CA). While floating L1 cells were eradicated by 1 μM imatinib, a portion of adherent L1 cells could survive even at 10 μM imatinib. Similarly, L2 cells forming CA beneath the HESS-5 cell layer considerably resisted prolonged exposure to 10 μM imatinib. Leptomycin B (LMB), a potent inhibitor of CRM1/exportin-1, can trap BCR -ABL in the nucleus and can aggressively eliminate BCR-ABL^+ cells in combination with imatinib. We tested LMB for its ability to eliminate CA or adherent Ph-ALL cells in combination with imatinb. Dramatically, combined use of 10 μM imatinib and 1 nM LMB for 7 days exerted a synergistic effect on reduction in the number of CA. L1 cells were also susceptible to the combination of imatinib and LMB. Our results suggest that nuclear entrapment of BCR-ABL may be a promising strategy for overcoming imatinib resistance mediated by stromal cells as well as a certain BCR-ABL mutant.

4. Potent receptor-mediated cytotoxicity of granulocyte colony-stimulating factor-Pseudomonas exotoxin, a fusion protein against myeloid leukemia cells.

Oshima Y, Tojo A.

A chimeric toxin in which the cell-surface binding domain of Pseudomonas exotoxin A was replaced with mature human granulocyte colony-stimulating factor (G-CSF) was produced in Escherichia coli, purified and tested for its biological activity on the human G-CSF-responsive myeloid leukemia cell line, UT7/GR. This fusion protein, termed G-CSF-PE40, showed potent cytotoxicity in the cell line in a dose-dependent manner. G-CSF-PE40 displaced binding of biotinylated G-CSF to its receptor, and the cytotoxicity of G-CSF-PE40 was neutralized by an excess of wild-type G-CSF, indicating the receptor-mediated effects of this chimeric toxin. When G-CSF-PE40 was injected into normal mice, they showed transient neutropenia but no significant changes in the numbers of red blood cells or platelets. Furthermore, G-CSF-PE 40 prolonged the survival of mice transplanted with syngeneic myeloid leukemia cells. These observations suggest that G-CSF-PE40 may be useful in targeted therapy of myeloid leukemia cells expressing G-CSF receptors.

5. Hierarchical Organization of Acute Myeloid Leukemia Characterized by Promoter Activity of the Human Telomerase Reverse Transcriptase Gene

Kobayashi S, Soda Y, Tojo A.

To gain insight into the hierarchical organization of AML, a lentiviral reporter assay, based on the expression of novel yellow fluorescent protein variant (Venus) using flow cytometry, was applied to the promoter activity of the human telomerase reverse transcriptase (hTERT) gene in primary AML cells. hTERT promoter activity correlated well with endogenous telomerase activity in experiments inducing apoptosis and differentiation of leukemia cell lines. AML

cells or purified cord blood (CB) $CD34^+$ cells transduced with hTERT-Venus (the lentiviral vector containing the expression cassette of the Venus gene driven by the hTERT promoter) were cultured in the presence or absence of colony-stimulating factors (CSF) mixtures for 48 hrs before analysis. In primary AML cases, hTERT promoter activity revealed considerable patient-to-patient variation, regardless of stimulation by CSF, but its distribution appeared as a single peak or two narrowly separated peaks, suggesting that hTERT expression occurs along a continuous gradient in the leukemia cell population. The mean promoter activity of the 19 AML cells was 2-fold higher than that of CB CD 34^+ cells without CSF, but almost equal to that with CSF. In four cases, the MFI was not responsive to CSF stimulation, and in three cases, the basal and stimulated MFI were prominent. In contrast, three lots of CB $CD34^+$ cells uniformly revealed very low activity (MFI) without stimulation (mean = 1.2), but 5-fold up-regulation with stimulation (mean = 5.8). Next, hTERT-Venus transduced leukemia cells were maintained with CSF mixtures, and Venus expression was analyzed using flow cytometry at Days 2, 6, and 10. Both the viable cell population and its hTERT promoter activity increased at Day 6. The viable cell population then decreased, but the fluorescence intensity of this fraction still increased markedly at Day 10. This might be due to the gradual disappearance of the cell fraction with lower hTERT promoter activity, which suggests that leukemia cells with higher telomerase activity survive in a mass population. In conclusion, we presented here novel evidence for a hierarchy of telomerase activity, deduced from hTERT promoter activity, among individual leukemia cells in AML.

6. Hematopoietic activity of common marmoset CD34 cells isolated by a novel monoclonal antibody MA24.

Izawa K, Tani K, Soda Y, Tojo A.

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 to 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. Multilineage differentiation of marmoset $CD34^+$ cells in NOD/SCID mice was confirmed by flow cytometry 1 month after xenotransplantation. These results demonstrated that MA24 is useful for the analysis and enrichment of hematopoietic progenitor cells in the marmoset model for preclinical experiments.

