Advanced Clinical Research Center
Division of Molecular Therapy

Our department is concerned primarily with basic and clinical research on hematopoiesis, signal transduction, cell cycle, cell transplantation and gene therapies, focused on immunohematopoietic disorders. In this year the strong clinical base coupled with the application of immuno-molecular techniques has led to the following achievements.

1. Very high gene transduction and integration efficiency of a new third-generation lentiviral vector in human leukemia cells, lymphocytes and hematopoietic cells

Y.Soda, Y.Bai, K.Tani, et al.

Various kinds of gene transduction systems have recently been developed, but the transduction efficiencies in human hematopoietic cells were generally low. As the difficulty of gene transduction is an obstacle to develop gene therapy targeting hematopoietic disorders, the development of new systems has been desired. To improve transduction and integration efficiency complying with the strictest safety standards, we used a novel third-generation lentiviral vector system based on human immunodeficiency virus type 1 (HIV-1). For comparison, Moloney murine leukemia virus (MLV) SIN vector (pMX-based 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 in colony-forming cells (CFC) derived from hematopoietic cells. Furthermore, we examined integration of these vectors in these cells. Transduction efficiencies of HIV and MLV vectors in the majority of leukemia cell lines were almost 100% and less than 50%, respectively. Expression of the transgene was maintained 8 weeks in cells transduced with HIV vectors, but not with MLV vectors. We also examined 11 primary cells from leukemia or myeloma patients, and the transduction efficiencies of HIV and MLV vectors were 50-90% and 30-80%, respectively. Expression of the transgene was maintained 4 weeks in the cells transduced with HIV vectors, but not with MLV vectors. Resting peripheral blood lymphocytes (PBL) were successfully transduced with HIV vectors, but not MLV vectors, although activated PBL could be transduced with both vectors. More than 90% of human CD34+ cells from cord blood (CB) could be transduced with HIV vectors and almost all colonies derived from these cells were positive for hrGFP. We also confirmed integration of HIV vectors in almost all of these colonies, but the efficiency was much lower with MLV vectors. These data indicate that this lentiviral vector system, compared with the MLV vector system, is an excellent gene transduction and integration system for human blood cells, including hematopoietic stem cells, and this system is considered to become a useful tool for clinical gene therapy.

2. Enhancement by VCAM-Ig of Granulocyte Colony Stimulating Factor-Induced Progenitor Cell Mobilization in Mice

T. Tsuruta, N. Ohno, et al.

a4b1 integrin (VLA4) mediates hematopoietic progenitor cell (HPC) adhesion to bone marrow (BM) stroma by interacting mainly with vascular cell adhesion molecule-1(VCAM-1), and plays an important role in the behavior of HPCs in BM. In fact, a number of preclinical studies revealed that *in vivo* administration of anti-VLA4/VCAM-1 antibodies resulted in efficient mobilization of HPCs into peripheral blood(PB). In the present study, an immunoadhesin consisting of N-terminal 4 immuno-

globulin (Ig)-like domains of human VCAM-1 and Fc fragment of human IgG1 (VCAM-Ig) was produced in a baculovirus expression system and was assessed for its ability to mobilize HPCs by competitive inhibition of VLA4 binding to VCAM-1. As previously described, FACS analysis confirmed that purified insect cell-derived VCAM-Ig colud bind to VLA4 in its active state, that is, only in the presence of Mn++. For HPC mobilization, either VCAM-Ig or control human IgG (300mg/head, day 3-5) was subcutaneously injected into Balb/c mice with or whithout G-CSF (125mg/kg, day 1-5) and cyclophosphamide (CY; 200mg/kg, day 0). Peripheral blood was taken at day 6-8 for white blood cell count and day 7-CFU-C assay. In comparison with control IgG, VCAM-Ig affected neither white blood cell counts nor their differential counts even in combination with G-CSF. Results for the cumulative number of day 7-CFU-C in PB for 3 consecutive days were as follows;

These results suggest that administration of VCAM-Ig results in 2-2.5 fold increase in the cumulative number of mobilized HPCs, especially in combination with G-CSF, and that this effect is not associated with increase in white blood cell counts. Preclinical studies with complete murine-type VCAM-Ig are ongoing.

