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. Efficient gene transduction and integration into human blood cells with a new third-generation lentiviral vector

YS Bai, Y Soda, et al.

As the difficulty of gene transduction of human blood cells including hematopoietic stem cells is an obstacle to develop gene therapy targeting hematological disorders, the development of new systems is strongly desirable. Under these circumstances, some reports demonstrated promising results by using lentiviral vectors, although more room for improvement has been left. In this study, we developed a novel third-generation self-inactivation (SIN) lentiviral vector system based on HIV-1 to improve the transduction efficiency and prevent vector-related toxicity and production of replication-competent lentivirus. The VSV-G-pseudotyped HIV vector was produced by cotransfection of the vector and packaging constructs together with the VSV-G- and Rev-expressing constructs into 293T cells. For comparison, VSV-G-pseudotyped SIN MLV vector was used. Transduction efficiencies were assessed by fluorescence microscopy and flow cytometry, detecting hrGFP transgene expression. PCR for hrGFP was also performed to determine transduction efficiencies of colony-forming cells derived from hematopoietic cells. We examined integration of these vectors in these cells by Alu-PCR method. Transduction efficiencies of leukemia cell lines with the HIV and MLV vectors were almost 100% and less than 50%, respectively. The expression of the transgene persisted for eight weeks in cells transduced with the HIV vector, but not with the MLV vector. Similar results were obtained in 11 kinds of primary cells obtained from leukemia or myeloma patients. In addition, resting peripheral blood lymphocytes and CD34-positive hematopoietic cells were successfully transduced with the HIV vector, but not with the MLV vector. Moreover, we confirmed integrations of vector genes in almost all colony-forming cells transduced with the HIV vector, but not with the MLV vector. Our lentiviral vector system is considered to be an excellent gene transduction system for human blood cells because of its high gene transduction and integration capability into host chromosome.

2. Influence on gene transduction with a third-generation sin lentiviral vector: DNA microarray analysis of gene-transduced hematopoietic progenitor cells

YS Bai, Y Soda, et al.

In the development of gene therapy targeting various kinds of disease including congenital enzyme

deficiency, it is thought that gene transduction into hematopoietic stem cells is a very important method. A lot of reports have recently described that VSV-pseudotyped lentiviral vector is promising as a gene transduction method to hematopoietic stem cells because of its excellent transduction efficiency and ability of long-term gene expression. However, the effect and safety of this vector system has not yet been fully evaluated, despite improvements of the vectors including the self-inactivation (SIN) of vectors to avoid the production of replication-competent virus and removal of cytotoxic accessory genes derived from HIV. Therefore, we studied the influence of gene transduction with a third-generation SIN lentiviral vector on the gene expression of human hematopoietic stem cells. CD34-positive cord blood cells (CD34+CBC) were obtained from cord blood using magnetic bead method, and transduced with the third-generation VSV-pseudotyped SIN HIV vector [HIV(VSV)] at M. O. I. 20. Cells were cultured for five days in the presence of stem cell factor, granulocyte-colony stimulating factor, and thrombopoietin. Cell growth and viability was examined by trypan blue exclusion test and expression of surface antigen was analyzed by flow cytometry to access the cell differentiation. Gene expression in transduced cells was compared with non-transduced cells by DNA microarray analysis. The gene transduction efficiencies of cord blood CD34+CBC were 80%, and cell number was increased seven times for five days. In the transduced cells and the non-transduced cells, there were no significant difference in cell number, viability, and expression of surface antigens. DNA microarray analysis demonstrated that there was no significant change on the expression of 23,000 genes. Our HIV(VSV) vector did not influence on the gene expression of CD34+CBC, and may be a useful and safe vector system in clinical gene therapy.

3. Development of HIV (VSV) vectors targeting neutrophil disorders: study of promoter activities in myeloid cells

XJ Li, Y Soda, et al.

Neutrophils are non-dividing effector cells of innate immunity. To treat neutrophil disorders, supplementation of defected gene in neutrophil is thought to be a promising approach. However, previous trials using MLV vectors could not obtain satisfactory results because of low transduction efficiency and low activity of promoters. Recently, VSV-pseudotyped HIV [HIV(VSV)] vectors was reported as a powerful tool for gene transduction into human blood cells, but the optimization of these vector systems for mature granulocytes has been left. In this study, we determined the optimal internal promoter for the gene expression in myeloid cells. In this study, we cultured CD34-positive cellsisolated from umbilical cord blood (CD34+CBC) by two-step culture method using 10ng/ml G-CSF, 5ng/ml SCF, 5ng/ml GM-CSF and 5ng/ml IL-3. To determine the most efficient promoters for neutrophils, we constructed six kinds of HIV(VSV) vectors that contain internal promoter such as CMV, PGK, EF-1α, CAG, MPO or defensin promoter. These vectors were inoculated to CD34+CBC at an M. O. I. of 100, and the expression levels of transgene in myeloid cells were determined by flow cytometry (FCM) to detect GFP. Microscopic observation and four color FCM detecting differentiaion antigens revealed that over 80% of differentiated cells were neutrophils. On day 14 of culture, GFP-positive rate of CD15-positive cells transduced with CMV, PGK, EF-1α and CAG promoter-harboring vectors is 26. 0±5. 0%, 5. 7±2. 9%, 3. 1±0. 6% and 3. 6±0. 6%, respectively. MPO and defensin promoter did not induce detectable GFP expressions. Granule proteins, alkaline phosphotase, myeloperoxidase and esterase were comparably expressed in transduced and non-transduced cells. Gene transduction with HIV vector did not affected cell growth and function of differentiated cells, e. g. chemotaxis, phagocytosis, and the expression of myloperoxidase and defensin. CMV promoter is the most suitable internal promoter in HIV (VSV) vector for gene modification of neutrophils and may be useful for gene therapy of neutrophil disoders.

4. Maxizyme, a novel ribozyme, induces cell death of Philadelphia chromosome-positive acute lymphoblastic leukemia (Ph+ ALL)

Y Soda, YS Bai, et al.