7. Anti-NK cell treatment induces stable mixed chimerism in MHC-mismatched, T cell-depleted, nonmyeloablative bone marrow transplantation.

Cho SG, Soda Y, Izawa K, Tojo A.

To clarify natural killer (NK) cell-mediated resistance under cytoreductive conditioning and T cell-depleted bone marrow transplantation, we investigated the effects of host NK cell depletion on engraftment and induction of stable mixed chimerism. BALB/c mice (H-2kd) were injected intraperitoneally with anti-asialoGM1 antibody (anti-NK Ab) on day-1. On day 0, they received total body irradiation (TBI) at a dose of 500 cGy, followed by intravenous infusion of 2×10^7 T cell-depleted (TCD) bone marrow cells from C57 BL/6 mice (H-2kb). Early engraftment and chimerism were determined by the relative ratio of peripheral blood (PB) lymphocytes expressing either H-2kd or H-2kb on day +21. Long-term engraftment and chimerism were evaluated on PB and spleen by multicolor flow cytometry. Although no recipients treated with TBI alone showed engraftment, all the recipients conditioned with anti-NK Ab and TBI showed successful engraftment as well as a donor-dominant pattern of mixed chimerism in both PB and spleen. Spleen cells from recipients with mixed chimerism showed specific tolerance to both host and donor strains, but not to a third party (C3H/He). None of the reconstituted mice showed signs of graft vs host disease, and all survived up to day +330. These observations indicate that host NK cell depletion may be used to reduce the intensity of conditioning regimens for engraftment of TCD grafts, and can contribute to establishment of stable mixed chimerism in major histocompatibility complex-mismatched nonmyeloablative transplantation.

8. A mechanism of transcriptional regulation of the CSF-1 gene by interferon-gamma.

Tsuchimoto D, Tojo A.

Interferon (IFN)-gamma exerts multiple functions including antiviral, anti-proliferative and immunomodulatory activities, which are mediated through the JAK-STAT pathway. We observed that IFN-gamma significantly increases the production of colony stimulating factor-1 (CSF-1) by a human lung carcinoma cell line, A 549. To gain insight into this mechanism, we determined the unknown nucleotide sequences of 5'-flanking region of human CSF-1 gene. About 60 bases upstream of the transcription start site of the CSF-1 gene contains a possible gamma interferon activated site (GAS), TTCCCATAA. The promoter assay and the electrophoretic mobility gel shift assay revealed that IFN-gamma stimulates transcription of the CSF-1 gene through this sequence, which binds STAT1.

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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 in the control of cell division and differentiation, (4) molecular mechanism of leukemogenesis, (5) identification of self-renewal factor for embryonic stem cells, (6) characterization of hematopoietic stem cells, (7) molecular mechanism regulating hematopoiesis, and molecular targeted therapy of leukemia and cancer.

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], and Toshio Kitamura: [4]Heinrich-Pette Institute, Hamburg University

We previously developed a MuLV-derived efficient retroviral 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 effi-

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

Toshiyuki Kawashima[3], Yukinori Minoshima, Koichi Hirose[3], Yukio Tonozuka, Takaya Satoh[5], Yoshito Kajiro[6], Hideaki Nakajima, Tetsuya Nosaka[3], and Toshio Kitamura: [5]Kobe University, [6]Kyoto University.

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

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], Tetsuya Nosaka[3], and Toshio Kitamura:

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 corresponding 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, Atsushi Urano, Fumi Shibata, Tetsuya Nosaka[3], H Nomura[6], and Toshio Kitamura: [6]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. 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 Kogan1[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/EBPε 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

9. 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.

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Advanced Clinical Research Center

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

1. Efficient antigen presentation to HIV-1 specific CD8T cells using epitope-linked β2m and TAP inhibitor..

A. Kawana-Tachikawa et al.

In an effort to manufacture an effective vector for immuno-gene therapy, we previously reported Sendai virus vector (SeV) to stimulate HIV-1-specific CD8T cells. When an A24-restricted HIV-1 CTL epitope linked to the beta 2 microglobulin (β2m) was expressed in A24-positive LCLs by SeV, the epitope was presented as MHC class I complex and recognized by a specific CTL clone. To minimize the rejection of antigen presenting cells due to vector-derived antigens and also to lower the presentation of self antigens, we now report a SeV which expresses both the epitope-linked β2m and a TAP inhibitor, ICP47 derived from Herpes simplex virus type 1 (HSV-1). ICP47 down-regulates MHC class I molecules on the cell surface by inhibiting peptide transport into rough endoplasmic reticulum (ER) via TAP. Since the expression of epitope-linked β2m in ER should be TAP independent, high expression of MHC class I molecules presenting epitope-linked b2m would be expected. We show that ICP47 encoded by SeV actually down-regulated MHC class I molecules on the cell surface, while epitope-linked β2m could be expressed as MHC class I complexes and recognized by a specific CTL clone. This stealth SeV may prove to be a useful tool for immuno-gene therapy.