3. Suppression of Myeloblastoma Growth by Local Injection with VEGF Antagonist, Flt-1/Fc; A Model for Antiangiogenic Therapy of Hematologic Malignancy

A Tojo, N Ohno, et al.

Anigiogenesis plays an important role in *in vivo* tumor growth even in hematological malignancies. Recent studies have suggested that autocrine and/or paracrine loop of vascular endothelial growth factor (VEGF) may support survival and proliferation of leukemia/lymphoma cells. In reality, a major part of freshly isolated leukemias and lymphomas expressed mRNA and protein for VEGF and at least one of its receptors, suggesting that the blockade of VEGF loop may contribute to *in vivo* tumor suppression. Considering that Flt-1 (VEGFR-1) has much higher affinity for VEGF than Flk-1/KDR (VEGFR-2), we produced a fusion protein consisting of the extracellular domain of human Flt-1 and Fc fragment of human IgG1 (Flt-1/Fc) in a baculovirus expression system and used as a VEGF antagonist. *In vitro* experiments confirmed that Flt-1/Fc, purified through protein-G column chromatography, specifically recognized VEGF and inhibited the VEGF-dependent growth of human umbilical vein endotherial cells in a dose dependent manner. U937 cells expressed several species of VEGF mRNA, but Flt-1/Fc did not suppress its *in vitro* growth. We inoculated 1x107 U937 cells into Balb/c nude mice subcutaneously at day 0. Local injection of 100mg of Flt-1/Fc around the tumor site was performed every 2 days from day 6. Control mice were similarly injected with 100 mg of human IgG. Treatment with Flt-1/Fc did not regress but significantly suppress the growth of U937-derived blastoma, compared with control IgG.

Pathological and immunohistochemical analysis of excised tumor tissues disclosed central massive necrosis and paucity of blood vessels in Flt-1/Fc-treated mice, but not in control mice. These findings suggest that Flt-1/Fc may be a candidate therapeutic agent for hematological malignancies.

4. *In Vitro* Anti-Myeloma Activity of IFNα/VCAM-Ig; Bispecific Immunoadhesin for Targeting IFNα to Cells Expressing Activated VLA-4

N Ohno, A Tojo, et al.

Although interferonα(IFNα) is an effective therapeutic agent for hematological malignancies including chronic myeloid leukemia (CML) and multiple myeloma (MM), it also binds various nonhematological tissues and causes many adverse effects, making it difficult to be given in higher doses. A strategy to make the best clinical use of IFNα against malignancies is its targeting to particular neoplastic cells and/or immune effector cells. From this viewpoint, we produced a bispecific immunoadhesin, in which both IFNα2β and N-terminal 4 immunoglobulin (Ig)-like domains of vascular cell adhesion molecule-1 (VCAM-1) are fused to the Fc region of IgG1, by simultaneous infection of insect High-Five cells with baculovirus which carries cDNA encoding each molecule. The resulting heterodimer, IFNα/VCAM-Ig, was purified to nearly homogeneity from the culture media by sequential ion-exchange and gel-filtration column chromatography and migrated to the apparent molecular mass of 148κDα in SDS-PAGE, confirmed by immunoblot analysis using both anti-IFNα2β and anti-VCAM-1 antibodies. IFNα/VCAM-Ig retained comparable antiviral activity to recombinant IFNα2β and also inhibited the growth of Daudi lymphoma cells in a comparable dose-dependent manner. Since VCAM-Ig homodimer binds only activated VLA4 which is expressed by a substantial portion of primary MM cells, the biological properties of IFNα/VCAM-Ig were further examined in a series of MM cell lines. As a result,growth inhibition by IFNα/VCAM-Ig was observed in 7 cell lines, which were all positive for VLA4 expression but could not bind VCAM-Ig homodimer in the absence of Mn++, indicating that VLA4 was not activated. Among those, 4 cell lines were also sensitive but the remaining 3 cell lines(KMS-12PE, -21PE, and - 28PE) were resistant to growth inhibition by IFNα2β. These results suggest that IFNα/VCAM-Ig may exert more potent in vitro anti-myeloma activity than IFNα2β by the unidentified mechanism besides simply targeting VLA4.

5. Autologous antileukemic immune responses induced by chronic lymphocytic Leukemia B cells expressing the CD40 ligand and interleukin-2 transgenes

S.Takahashi, J.Ooi et al.