Patients with Ph+ ALL consisting about 30% of ALL have poor prognosis despite intensive treatment including hematopoietic cell transplantation. We previously demonstrated that an allosterically controllable novel ribozyme, designated as Maxizyme (Mz), induced cell death of chronic myelogenous leukemia (CML) cells expressing b2a2 type bcr-abl transcripts, and proposed that it might provide a new strategy for future treatment of CML. Similar to CML, Ph+ ALL cells harbor e1a2 type bcr-abl fusion gene (e1a2) encoding the 190kD fusion protein (p190) and is involved in the pathogenesis of this disease. To develop a new therapeutic approach for Ph+ ALL, we have designed a Mz specifically cleaving e1a2 mRNA (e1a2Mz). We inserted Mz-coding gene downstream of human transfer RNA ($tRNA^{Val}$) gene having polIII promoter activity. This Mz expression unit was inserted in a third-generation self-inactivating lentiviral vector carrying a reporter gene of human CD2 to achieve high transduction and integration efficiency complied with strict safety. The virus particles were produced by cotransfection of the vectors and packaging constructs together with the VSV. G- and Rev-expressing constructs into 293T cells. We trans-

duced Mz into various types of cell lines including Ph+ ALL cells and primary cells from Ph+ ALL patients with this lentiviral vector. In all types of cells, transduction efficiencies detected by flow cytometry were almost 100%, and Mz expression was detected by RT-PCR. In three of five Ph+ ALL cell lines, Mz transduction resulted in significant decrease of viability (about 20% on day 14), and increase of apoptotic cells. However, remaining two Ph+ ALL cell lines did not show cell death similar to p190-negative K562 and NB4 cells. To elucidate the cause of the Mz-resistance of Ph+ ALL cell lines, we measured the e1a2 mRNA level by quantitative RT-PCR. We observed decreased level of e1a2 mRNA in all Ph+ ALL cells transduced with Mz, however, the e1a2 mRNA level was higher in Mz-resistant cells than Mz-sensitive cells. In all three primary Ph+ ALL cells tested, Mz induced growth inhibition and cell death comparable to Mz-sensitive Ph+ ALL cell lines. Furthermore, Mz did not influence the growth and colony formation of normal $CD34^+$ cord blood cells. In conclusion, e1a2Mz will be a useful tool for the gene therapy of Ph+ ALL. In vivo study to see the effect of e1a2Mz on Ph+ ALL cells is underway.

5. Development of a new targeting therapy for Philadelphia chromosome-positive acute lymphoblastic leukemia (Ph+ ALL): anti-CD19 antibody-binding liposome (CD19-liposome) containing STI571 efficiently killed Ph+ ALL cells

M Harata, Y Soda, et al

Patients with Ph+ ALL have poor prognosis despite intensive treatments including hematopoietic cell transplantation. Recently, bcr-abl tyrosine kinase inhibitor, STI571, proved to be a useful agent for Ph+ ALL. However, most patients will soon acquire the resistance to STI571. To overcome this problem, high dose administration, which leads to high intracellular concentration of STI571, is though to be a promising approach, although various toxicities will not allow this approach. Therefore, dose escalation of STI571 only in target cells may resolve this problem. Since almost all Ph+ ALL are derived from precursor B cells and express CD19 specifically on the cell surface, targeting of Ph+ ALL cells using CD19 monoclonal antibody is exploited. In the present study, we developed STI571 encapsuled anti-CD19 antibody binding liposomes for the purpose of developing a new cell targeting therapy of Ph+ ALL. The binding of anti-CD19 antibody to the surface of liposomes was made via polyethyleneglycol (PEG). Transferrin (TF) binding PEG-liposomes (TF-liposome), PEG-liposomes and bare (DSPC/CH) liposomes were used as controls. Eight kinds of cells, such as OM9;22 Ph+ ALL, CML and APL cell lines, etc. were used. The efficacy of induction to the target cells was examined by FCM and fluorescent microscopy using calcein or DiI pigment encapsuled liposomes. The specificity of pigment induction via CD19 was examined by a competitive inhibition experiment using anti-CD19 antibody. The effect of STI571 encapsuled liposomes(STI571-CD19-liposomes) was determined by observing viable cell number and survival rates in the time course. We also examined apoptosis of cells by Annexin-V assay. The induction efficiency by calcein encapsuled liposomes was almost 100% in all Ph+ ALL cell lines and was significantly enhanced by CD19-liposomes compared with bare liposomes and PEG-liposomes. This enhancement was inhibited by the addition of an anti-CD19 antibody in a dose dependent manner. In contrast, in CD19 non-expressing cells, the enhancement effect of CD19-liposomes was not observed. When STI571-CD19-liposomes were added, Ph+ ALL and CD19-positive CML lymphoid crisis cell line, BV173, died in a shorter period compared with other STI571 containing liposomes or free STI571. This effect was observed proportionally to the STI571 concentration in liposomes, but the amount of STI571 required to exert the effect was 0. 1μM or less in STI571-CD19-liposomes, being 1/10 or less of free STI571. A further study is now underway targeting STI571 resistant cell lines, primary leukemia cells from Ph+ ALL patients, and normal hematopoietic cells, in vitro and in animal model. STI571-CD19-liposome was demonstrated to induce cell death specifically to Ph+ ALL cells. This new therapeutic approach might be useful to obtain specific cell killing effects for Ph+ ALL cells with much less side effects to other organs in vivo than administration of free STI571.

6. Isolation from phage-display libraries of three candidate peptides which potentially target hematological malignancies

MH Chen, Y Soda, et al.