2. Gene Transfer to Dendritic Cells

N. Hosoya et al.

Immuno-gene therapy using dendritic cells (DCs) can be applied to HIV-1 infection. We examined gene transfer efficiencies of Sendai virus vectors (SeVs) and adenovirus vectors (AdVs). We prepared SeVs with the wild-type F protein, SeVs deficient of the F-protein, or AdVs deficient of E1 and E3. They carried a green fluorescent protein gene and were named F (−) SeVGFP, F (+) SeVGFP, or AdVGFP, respectively. We infected DCs that were obtained from human monocytes cultured with IL-4 and GM-CSF for 7 days with one of those vectors at various multiplicity of infection (moi) and cultured

for 24-72hrs. GFP expression and viability were quantified by FACS analysis. DCs infected with either F (−) SeVGFP or F (+) SeVGFP showed the maximum expression at moi of 2 and viablity over 80%. DCs infected with AdVGFP showed the maximum expression at moi 1000 and viablity over 80% even at moi of 1000. The maximum GFP expression was observed 48hrs after infection with AdVGFP and 24hrs after infection with both F (−) and F (+) SeVGFP. DCs were also infected with a SeV or AdV encoding HIV-1 *gag* and *env* genes, and the expression of each gene was quantified by western blot analysis. Env protein expression with the SeV was 3.8 times more than that with the AdV. Both SeV and AdV infections upregulated the expression levels of CD83, CD86, CD40, MHC class I, and MHC class II on the DC surface. Although the SeV showed cytopathic effect on DCs a little more than the AdV, it expressed exogenous genes (either GFP or HIV-1 genes) earlier at much lower moi. Although further studies are required to investigate the changes of cell surface markers and their abilities to induce CTL, SeVs are good candidates for immunotherapy of HIV-1 infection.

3. HIV vectors

R. 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; this parental prototypic vector was HXN. Insertion of a fragment containing cPPT, either 282bp or 178bp long, into HXN resulted in good production vector particles comparable to HXN. Insertion of the longer fragment resulted in decrease in transduction efficiency in about eightfold, whereas insertion of the shorter resulted in a slight increase in transduction efficiency independently of the orientation of the insert. The disparity of these two fragments was the 104bp region just upstream of the cPPT and was named dZ. Transfection of 293 cells with DNA of HXN carrying dZ did not result in infectious vector particles. We found that the released particles contained no vector genomic RNA . Furthermore, we found that HXN-dZ RNA was not present in cytoplasm but nucleus. This implies that the dZ sequence on RNA is involved in RNA tansport to cytoplasm.

4. Diagnosis of Respiratory Infections by LAMP and RT-LAMP.

I. Miyata et al. et al.

Although many pathogens such as influenzavirus cause acute respiratory infections and pneumonia, few of those infections have been diagnosed for their causative pathogens. To develop a rapid and specific method for identifying pathogens causing acute respiratory infections, we employed LAMP™ (Eiken Chemical), a method for amplifying DNA at 60-65℃ only for 5-30 min. For RNA viruses, we converted RNA to DNA with reverse transcriptase and amplified it by LAMP™ (RT-LAMP™). Our current target pathogens include SARS coronavirus, influenzaviruses, mycoplasmas, and so forth. As LAMP™ requires four primers as a set for amplifying one specific region of DNA, we prepared several sets of primers for several regions in target pathogen genomes, which were chosen in a bioinfomatic fashion. Among those primer sets, we found some sets were eligible for RT-LAMP/LAMP™ and others were not. Our goal is to get a large assortment of primer sets lined-up for identifying pathogens.

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Advanced Clinical Research Center

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Our department has a major goal in basic research that is development of innovative cancer therapy using immunologic approaches and gene therapy strategies.

Development of innovative cancer therapy for gene therapy strategies

a. Essential roles of type-1 helper T-cells in anti-tumor effects of IL-23

Teruo Kaiga, Marimo Sato, Hideaki Tahara

Appropriate induction of the T-helper 1 (Th1) immune response plays significant roles in the induction of potent anti-tumor immunity. Interleukin (IL)-23, a recently discovered cytokine, which is composed of the IL-12 p40 subunit and the IL-23-specific p19 subunit, has been shown to preferentially act on Th1 effecter/memory CD 4^+ T cells and induce their proliferation and interferon (IFN)-γ production. Thus, IL-23 might be useful in promoting therapeutic anti-tumor immune responses. In this study, we examined the extent and the mechanism of anti-tumor effects of IL-23 using systemic administration of IL-23 in mouse tumor systems and compared with those of IL-12, which has structural and functional similarities with IL-23. High concentration of IL-23 protein and IL-12 protein in systemic blood stream were achieved using in vivo electroporation (IVE) of expression plasmid DNA into the pre-tibial muscles of mice. We treated C57BL/6 mice bearing established MCA 205 fibrosarcoma or B16F10 melanoma with IVE of IL-23 plasmid DNA, and compared with the treatment using IVE of IL-12 plasmid DNA or EGFP plasmid DNA. The IL-23 treatment resulted in significant suppression of the growth of MCA205 tumors and B16F10 tumors respectively compared with the EGFP treatment. Although the therapeutic outcomes were similar to those with the IL-12 treatment, the IL-23 treatment was associated with characteristic immune responses. These include very low serum IFN-γ concentration during the treatment, significantly increased IFN-γ response from $CD4^+$ T cells in the lymph nodes on day 20 or 30 after tumor inoculation, and the critical need for $CD4^+$ T cells in anti-tumor effects in vivo or in vitro. Moreover, anti-tumor activity of IL-23 treatment was completely abrogated with $CD4^+$ depletion. These results strongly suggest that the IL-23 has potent anti-tumor activity mediated by characteristic Th1 immune responses significantly different from those of IL-12.