Selection of peptides which recognize specific cell types is a promising strategy both in basic research and clinical applications. Cell type-specific peptides can be applied to delivery vehicles for small molecule compounds, nucleotides, genes as well as diagnostic agents. They can also be useful as affinity reagents for cell purification and research tools for cell surface profiling. Recently, a number of cell or tissue-specific targeting peptides have been identified by screening recombinant phage-display libraries *in vitro and/or in vivo*. Using the similar method, we isolated 3 distinct peptide-presenting phages which can bind to several human cancer cell lines including myeloid/lymphoid leukemia cell lines. We screened two types of phage-display random peptide libraries containing linear 12-mer ligands or disulfide constrained 7-mer ligands by the

subtraction method. Daudi lymphoma cells were at first used to exclude non-specific binding and/or lymphoid specific binding of peptide-presenting phages from libraries, and next AML-M2 derived Kasumi-1 cells were used to isolate peptides potentially targeting myeloid leukemia cells. Screening of libraries was performed virtually according to the standard protocol. Cells were incubated with 100 library equivalent phages at 37°C for 1 hr. After extensive washing, cell-surface binding phages were recovered by acid treatment of cells and neutralized. Internalized phages were prepared by lysing cells. After five rounds of screening procedures, we isolated a single phage presenting 12-mer peptides (WAPPLFRSSLFY), which was specifically internalized by Kasumi-1 cells. In another selection, we identified two Kasumi-1 cell-surface binding phages containing 12-mer inserts (GIQLANPPRLYG and LQAAYKGFARAG) after eighth rounds of screening. Specific binding of the purified three phages to Kasumi-1 cells at 4°C was confirmed by flowcytometry using anti-M13 monoclonal antibody with phages containing random 12-mer inserts as control. We screened a panel of cancer cell lines to examine the cell-type specificity of these three peptides. We selected three 12-mer peptide sequences which can definitely bind to several leukemia cell lines but not to normal lymphocytes. Further characterization of the peptides is ongoing.

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

SG Cho, Y Shuto, K Izawa et al.

Natural killer (NK) cells have been reported to be involved in resistance to engraftment of transplanted allogeneic stem cells. We investigated the effects of NK cell depletion on engraftment and induction of stable mixed chimerism after MHC-mismatched, nonmyeloablative bone marrow transplantation (NMT). Recipient mice (BALB/c, H-2k^d) were injected intraperitoneally with anti-asialoGM1 antibody (anti-NK Ab) on day -1. On day 0, they received TBI at a dose of 500 cGy, followed by intravenous infusion of 2 x 10^7 T-cell-depleted (TCD) bone marrow (BM) cells from allogeneic donors (C57BL/6, H-2k^b). Early engraftment and chimerism were determined by the relative ratio of peripheral blood (PB) lymphocytes expressing either recipient or donor MHC class I molecules (FITC-H-2k^b vs. PE-H-2k^d) on day +21. Long-term engraftment and chimerism were evaluated by examination of PB and spleen by flowcytometry. All the recipients conditioned with a single injection of anti-NK Ab and 500 cGy TBI showed successful engraftment. In contrast, no recipients treated with TBI alone showed engraftment. Furthermore, a donor-dominant pattern of mixed chimerism was observed in both PB and spleen. Spleen cells from recipients showing mixed chimerism revealed specific tolerance to both host and donor strains, but were reactive to third-party cells (C3H/He). None of the reconstituted mice showed signs of GVHD, and all survived up to day +330. These observations indicate that host NK cell depletion may be used to reduce the intensity of the conditioning regimen for engraftment of TCD grafts, and can contribute to the establishment of stable mixed chimerism in MHC-mismatched NMT.

8. Multi-lineage differentiation and lethal GVHD-like syndrome after transplantation into NOD/SCID mice of non-human primate common marmoset CD34$^+$ cells

K Izawa, et al.

We have been trying to establish a non-human primate model of preclinical studies such as gene therapy toward hematological disorders, using the small New World monkey, common marmoset (Cm) (*Callithrix jacchus*). However, handling with Cm stem/progenitor cells has been hampered by the lack of procedures for isolating those populations. Discrimination of stem/progenitor cells from mature blood cells usually depends on their surface expression of CD34 antigens in human and rodents. Therefore, we developed for the first time anti-Cm CD34 monoclonal antibody (MoAb) and tested cell populations isolated by this MoAb for their colony-forming capacity as well as repopulating activity in NOD/SCID mice. Cm cDNA encoding a human and rodent CD34 homologue was cloned from bone marrow (BM)-derived RNA. NIH3T3 cells were engineered to express CM CD34 cDNA and immunized into BALB/c mice. The resulting MoAb was used to isolate CD34$^+$ cells in combination with a magnetic cell sorting system. Purified CD34$^+$ cells were subjected to colony-forming assays in methylcellulose and transplantation into sublethally irradiated NOD/SCID mice. A series of murine MoAbs against human CD antigens, which cross-reacted with corresponding Cm antigens, were used to detect mature Cm blood cells in peripheral blood (PB) and BM of NOD/SCID mice. The open reading frame of Cm CD34 cDNA had 89% homology with the human sequences. One of the five available MoAbs, MA24 (IgM), recognized 1~2% of BM mononuclear cells (MNCs), and purified CD34$^+$ cells were approximately 11 to 30 fold enriched in CFU-GM content, compared with BM MNCs. Around one month after transplantation of up to 10^5 Cm CD34$^+$ cells into NOD/SCID mice, CD2$^+$, CD4$^+$, CD8$^+$, CD11b$^+$ as well as CD14$^+$ Cm cells could be detected in PB and BM. Surprisingly, graft versus host disease (GVHD)-like syndrome including scleroderma, weight loss or hunched posture developed

around 3 weeks after transplantation and thereafter worsened. Such a syndrome inevitably occurred in every mouse which had Cm blood cells in PB. Diseased mice were sacrificed for histological examination of various tissues, indicating massive T($CD2^+$) cell infiltration into , liver, spleen. Cm $CD34^+$ cells isolated by MA24 demonstrate a repopulating activity in a NOD/SCID transplantation model. However, engraftment of Cm $CD34^+$ cells coincided with GVHD like-syndrome. In this xenogeneic transplant model, the behavior of progenies of Cm $CD34^+$ is quite different from that of human $CD34^+$ cells which could not induce GVHD in the comparable experimental conditions.

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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

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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 which 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 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 (STAT5A1*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

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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 (VPP1) (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, Yuseok Moon 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 in normal hematopoiesis and leukemogenesis

Hideaki Nakajima, 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 post-coitum (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 multi-potent 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. Development of human lymphohematopoiesis defined by CD34 and CD81 expressions.

Feng Ma, Kohichiro Tsuji

Cord blood (CB) $CD34^+$ cells expressed CD81, a member of transmembrane 4 superfamily, and were classified into three subpopulations based on their expression levels: $CD34^+CD81^+$, $CD34^{Low}CD81^+$ and $CD34^+CD81^{High}$ cells. We then examined the lymphohematopoietic activity of each subpopulation, using suspension and clonogenic cultures for hematopoietic potential, coculture with MS-5 cells for B cell potential, NOD/SCID mouse fetal thymus organ culture for T cell potential, coculture with NOD/SCID mouse fetal liver-derived stromal cells for natural killer (NK) cell and mast cell potentials, and xenotransplantation into NOD/SCID mice for long-term repopulating ability. $CD34^+CD81^+$ cells represented a heterogeneous population that had all lymphohematopoietic activities, including NOD/SCID mouse-repopulating ability. $CD34^{Low}CD81^+$ cells were more enriched in erythroid, megakaryocytic and NK lineage potentials but already lost T and B potentials and long-term repopulating ability. $CD34^+CD81^{High}$ fraction had depleted of most lymphohematopoietic potential except for NK cell and mast cells potentials. Thus, along the differentiation cascade from $CD34^+CD81^+$ lymphohematopoietic stem cells, an up-regulation of CD81 or a down-regulation of CD34 means the change of lymphohematopoietic property. CD81 may serve as a marker to define developmental stages of lymphohematopoietic stem cells.

14. 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 x 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 x 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.

15. 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 CD34$^+$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.

16. 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. Analysis of human immunodeficiency virus type 1 Nef, focusing on the proline-rich domain using a Sendai virus vector.

Takeshi Yamada et al.

Human immunodeficiency virus type 1 (HIV-1)Nef protein plays a role in the down-regulation of human-leukocyte antigen class I (HLA-I) molecules on the surface of T cells, which is likely to be related with its evasion from cytotoxic T lymphocytes (CTLs). To investigate in detail such a function of Nef, we have designed Sendai viruses (SeVs) to highly and readily express recombinant proteins in the floating cells, for example, human T-lymphocytes. All of the generated recombinant SeVs achieved high levels of gene expression in CEM cells by 24 hours of culture after infection at a multiplicity of infection(MOI) of 10. By flow cytometric analysis, it was confirmed that HLA-class I and CD4 molecules on CEM cells were efficiently down-regulated by Nef. We applied this new system using recombinant SeVs to analyze the proline-rich domain (amino acid 69-78)of Nef by site-directed mutagenesis. As a result, we elucidated that the amino acid proline at position 78(Pro-78) was the most crucial amino acid in the downregulation of HLA-class I. However, the conversion of Pro-78 into alanine did not alter the down-regulation of CD4 molecules. These results suggest that a single amino acid, Pro-78 in Nef distinguishes the down-regulation of HLA-class I from CD4.

2. 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 efficient-

ly 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.

3. Dihydrofolate Reductase Gene Polymorphisms in Pneumocystis carinii f. sp. hominis in Japan

Takashi Takahashi et al.

Pneumocystis carinii f. sp. *hominis* (*P. carinii*) is an important causative pathogen of morbidity and mortality in immunocompromised patients with human immunodeficiency virus type-1 infection, hematological malignancies, organ transplantation state or connective tissue diseases. We examined polymorphisms in the dihydrofolate reductase (DHFR) gene of Pneumocystis carinii isolates from 27 patients with P. carinii pneumonia in Japan. Four substitution sites with two synonymous and two non-synonymous changes were found. Two synonymous substitutions at nucleotide positions 540 and 312 were identified in one and 13 patients, respectively. Two amino acid substitutions (Ala67Val, Cysl66Tyr) were found in two different patients. No linkage of amino acid substitutions in DHFR to those in dihydropteroate synthase was observed. The two patients whose isolates showed non-synonymous DHFR mutations were not exposed to DHFR inhibitors before they developed PCP and were treated successfully with co-trimoxazole.

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

Noriko Kobayashi and Hitomi Taguchi-Nakamura et al.

The human liver-specific intercellular adhesion molecule 3 (ICAM-3) -grabbing nonintegrin protein (CD209L, L-SIGN, DC-SIGNR) is a type II membrane protein homologous to the dendritic cell (DC)-specific counterpart (CD209, DC-SIGN) and is expressed in liver and lymph nodes but not in DCs. Reportedly, CD209 is required for activation of resting T cells through its binding to ICAM-3, which stabilizes the DC/T-cell contact zone. CD209L may have a similar function. CD209L as well as CD209 were shown to capture human immunodeficiency virus type I (HIV) virions by binding HIV gp120 with carbohydrate recognition domains (CDRs) and to transmit them to T lymphocytes. Therefore, the genetic polymorphisms of *CD209L* and *CD209* may affect HIV pathogenesis. Studies to test such a hypothesis have not been conducted, so far, at least on *CD209L*. The human *CD209L* gene consists of 8 exons and spans about 6. 5 kb on chromosome 19. Exon 4 encodes the neck domain and exons 5-7 CRDs. IMSUT/JST (http://snp. ims. u-tokyo. ac. jp) has discovered 5 single-nucleotide polymorphisms (SNPs; one in intron 4, one in exon 5, and three in intron 5) in the *CD209L* gene in a Japanese population (IMS-JST025120 to IMS-JST025124). Soilleux et al. described a variable-number-of-tandem-repeats (VNTR) of a 69 bp-sequence in exon 4; this VNTR results in 23 aa repeats in the neck domain of CD209L. We typed the 5 SNPs in DNA samples from 59 HIV-infected patients. We also typed the VNTR polymorphism in the same samples. Among 44 HIV-infected patients, whose CD4+ cells were counted on more than three occasions before the start of anti-retroviral treatment, (5 untreated, and 54 treated), the A allele in intron 5 at IMS-JST025124 was associated with the higher number of lowest $CD4^+$ cell counts during untreated periods (statistical significance: A/A vs. G/G and G/A, $P = 0.0069$ by Mann-Whitney's U Test).