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

Dendritic cells (DCs) are potent antigen-presenting cells which play pivotal roles in immunological response. The clinical application of DCs induced from peripheral monocytes in vitro has been initiated as a promising immunological therapy against cancer. If the same type of immuno-stimulator could be achieved without in vitro manipulation, it might be very convenient in clinical settings. In this study, we performed systemic gene transfer of Flt3L using in vivo electroporation of Flt3L plasmid DNA (Flt3 L-IVE) in pretibial muscles in order to determine the effects on DCs in situ. After Flt3L-IVE, Flt3L was detected in the serum for 10 days after IVE at significant levels. The peak concentration of 5326±920 pg/ml in the serum was observed 4 days after Flt3L-IVE. The number of DCs was significantly increased and showed highly co-stimulatory molecules expressions both in spleen and bone marrow after Flt3L-IVE compared to those of control groups. Immunohistochemical evaluation revealed that not only DCs but also CD8 and CD4 positive cells were significantly infiltrated into the local tumor site compared with those of control and remained in the tumor 21 days after a single Flt3L-IVE. However, anti-tumor effects of Flt3L-IVE were not significant in MCA205 established tumor. When the local tumor environment was examined using immunohistochemical staining, the number of DCs in tumor was significantly higher when compared with that of controls. However, most of the tumor infiltrating DCs had immature phenotype. Only the small number of DCs in the peripheral areas had mature phenotype. These results suggest that Flt3L gene transfer using in vivo electroporation could mobilize DCs into tumor site. Additional means to induce maturation of these DCs could have positive impact on anti-tumor effects of this strategy.

c. Induction of systemic anti-tumor immunity using intra-tumor administration of adenoviral vector expressing biologically active IL-18 in combination with Flt3-Ligand

Takuya Takayama, Tetsuya Saitoh, Shigenori Nagai, Hideaki Tahara

We have reported that interleukin (IL)-18 has potent antitumor effects mediated by CD4+ T cells and NK cells, but in IFN-γ and IL-12-independent pathways. However, we also found that it is hard to induce systemic anti-tumor immunity by IL-18 treatment alone. 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 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 in combination with Flt3-Ligand. Substantial anti-tumor effects were observed when established MCA205 fibrosarcoma was treated in syngeneic immunocompetent mice with intratumoral injection of Ad. PTH. IL-18. Interestingly, the anti-tumor effects was also observed on distant tumors inoculated in the contralateral flank of the animal by this combination therapy. This study suggests that intratumoral administration of an adenoviral vector expressing biologically active murine IL-18 in combination with Flt3-Ligand could be a new strategy of gene therapy in the clinical setting to treat patients with cancer.

Development of innovative cancer therapy for immunologic approaches

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

Hiroyuki Mushiake, Takuya Tsunoda, Hideaki Tahara

Except in the early stages of and in certain types of cancer, we have not been satisfied with the outcome of anticancer therapy. To overcome this situation, new strategies for cancer treatment are required. One of them, chemoimmunotherapy has been advocated as a modality of combination chemotherapy and immunotherapy since the 1970's. Recent attention has been paid to their combinatorial effect on the host immunological response. Actually, some cytokines (IL-2, GM-CSF, IFN-α and IFN-β, etc) or biochemical response modifiers (BRM; BCG, LNT, etc.) have been used clinically, and these have advantages in eliciting antitumor-immunological response. Especially, the elucidation of immunological molecular mechanism has provided us strategies to utilize them as a weapon to treat cancer patients. When addressing the priming immune response, we could not disregard the role of dendritic cells (DCs), professional antigen presenting cells (APC). In immune response to tumors, immature DCs acquire tumor antigens in tumor tissues, process the antigens and then migrate into regional lymph nodes. They differentiate into mature DCs and present the tumor antigens on their surface MHC class I and class

II molecules to prime naive T cells[5]. The activated T cells show specific cytotoxic activity to the target cells and mediate tumor destruction.