We also look for association of frequency and extent of adverse effects by anti-retroviral drugs with polymorphisms of gene involved in lipid metabolism and drug transporters.

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

Tae Furutuki 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*, *env*, and *pol* regions that encode CTL epitopes restricted by HLA-A24. We are likely to be finding some evidence of immunological pressure involved in viral evolution in human individuals.

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Advanced Clinical Research Center

Division of Bioengineering

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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. Critical Involvement of Nitric Oxide Production in the Establishment of Tumor Specific Cellular Immunity Induced with Dendritic Cells Activated with Streptococcal Preparation OK-432.

Yong-kook Kim, Naoya Ichikawa, Hideaki Tahara

Dendritic cells (DCs) are potent professional antigen presenting cells and play a critical role in cellular immunity. We have previously demonstrated that specific antitumor immune response can be induced with intratumoral (i. t.) injection of bone marrow-derived DCs genetically engineered with interleukin (IL)-12 genes. In this study, we examined whether DCs stimulated with OK-432 could be used in the same manner. *In vivo* administration of recombinant IL-12 causes NK cells and T cells to secrete IFN-γ and enhances the cytolytic functions against tumor cells. These cytokines, however, induce abundant secretion of nitric oxide (NO) from activated macrophages, and appear to suppress cellular immunity in murine system. OK-432, a penicillin inactivated and lyophilized preparation of low-virulence strain (i. e. Su) of *Streptococcus pyogenes*, has been clinically used as a potent biological modifiers for treating cancer patients and is known to induce multiple cytokines including IFN-γ and IL-12 *in vitro*. In tumor models of C57BL/6 mice inoculated with MCA205 (fibrosarcoma) or MC38 (colon adenocarcinoma) intradermally, i. t. injection of bone marrow-derived DCs with or without OK-432 showed marginal antitumor effects on day 7 established tumors. However, significant antitumor effects were observed when the tumor bearing mice were treated with the combination of DCs, OK-432, and N-nitro-L-arginine methyl ester (L-NAME), which inhibits inducible nitric oxide synthase (iNOS)-mediated NO production. Furthermore, tumor specific and potent cytotoxic T lymphocytes (CTLs) could be obtained from the splenocytes of the mice treated with the combined therapy. In contrast, the tumors treated with DCs, OK-432, and N-nitro-D-arginine methyl ester (D-NAME), which is an inactive optical isomer of L-NAME and has no inhibitory effects on iNOS, showed only marginal effects at the levels similar to those of mice treated with DCs with or without OK-432 alone. These results suggest that tumor specific cellular immunity in our murine models could be strongly suppressed by NO, which is abundantly secreted by macrophages and DCs stimulated with OK-432. Thus, i. t. injection of DCs and OK-432 could be a promisingly cancer immunotherapy if NO production is properly regulated.

b. Immunomonitoring for cancer vaccine using HLA- tetramer

Toshiyuki Baba, Takuya Tsunoda, Hiroyuki Mushiake, Hideaki Tahara

The HLA tetramer is a powerful tool to quantita-

tively evaluate the CTLs specific to antigen peptide presented on particular HLA. Although HLA-A24 is the most common MHC class I allele in Japanese, A24 tetramer assay has not been established yet. We established CTL lines using CE3 that was nonamer peptide encoded in CEA and CMVpp65 A24 peptide. We also prepared A24/CE3 and A24/CMV tetramers, and succeeded in evaluations the frequency of CE3 and CMV peptide specific CTL using A24/CE3 and A24/CMV tetramer sufficiently. Still more, we evaluated the frequency of antigen specific CTLs in the PBMC from the melanoma patients treated with gp100 peptide vaccination.

c. Penicillin-killed Streptococcus pyogenes (OK-432) promote dendritic cell maturation neither through TLR2 nor 4 but through beta 2 integrin

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 (10μg/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 CD8+ 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 was the stimulation. These results strongly suggest that OK-432 could be a useful agent for peptide-based cancer vaccine using DCs.

d. 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.

e. In vivo electroporation of human FLT3-Ligand plasmid DNA induce anti-tumor specific immunity by mobilization of dendritic cells in situ.

Takuya Takayama, Kazuya Shimao, 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 also 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 transfection 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 anti-tumor effects that were 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.

f. Vaccination of SLC gene-Modified tumor cells induce anti-tumor immunity modulating dendritic cell's functions in situ

Kazuya Shimao, Takuya Takayama, Hideaki Tahara

Secondary lymphoid –tissue chemokine (SLC), which is a member of CC chemokine, promotes the migration of mature dendritic cell (DC) expressed CCR7. In this study, we are going to examine the efficacy of SLC on DC mobilization in vivo and anti-tumor immunity by SLC gene modified tumor vaccination.

2. Mechanistic study on transplantation immunology

a. The promotion of Donor engraftment with Non-lethal irradiation, G-CSF and Tolerogenic DC in allergenic BMT.

Kaname Yamamoto, Yoshifumi beck, Yuichi Ando, Hideaki Tahara

We analyzed the effect of G-CSF and vitamin D_3 on maturation and character of DC in vitro mice BM culture with GM-CSF and IL-4. The addition of G-CSF (10 - 100ng/mL) in BM-DC culture induced expansion of the percentage and the number of $CD11c^+$ CD86-cells with G-CSF receptor. It is reported that G-CSF polarize to Th-2 in helper T balance. So we evaluate the effect of pre/post G-CSF injection into major mismatched BMT recipient. G-CSF injection enhanced donor engraftment after BMT with sub-lethal irradiation (700R), but not with non-lethal irradiation (500-600R). It is unclear that how G-CSF promote the donor engraftment. Next we demonstrated that the addition of vitamin D_3 (10-100nM) in BM-DC culture suppressed the expression of costimulatory molecule (CD86, CD40) on $CD11c^+$ cells and DC derived with vitamin D_3 had no stimulatory activity on MLR with allo-splenocytes. Furthermore we separated $CD11c^+$ CD86-cells from vitamin D_3 derived DC by MACS. But pre-injection of Macs separated this tolerogenic DC into allo-BMT recipient had no effect of the promotion of donor engraftment. We examine the effect of injection of vitamin D_3 into allo-BMT recipient.

b. Evaluation of in vivo tolerogenicity of genetically modified recipient dendritic cells (syngeneic DC) pulsed with immunogenic peptides (allopeptides) derived from donor HLA molecule in HLA class I transgenic mouse.

Sumito Tamura, Yoshifumi beck, Yuichi Ando, Hideaki Tahara

Foreign tissue allografts are rejected because the histocompatibility antigens they express stimulate a response from the host's immune system. The predominant cell type involved in this response is the T lymphocyte. Before T cell causes rejection, naive alloreactive T cell must be activated by antigen presenting cells (APCs) that bear both allogeneic MHC molecule and co-stimulatory signals. DC, a professional APC, plays a critical role in this initiation and modulation of immune response. Regulating the activity of DC is, in theory, an ideal approach to establish donor specific tolerance. Our goal is to establish a state of alloantigen specific tolerance by manipulating the interaction of DC and reactive T cell.

T cells of C3H. B51 recognize HLA-B*3501 molecules expressed on C3H. B35 as allo-MHC class I antigens and rejects vascularized C3H. B35 grafts by cellular mechanism. (Ando and Beck et al., Transplantation 68, 904-908, 1999.) HLA-B*3501 derived peptide induces long-term heart graft survival in C3H. B35 into C3H. B51 cardiac transplantation model (Ando and Beck et al., Transplantation 68, 904-908, 1999) by intra-thymic injection. (Transplant proceedings, 30, 3890-3891, 1998). As explained in the last annual report, we initially planned to clarify the tolerogenic capability of syngeneic DC involved with allo-MHC molecules based on this HLA TGM heart transplant model. The attempt, however, was faced by difficulties in creating an adequate allo-peptide pulsed syngeneic bone marrow DC with allogeneic effect for further in vitro assay planned prior to in vivo application. In order to circumvent the stagnation, we have decided to alter our approach. We have decided to study the mechanism of tolerance in this model, hoping that this approach will give us the "lead" to the establishment of peripheral allo-specific tolerance.

Results obtained so far suggest that B35 allo-peptide seems to be involved with class II rather than class I in inducing tolerance. T cell obtained from tolerant recipients showed both proliferation and CTL generation following stimulation by alloantigen, a state so called "split tolerance". This means that simple deletion based on MHC-allo-peptide molecular mimicry theory is unlikely as the cause of graft specific tolerance. On the other hand, adoptive transfer of bulk splenocytes obtained from sygeneic donors that received intra-thymic administration of allo-

peptide resulted in prolongation of graft survival, suggesting that regulatory T cell may be involved in tolerance induction. We are currently planning to study the role of regulatory cells in the HLA. TGM model.

Publication

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Advanced Clinical Research Center

Division of Clinical Immunology

免疫病態分野

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Our long term goal is to define the molecular 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.

1. Crk-associated substrate lymphocyte type (Cas-L) project

Satoshi Iwata, Hiroshi Kobayashi, Akiko Souta-Kuribara, Takahiro Sasaki, Rikako Miyake-Nishijima, Seiji Kobayashi, Masahiko Uchiyama, Mamoru Nori, Osamu Hosono, Hiroshi Kawasaki, Hirotoshi Tanaka, and Chikao Morimoto

β1 integrins play crucial roles in a variety of cell processes such as adhesion, migration, proliferation, and differentiation of lymphocytes. Previously we showed that co-immobilized ligand or anti-β1 integrin mAbs 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 integrin/ligand binding in T cell activation. Furthermore, we showed that the ligation of β1 integrins 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), and thus designated Cas-L (Crk-associated substrate lymphocyte type).

Cas family proteins (p130Cas, Cas-L/HEF-1, Efs/Sin) have common structural features. From N-terminal end, they have SH3 domain, SD (substrate domain) that is a cluster of YXXP motifs, serine-rich domain, YDYVHL motif, Helix-loop-helix domain, and coiled-coil domain. Based on this structural analysis, we showed that 1) FAK binds SH3 domain of Cas-L, 2) Crk, Nck, and SHP-2 bind YXXP motifs in SD, and 3) Src family PTKs (Fyn, Lck, and Src) bind YDYVHL motif, respectively. Furthermore, we have recently identified a new Cas-L binding protein, and designated it as MICAL (Molecule Interacting with Cas-L). MICAL binds SH3 domain of Cas-L through its proline rich sequence, and bind vimentin, through its C-terminal portion, suggesting its role in cytoskeletal reorganization.

Our present projects aim at 1) identifying novel

Cas-L binding proteins by Two-Hybrid screening, 2) investigating the biological significance of those protein-protein interactions in vitro, and 3) evaluating the clinical relevance of those interactions in a variety of disorders, including inflammatory diseases and malignancies.

a. Cloning and characterization of binding molecules for Cas-L by Two-Hybrid screening

Although our previous analysis revealed that Cas-L plays a crucial role in T cell costimulation and migration through the engagement of β1 integrins and TCR/CD3, exact biological roles of their Cas-L in these processes has not fully understood. To gain an insight in this issue, we have screened several millions of cDNA clones from a HTLV-I (human T-lymphotropic virus type I) transformed human T cell line (SLB-I) and human fetal brain by Two-Hybrid assay in search for Cas-L binding molecules. As a result, we have successfully isolated some uncharacterized clones as well as the clones for known proteins. Two cDNAs for FAK were among those clones, which have been already reported as Cas-L binding proteins. Interestingly, we identified two cDNAs for HTLV-I tax and one for Smad7. GFP-tax and GFP-Smad7 expressed in 293T cells show co-precipitation with Cas-L. These results were further confirmed by full length cDNAs for tax and Smad7 subcloned in mammalian expression plasmid.