In this study, we demonstrated that chemoimmunotherapy using S-1, a novel oral fluoropyrimidine anti-cancer drug, combined with lentinan (LNT) a β (1→3) glucan was effective *in vivo* and clarified the augmentation of the function of the dendritic cells *in vivo* and *in vitro*. The survival period of Colon-26 bearing mice treated with S-1 + LNT was significantly prolonged than those treated with S-1 alone ($P < 0.05$). On the other hand, LNT did not prolong the survival periods when combined with S-1 in Colon-26 bearing athymic mice. The frequency of CD86+ dendritic cells (DCs) infiltrated into Colon-26 was increased in mice treated with S-1 + LNT, and splenic DCs harvested from treated with S-1 + LNT showed more potent T cell proliferation activity than that with S-1 alone ($P < 0.05$). Furthermore, the activity of cytotoxic T lymphocytes (CTLs) in splenocytes of S-1 + LNT treated mice was specific and more potent than those treated with S-1 alone ($P < 0.05$). These results suggest that modulation of specific immunity with LNT has a significant role in the enhanced anti-tumor effects through the modification of DC function. We demonstrated that DCs might play an important role in chemotherapy, and the combination therapy of S-1 and LNT presents a promising chemoimmunotherapy, which might lead to better survival for cancer patients.

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

Naotaka Uchida, Takuya Tsunoda, Satoshi Wada, Yoichi Furukawa, Yusuke Nakamura, Hideaki Tahara

It has been demonstrated that $CD8^+$ cytotoxic T lymphocytes (CTLs) recognize epitope peptides derived from tumour-associated antigens (TAAs) presented on MHC Class I molecules and then lyse the tumor cells. After the discovery of MAGE family as the first example of TAAs, other TAAs have been discovered using similar immunological approaches. Although the significant progress has been made in the development of cancer vaccine using specific epitope peptides, usefulness of this strategy is still greatly hampered by the fact that limited numbers of TAAs are currently available for the treatment of cancer patients. Although new TAAs have been successfully identified after the extensive efforts using these measures, one might need to admit that useful new TAAs are still in need for clinical application. Development of cDNA microarray technologies, coupled with genome information, has enabled us to obtain comprehensive profiles of gene expressions of malignant cells comparing with those of normal cells. This approach discloses the complex nature of cancer cells and leads to identification of genes of which expression patterns are different in tumors when compared with those of non-transformed cells. Since TAAs should theoretically be expressed excessively and preferentially by the tumor cells but not by the normal tissues, gene expression profiling using cDNA microarray technologies is useful to identify TAAs. We analyzed the expression profiles of the newly identified genes with a genome-wide cDNA microarray technology, selected TAA candidates from these genes using the information, and examined whether they contain antigenic T-cell epitope peptides to prove that they are indeed TAAs.

In our preceding study, we identified multiple new genes using gene expression profiling with a genome-wild cDNA microarray containing 23040 genes. Among them, we selected RNF43 (Ring Finger Protein 43) as a promising candidate for a TAA expressed by colon cancer cells. We examined in this study whether the RNF43 protein contains antigenic epitope peptides restricted to HLA-A*0201 or HLA-A*2402. The CTL clones were successfully induced with stimulation using the peptides binding to HLA-A*0201 (ALWPWLLMA and ALWPWLLMAT) and HLA-A*2402 (NSQPVWLCL), and these CTL clones showed the cytotoxic activity specific to not only the peptide-pulsed targets but also the tumor cells expressing RNF43 and respective HLAs. Lytic activities mediated by two HLA-A2-restricted epitopes were marginal, whereas tumor lysis mediated by the HLA-A24 epitope was clearly better. These findings might be caused by the poor natural presentation of RNF43-11 (IX) and RNF43-11 (X) by tumors or poor TCR avidity for these specific epitopes. These results strongly suggest that RNF43 is a new TAA of colon cancer. Furthermore, these results also suggest that our strategy might be a promising one to efficiently discover clinically useful TAAs.

c. Development for anti-angiogenic cancer therapy with vaccination using epitope peptides derived from human vascular endothelial growth factor receptor 2 (VEGFR 2)

Satoshi Wada, Takuya Tsunoda, Toshiyuki Baba, F. James Primus, Masabumi Shibuya, Hideaki Tahara

Angiogenesis has been shown to be a critical mechanism for tumor progression. Multiple studies have suggested that tumor growth can be suppressed if tumor angiogenesis can be inhibited using various types of anti-angiogenic agents. Recent studies in mouse systems have shown tumor-angiogenesis can also be inhibited if cellular immune response could be induced against vascular endothelial growth factor receptor2 (VEGFR2), which has been shown to be one of the key factors in tumor angiogenesis. In this study, we examined the possibility of developing this novel immunotherapy in clinical setting. We first identified the epitope peptides of VEGFR2 and showed that stimulation using these peptides induces CTLs with potent cytotoxicity in the HLA class I restricted fashion against not only peptide-pulsed target cells but also endothelial cells endogenously expressing VEGFR2. In A2/Kb transgenic mice which express α1 and α2 domain of human HLA-A*0201, vaccination using these epitope peptides *in vivo* was associated with significant suppression of the tumor growth and prolongation of the animal survival without any adverse effects. In anti-angiogenesis assay, tumor-induced angiogenesis was significantly suppressed with vaccination using these epitope peptides. Furthermore, CTLs specific to the epitope peptides were successfully induced in cancer patients, and the specificities of the CTLs were confirmed using functional and HLA-tetramer analysis. These results *in vitro* and *in vivo* strongly suggest that the epitope peptides derived from VEGFR2 could be used as the agents for anti-angiogenic immune-therapy against cancer in clinical settings.