Tax was originally identified as cis-activator of LTR-mediated HTLV-I replication. It has been reported that Tax transactivate a variety of host genes, especially downstream of NF-κB.

Smad7 is an endogenous inhibitor of TGF-β signaling pathway. Golemis et al. have recently reported that Smad 3 is also one of the binding partners of Cas-L/HEF1, which is involved in TGF-β signaling pathway as a positive regulator (Co-Smads). Since Cas-L binds both positive and negative regulator of TGF-β signaling pathway, we evaluate overall biological outcome of Cas-L-Smads interaction.

At this moment, we focus on these clones as putative Cas-L binding proteins.

b. Induction of Cas-L by HTLV-I tax; Role of Cas-L in ATL (adult T cell leukemia)

Since Cas-L is overexpressed in various HTLV-I-infected T cell lines, we evaluated the effect of p40tax protein on expression and phosphorylation of Cas-L using JPX-9 cells in which tax is induced by addition of $CdCl_2$. Surprisingly, tyrosine phosphorylation as well as the expression of Cas-L was markedly enhanced through the induction of tax protein in JPX-9 cells. Furthermore, these cells showed markedly enhanced motile behavior on FN-coated Transwell™ insert, confirming that Cas-L play an important role in cell migration in those cells.

In the clinical setting, we have shown that leukemic cells from ATL patients express significantly higher amount of Cas-L compared to the healthy individuals. Furthermore, we have shown that tyrosine phosphorylation of Cas-L is spontaneously elevated in the case with ATL patients, suggesting that extremely invasive nature of ATL cells may be attributed to the enhanced expression and phosphorylation of Cas-L.

c. Role of Cas-L in pathogenesis of arthritis in HTLV-I tax transgenic mice and human rheumatoid arthritis

It has been reported that the expression of β1 integrins and their ligands are elevated in the inflammatory sites in rheumatoid arthritis (RA), suggesting that β1 integrins play an important role in triggering and maintaining the inflammatory response of such diseases.

We have shown that Cas-L plays a crucial role in T cell costimulation and migration through the engagement of β1 integrins and TCR/CD3 complex. To investigate the role of Cas-L in pathophysiology of RA, we utilized HTLV-I tax transgenic mice, since they develop RA-like disease. We compared littermate control mice (Ct), tax transgenic mice without arthritis (Ntg) and those with arthritis (Atg) at 4-14 weeks after birth. Transendothelial migration assay revealed that migratory activity of spleen cells from Atg and Ntg mice was much higher than that of Ct mice. Biochemical studies using the lysate of spleen cells showed that Cas-L protein and its spontaneous tyrosine phosphorylation were increased in both Atg and Ntg mice compared to Ct mice. Interestingly, the extent of elevation in Cas-L protein and its phosphorylation level was higher in Atg mice than that of Ntg mice, which paralleled with the migratory capability of spleen cells. To define the kinases that phosphorylated Cas-L, we analyzed Src family PTKs (Fyn and Lck) and β1 integrin-associated PTKs (FAK and Pyk2). Subsequently we found that spontaneous tyrosine phosphorylation of Fyn and Lck were observed in spleen and lymph node of Atg mice, whereas FAK and Pyk2 were only marginally phosphorylated. Immunohistochemical analysis showed a large number of Cas-L positive lymphocytes and leukocytes migrating into the affected joints with RA. These results strongly suggest that increased expression as well as tyrosine-phosphorylation of Cas-L protein is involved in the development of arthritis in HTLV-I tax transgenic mice.

Finally, we investigated the human materials from patients with rheumatoid arthritis, and have shown that tyrosine phosphorylation of Cas-L are markedly enhanced in SFMC (synovial fluid mononuclear cells) from RA patients. These cells exhibited increased capability of transendothelial migration compared to PBMC from RA patients and healthy volunteers. Since

β1 integrins play a key role in triggering and maintaining inflammation of RA, the above results strongly suggest that Cas-L protein appears to play an important role in the pathophysiology of RA.

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

Chikao Morimoto, Kei Ohnuma, Masahiko Uchiyama, Hiroshi Kobayashi, Satoshi Iwata, Osamu Hosono, Hiroshi Kawasaki, and Hirotoshi Tanaka(in collaboration with Nam H Dang, MD Anderson Cancer Center, USA, and with Rafael Franco, University of Barcelona, Spain).

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 basis for CD26 mediated T cell activation signaling and enhancement of memory T cell response by sCD26. Furthermore , we are focusing on the translational research of utilization of anti-CD26 mAb as well as soluble CD26 for malignant tumors, immune-mediated disorders and immune deficiency diseases.

a. Presence of CD26/DPPIV is associated with enhanced topoisomerase II alpha expression and increased sensitivity to drug-induced G_2-M arrest.

CD26 is a 110kD surface-bound ectopeptidase with dipeptidyl peptidase (DPPIV) activity that has multiple functions, including a role in T cell activation and we demonstrated that the expression of CD26 and its DPPIV activity enhanced the sensitivity of CD26 Jurkat transfectants to doxorubicin-induced cell cycle arrest at the G_2-M checkpoint. We extend our previous findings by showing that surface expression of CD26/DPPIV enhanced sensitivity of CD26 Jurkat transfectants to G_2-M arrest mediated by the antineplastic agent etoposide. Similar to the case found with doxorubicin, the increased sensitivity to etoposide-induced G2-M arrest was associated with disruption of cell cycle-related events, including hyperphorylation of $p34^{cdc2}$ kinase, prophorylation of cdc25C and alterration in cyclin B1 expression. CD26/DPPIV-associated enhancement of doxorubicin and etopioside-induced G_2-M arrest was also ovserved in serum-free media. Consistent with the fact that CD26 Jurkat transfectants displayed greater sensitivity to the topoisomerase II-targeted drugs doxorubicin and etoposide, as well as suggesting a potential mechanism for this enhanced susceptibility, our findings strongly suggested that CD26/DPPIV surface expression was associated with increased topoisomerase II alpha levels.

b. G1/S cell cycle arrest provoked in human T cells by antibody to CD26

The expression of CD26 is enhanced after activation of T cells, while it is preferentially expressed on a subset of CD4+ memory T cells in the resting state. We demonstrate that binding of the soluble anti-CD26 monoclonal antibody (mAb) 1F7 inhibits human T-cell growth and proliferation in both CD26-transfected Jurkat T-cell lines and human T-cell clones by inducing G1/S arrest, which is associated with enhancement of p21Cip1 expression. This effect depends on the DPPIV enzyme activity of the CD26 molecule. Moreover, we show that expression of p21Cip1 after treatment with the anti-CD26 mAb 1F7 appears to be induced through activation of extracellular signal-regulated kinase (ERK) pathway. These data thus suggest that anti-CD26 treatment may have potential use in the clinical setting involving activated T cell dysregulation, including autoimmune disorders and graft-vs.-host disease.

c. Reduction of serum soluble CD26/DPPIV enzyme activity and its correlation with disease activity in SLE

The soluble form of CD26 is present in serum and recombinant soluble CD26 (rsCD26) can enhance in vitro antigen-specific T cell responses. To determine

the role of soluble CD26 (sCD26) in the pathophysiology of patients with systemic lupus erythematosus (SLE), we measured levels of sCD26 and its specific DPPIV activity in serum. Serum sCD26 levels and DPPIV activity were measured by sandwich ELISA in 53 patients with SLE and 54 healthy controls. Serum sCD26 was identified by immunoprecipitation and immunoblot analysis. Expression of CD26 on T cells was analyzed by flow cytometry. Serum levels of sCD26 and its specific DPPIV activity were significantly decreased in SLE and were inversely correlated with SLE disease activity index score, but not with clinical variables or clinical subsets of SLE. Close correlation between sCD26/DPPIV and disease activity was observed in the longitudinal study. Our results showed that serum levels of sCD26 may be involved in the pathophysiology of SLE, and appear to be useful as a new disease activity marker for SLE.

d. Soluble CD26/DPPIV enhances transendothelial migration via its interaction with M6P/IGFIIR

In addition to its membrane form of CD26, CD26 exists in plasma as a soluble form (sCD26), which is the extracellular domain of the molecule thought to be cleaved from the cell surface. We demonstrate that sCD26 mediates enhanced transendothelial T cell migration, an effect that requires its intrinsic DPPIV enzyme activity. We also show that sCD26 directly targets endothelial cells and that mannose 6-phosphate/insulin-like growth factor II receptor (M6P/IGFIIR) on the endothelial cell surface acts as a receptor for sCD26. Our findings therefore suggest that sCD26 influences T cell migration through its interaction with M6P/IGFIIR.

e. Regulation of epithelial and lymphocyte cell adhesion by ADA-CD26 interaction

The extra-enzymic function of cell-surface adenosine deaminase (ADA), an enzyme mainly localized in the cytosol but also found on the cell surface of monocytes, B cells and T cells, has lately been the subject of numerous studies. Cell-surface ADA is able to transduce co-stimulatory signals in T cells via its interaction with CD26, an integral membrane protein that acts as ADA-binding protein. We attempted to explore whether ADA-CD26 interaction plays a role in the adhesion of lymphocyte cells to human epithelial cells. To meet this aim, different lymphocyte cell lines (Jurkat and CEM T) expressing endogenous, or overexpressing human, CD26 protein were tested in adhesion assays to monolayers of colon adenocarcinoma human epithelial cells, Caco-2, which express high levels of cell-surface ADA. Interestingly, the adhesion of Jurkat and CEM T cells to a monolayer of Caco-2 cells was greatly dependent on CD26. An increase by 50% in the cell-to-cell adhesion was found in cells containing higher levels of CD26. Incubation with an anti-CD26 antibody raised against the ADA-binding site or with exogenous ADA resulted in a significant reduction (50-70%) of T-cell adhesion to monolayers of epithelial cells. The role of ADA-CD26 interaction in the lymphocyte-epithelial cell adhesion appears to be mediated by CD26 molecules that are not interacting with endogenous ADA (ADA-free CD26), since SKW6.4 (B cells) that express more cell-surface ADA showed lower adhesion than T cells. Adhesion stimulated by CD26 and ADA is mediated by T cell lymphocyte function-associated antigen. A role for ADA-CD26 interaction in cell-to-cell adhesion was confirmed further in integrin activation assays. FACS analysis revealed a higher expression of activated integrins on T cell lines in the presence of increasing amounts of exogenous ADA. Taken together, these results suggest that the ADA-CD26 interaction on the cell surface has a role in lymphocyte-epithelial cell adhesion.

3. Therapeutically targeting transcription factors

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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.

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 by glucocorticoids in the 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 en-

zymes, 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 bullets.

(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 GR belongs to a nuclear receptor superfamily and acts as a ligand-dependent transcription factor. We demonstrated that the GR 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.

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 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.

(iv) Hypoxia-inducible Factor (HIF)-1a 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.

b. Transcriptional Network Controlling Angiogenesis in Health and Diseases

Angiogenesis is regulated by a combination of various factors including transcription factors. Recently, we have isolated cDNA encoding the novel protein IPAS which can squelch HIF-1a. 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-1a 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-3a, 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 for homeostatic control of tissue function. In this line, we are now identifying the molecular mechanism for such regulation of HIF-1α.

4. 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.

An abortive ligand-induced activation of CCR1-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, CCL3 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.

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.

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 responsiveness. 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

Professor Tetsuo Shimizu

教 授 清 水 哲 男

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, especially Translational Research, which is performed as application of genome science to clinical medicineÅD 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 started the construction pathological knowledge database including genome, protein, and their relation, i. e. pathway database for pathological application of genome science.

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 also started to introduce 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 prevented. This system will be hoped to contribute much to national health programs of Japan.

For the first case, 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.

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清水哲男:医科学のためのシステム論，放射線科学， vol. 45 No. 1, 13-23, 2002