d. *In vivo* anti-target response and immunological monitoring of stage IV melanoma patients treated with peptide-based vaccine using HLA-A*2402-restricted gp100 peptide

Toshiyuki Baba, Takuya Tsunoda, Satoshi Wada, Juichiro Konishi, Hiroaki Tanaka, Syogo Nakano, Hiroyuki Mushiake, Takuya Takayama, Fumiaki Beck, Yutaka Kawakami, Hideaki Tahara

A phase I clinical trial has been performed using epitope peptides derived from gp100; gp100-in4 (VYFFLPDHL) for HLA-A*2402-positive patients, to evaluate the toxicity, and the clinical and immunological responses. Six patients positive for HLA-A*2402 with stage IV melanoma were immunized with a vaccine consisting of the epitope peptide emulsified with incomplete Freund's adjuvant (IFA). No toxicity greater than grade I in the vaccinated lesion was observed. One patient showed the mixed response (a partial regression of liver metastases) and decrease of tumor marker after the vaccination. Furthermore vitiligo was observed after the vaccination in two patients. Specific cytotoxic T lymphocytes (CTLs) response and HLA-tetramer analysis were examined for peripheral blood mononuclear cells (PBMCs) from these patients for the immunological monitoring *in vitro*. PBMCs from the patients, who showed clinical and *in vivo* immunological response after vaccination, produced significant amounts of IFN-g on stimulation with gp100-in4. In gp100-in4-stimulated PBMCs obtained from patients with vitiligo, the positive subsets for both CD8 and HLA-A*2402/gp100-in4 tetramer were detected, furthermore gp100-specific CTL clones were established. These CTL clones lysed melanoma cell line endogenously expressing gp100 with HLA-restricted fashion. In conclusion, our data suggested that peptide-based vaccine using gp100-in4 was safe, well-tolerated, and this peptide might be immunogenic *in vivo*. Gp100-in4-specific CTLs could be detected with the immunological monitoring using the PBMCs of patients.

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

I. β1 integrins and tetraspanin projects

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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 its ligand or anti-tetraspanin mAbs (CD82 and CD9) with a submitogenic dose of anti-CD3 mAb induced a marked increase of IL-2 secretion and proliferative response of T cells, indicating the role of β1 integrins 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 (Crk-associated substrate 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 (survivor of motor neuron disease) 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.

a. HTLV-I Tax induces and associates with Crk-associated substrate lymphocyte type (Cas-L)

Crk-associated substrate lymphocyte type (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 PBMCs from ATL patients as well as ATL-derived T cell lines showed an elevation of Cas-L in these cells. We showed that tyrosine phosphorylation as well as expression of Cas-L was markedly elevated through the induction of human T-lymphotropic virus type I (HTLV-I) Tax in JPX-9 cells, with these cells showing marked motile behavior on the ligands for integrins. 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. 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 nuclear factor κB (NF-κB), while Tax-independent activation of NF-κB remained intact, hence indicating that Cas-L might specifically regulate Tax -NF-κB pathway.

b. NEDD9, a Cas-L homologue, is upregulated and involved in the differentiation of neurons after transient global ischemia in rats.

Some proteins involved in self-repair after stroke in adult brain 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 Crk-associated substrate lymphocyte type (Cas-L), a docking protein that associates with a variety of signaling molecules such as focal adhesion kinase (FAK), proline-rich tyrosine kinase 2 (Pyk2), and Crk. We investigated the involvement of these proteins in the pathophysiology of global cerebral ischemia. We found that Nedd9 was a splicing variant of Cas-L and selectively induced in neurons of cerebral cortex and hippocampus 1 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. Finally, it was demonstrated that Nedd9 promoted neurite outgrowth of PC-12 cells. Our study may support the potential of Nedd9 for participation in the differentiation of neurons after global ischemia in rats.

c. Crk-associated substrate lymphocyte type associates with Smad6 and Smad7

Crk-associated substrate lymphocyte type (Cas-L) is a docking protein that is heavily tyrosine phosphorylated by the engagement of β1 integrins in T cells. Cas-L consists of multifunctional domains that are involved in protein-protein interaction. To identify the Cas-L binding proteins, we performed yeast two-hybrid screening of cDNA library from a human T cell line (SLB-I), which resulted in the identification of Smad7 as a putative binding partner for Cas-L.

Smad7 is an inhibitory Smad (I-Smads) that represses signaling by cytokines of the transforming growth factor-β (TGF-β) superfamily. Co-precipitation experiments revealed that Smad2, Smad3, Smad6, Smad7 associated with Cas-L, whereas Smad4 failed to do so. It was shown that the Cas-L binding site in Smad7 was c-terminal half. On the other hands, domain deletion of SH3, SD (substrate domain), SR (serine-rich region) or c-terminal half of Cas-L abrogated the binding ability with Smad6 and Smad 7. Co-localization study showed that Smad6 as well as Smad7 partly merged with Cas-L in the cytoplasm. Finally, we found that overexpression of Cas-L restored Smad6 and Smad7-mediated transcriptional inhibition of SBE (Smad binding element)-Luc, and p3TP-Lux, both of which are under the control of the complex of common mediator Smads (e.g. Smad4) and receptor-regulated Smads (e.g. Smad2 and Smad3), hence indicating that Cas-L may regulate TGF-β signaling pathway through the interaction with I-Smads (Smad6 and Smad7).

d. The tetraspanin CD9 is preferentially expressed on human $CD4^+$ $CD45RA^+$ naive T cell population and is involved in T cell activation

Human $CD4^+$ T cells can be divided into reciprocal memory and naive T cell subsets based on their expression of CD45 isoforms and CD 29/integrin beta1 subunit. To identify unique

cell surface molecules on human T cells, we developed a new monoclonal antibody termed anti -5H9. 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 the tetraspanin CD9. We now 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 recombinant $beta_2$-glycoprotein I, as compared to $CD9^-CD45RA^+$ T cells. Furthermore, anti-5H9 inhibits both the recombinant $beta_2$-glycoprotein I-and the recall antigen tetanus toxoid-specific T cell proliferation. These results suggest that the tetraspanin CD9 plays an important role in T cell activation.

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 and structural basis for CD26 mediated T cell activation signaling and enhancement of memory T cell response by sCD26. Moreover, since the putative ligand for CD26 mediated costimulation has not yet been established, we are searching for its ligand directly involved in CD26-mediated T crell costimulation. Furthermore , we are focusing on the translational research of utilization of anti-CD26 mAb as well as recombinant soluble CD26 for treatment of malignant tumors, immune-mediated disorders and immune deficiency diseases.

a. CD26 up-regulates expression of CD86 on antigen-presenting cells by means of caveolin-1.

CD26 is a T cell costimulatory molecule with dipeptidyl peptidase IV activity in its extracellular region. We previously reported that recombinant soluble CD26 enhanced T cell proliferation induced by the recall antigen, tetanus toxoid (TT). However, the mechanism involved in this enhancement is not yet elucidated. We now demonstrate that CD26 binds caveolin-1 on antigen-presenting cells, and that residues 201-211 of CD26 along with the serine catalytic site at residue 630 contribute to binding to caveolin-1 scaffolding domain. In addition, after CD26-caveolin-1 interaction on TT-loaded monocytes, caveolin-1 is phosphorylated, which links to activate NF-kappaB, followed by up-regulation of CD86. Finally, reduced caveolin-1 expression on monocytes inhibits CD26-mediated CD86 up-regulation and abrogates CD26 effect on TT-induced T cell proliferation. Taken together, these results strongly suggest that CD26-caveolin-1 interaction plays a role in the up-regulation of CD86 on TT-loaded monocytes and subsequent engagement with CD28 on T cells, leading to antigen-specific T cell activation.

b. Regulation of p38 phosphorylation and topoisomerase II alpha expression in the B -cell lymphoma line Jiyoye by CD26/ dipeptidyl peptidase IV (DPPIV), associated with enhanced in vitro and in vivo sensitivity to doxorubicin

CD26 is a M_r 110,000 surface-bound glycoprotein with diverse functional properties, including having a key role in normal T-cell physiology and the development of certain cancers. In this paper, we show that surface expression of CD26, especially its intrinsic dipeptidyl peptidase IV (DPPIV) enzyme activity, results in enhanced topoisomerase II alpha level in the B-cell line Jiyoye and subsequent *in vitro* sensitivity to doxorubicin-induced apoptosis. In addition, we demonstrate that expression of CD26/DPPIV is associated with increased phosphorylation of p 38 and its upstream regulators MKK3/6 and ASK1, and that p38 signaling pathway plays a role in the regulation of topoisomerase II alpha expression. Besides demonstrating that CD26 effect on topoisomerase II alpha and doxorubicin sensitivity is applicable to cell lines of both B and T-cell lineages, the potential clinical implication of our work lies with the fact that we now show for the first time our *in vitro* results can be extended to a SCID mouse model. Our findings that CD26 expression can be an *in vivo* marker of tumor sensitivity to doxorubicin treatment may lead to future treatment strategies targeting CD26/DPPIV for selected human cancers in the clinical setting. Our result thus characterizes the biochemical linkage among CD26, p38 and topoisomerase II alpha, while providing evidence that CD26-associated topoisomerase II alpha expression results in greater *in vitro* and *in vivo* tumor sensitivity to the antineoplastic agent doxorubicin.

c. 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 CD 26+ 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.

III. Therapeutically targetting transcription factors

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

a. Glucocorticoid receptor project

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

(i) Redox Regulation of the Glucocorticoid Receptor

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

(ii) Development of Dissociating Ligand for the Glucocorticoid Receptor

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

On the other hand, receptor specificity is another important aspect of novel GR regulator. In this line, we have shown that cortivazol is extremely specific for GR and does not bind to MR. We are studying the molecular basis for this receptor specificity of the ligand using cortivazol as a model.

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

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

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.

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

IV. Immunobiology and clinical applications of innate and acquired immune systems.

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 proinflamatory cytokines and their receptor system in order to clearly address their roles in innate and acquired immune system. Molecules of our interest in this field at present are IL-12, IL-23 and TRAIL.

a. Structure and function of IL-12 receptors and IL-23 receptors.

Human CD4 T cells respond to antigenic stimuli to develop in two ways, Th1 and Th2 phenotypes. Th1-type CD4 T cells produce Interferon gamma to promote cellular immunity and Th2-type CD4 T cells produce IL-4 to promote humoral immunity. IL-12 is the key cytokine that preferentially lead antigen-stimulated T cells to mature into Th1-type. We previously analyzed the IL-12 receptors expressed in activated T cells with the aid of monoclonal antibodies elaborated in our hands against IL-12 receptor beta1 chain. In the course of the work, we verified the association of the beta 1 chain with the putative beta 2 chain., which was in line with the proposed structural model of IL-12 receptor system. Surprisingly, we identified a 75-Kd phosphorylated molecule that formed complex with IL-12 receptor beta 1 chain. Recently, IL-23 was identified and the IL-23 receptor was shown to form a dimer with the IL-12 beta 1 chain. We are in the process of newly establishing monoclonal antibodies raised against the IL-23 receptor to elucidate the identity of our 75-Kd molecule.

b. The Role of TRAIL in the prevention of Acute Graft-Versus-Host Dosease.

We report here the potential usefulness of tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) for the treatment of lethal acute graft-versus-host disease (GVHD) and leukemia relapse. Dendritic cells (DCs) genetically modified to express TRAIL showed more potent cytotoxicity than soluble TRAIL against both alloreactive T cells and leukemic cells mediated through TRAIL/death receptor (DR) pathway. In addition, cell gene therapy with genetically modified DCs expressing TRAIL was more effective than in vivo gene transfer of TRAIL for the protection against acute GVHD and leukemia relapse. Thus, gene transfer of TRAIL involving DCs is useful for the treatment of acute GVHD and leukemia relapse by selective targeting of the pathogenic T cells and leukemia relapse.

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

1. Integrated knowledge database system (E-pathfinder)

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

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

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

2. Clinical protocol management system for translational research

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

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

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

3. Development of a living cell-based high-throughput screening system and its application to candidate genes involved in megakaryocytic differentiation

In post-genomic era, innovative systems for analyzing gene functions and screening drug candidates are required. Microarray assay is one of the powerful tools to analyze gene expression profiles and a number of new findings have been reported using this technique. However, an appropriate cell-based analytic system has not been established so far. Therefore, we attempt to develop a new system for living cell-based high-throughput screenings of gene functions and drug candidates.

Because molecular mechanism of thrombopoiesis is poorly understood, we chose magakaryocytic differentiation as a model to develop a new screening system of putative genes involved in some functions. In this study, we transfected K562 and HEL cells with reporter plasmid expressing EGFP under the control of the CD9 or GPVI gene promoter, cultured them in the presence or absence of PMA, and then measured the transcriptional activity as a fluorescent intensity of EGFP. As a result, PMA significantly enhanced the intensity of EGFP in K 562 cells transfected with CD9 promoter reporter plasmid and in HEL cells transfected with GPVI promoter reporter plasmid compared with control. This result suggests that this system might be useful for screening of drug candidates mediating cell differentiation. We also apply this system, together with high-throughput transfection of cDNAs selected by information of gene expression profiles, for screening of putative genes involved in megakaryocytic differentiation.

4. Research and development of agent based simulation for epidemiological and biological analysis

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

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

agents properly by the present modeling platforms. So we developed SOARS as a multipurpose agent based modeling platform implemented in Java computational language.

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

Also, in biological view, a human body consists of various organs, and the organs consist of numerous cells. The ordinary biological analyses handle the cells by quantitative methods mainly using differential equation, but the analyses of some diseases caused by functional abnormality of cells need representation of qualitative changes and may properly described by the method of agent based simulation. We are also developing hybrid simulation platform of continuous and discrete agent based system using SOARS.

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