Although the B cells of chronic lymphocytic leukemia (B-CLL cells) express both tumor-specific peptides and major histocompatibility complex (MHC) class I antigens, they lack the capacity for co-stimulatory signaling, contributing to their protection against host antitumor immunity. To stimulate CLL-specific immune responses, we sought to transfer the human CD40 ligand (hCD40L) gene to B-CLL cells, using an adenoviral vector, in order to upregulate co-stimulating factors on these cells. Because efficient gene transduction with adenoviral vectors requires the expression of virus receptors on target cells, including CAR (the coxsackievirus B-adenovirus receptors) and α_v integrins, we co-cultured B-CLL cells with human embryonic lung fibroblasts (MRC-5 line). This exposure led to increased expression of integrin $\alpha_v\beta3$ on B-CLL cells, which correlated with higher transduction rates. Using this novel prestimulation system, we transduced B-CLL cells with the hCD40L gene. The Ad-hCD40L-infected cells had higher expression of B7 molecules and induced activation of autologous T cells *in vitro*, but these T cells could not recognize parental leukemic cells. By contrast, an admixture of Ad-hCD40L-positive cells and leukemic cells transduced with the human interleukin-2 (IL2) gene produced greater T-cell activation than did either immunostimulator population alone. Importantly, this combination generated autologous T cells capable of specifically recognizing parental B-CLLs. These findings suggest that the combined use of genetically modified CD40L- and in combination IL2-expressing B-CLL cells may induce therapeutically significant leukemia-specific immune responses.

6. Transgenic expression of CD40L and Interleukin –2 induces an autologous anti-tumor immune response in patients with non-Hodgkin lymphoma

S.Takahashi, K.Uchimaru, et al.

The malignant B cells of non-Hodgkin's lymphoma (B-NHL cells) express peptides derived from tumor specific antigens (such as immunoglobulin idiotypes), and also express major histocompatibility complex (MHC) antigens. However, they do not express co-stimulatory molecules, which likely contributes to their protection from host antitumor immunity. To stimulate NHL-specific immune responses, we attempted to transfer the human CD40 ligand (hCD40L) gene to B-NHL cells and enhance their co-stimulatory potential. We found an adenoviral vector encoding human CD40L (AdhCD40L) was ineffective at transducing B-NHL cells, because these cells lack CAR (the coxsackievirus B-adenovirus receptor) and α_v integrins. However, pre-culture of the B-NHL cells with the human embryonic lung fibroblast line, MRC-5 significantly upregulated expression of integrin $\alpha_v\beta3$ and markedly increased their susceptibility to adenoviral vector transduction. After pre-stimulation, transduction with AdhCD40L increased CD40L expression on B-NHL cells from 1.3±0.2% to 40.8±11.9%. Transduction of control adenoviral vector had no effect. Expression of transgenic human CD40L on these CD40 positive cells was in turn associated with upregulation of other co-stimulatory molecules including B7-1/-2. Transduced B-NHL cells were now able to stimulate DNA synthesis of autologous T cells. However, the stimulated T cells were unable to recognize unmodified lymphoma cells, a requirement for an effective tumor vaccine. Based on previous results in an animal model, we determined the effects of combined use of B-NHL cells transduced with AdhCD40L and AdhIL2 vectors. The combination enhanced initial T-cell activation and generated autologous T cells capable of specifically recognizing and killing parental (unmodified) B-NHL cells via MHC restricted cytotoxic T lymphocytes. These findings suggest that the combination of CD40L and IL2 gene-modified B-NHL cells will induce a cytotoxic immune response *in vivo* directed against unmodified tumor cells.

7. Selection of leukemia-targeting peptide using peptide-presenting phage libraries

S.Takahashi et al.

Tumor-specific ligands have potentially important applications for targeting diagnostic and therapeutic agents to tumors, including tumor-specific targeting viral vectors for cancer gene therapy. Previous work with random peptide-presenting phage libraries demonstrated that cell binding peptide ligands could be selected by direct panning on cells in culture (Barry et al. Nat Med 2:299 1996) or whole organs (Pasqualini et al.). We have applied this technique to identify specific targeting peptides for chronic lymphocytic leukemia (CLL). Phage libraries were selected against B-CLL cells collected from the peripheral blood of patients (CD5+/CD19+ >95%). Peptide selection was performed at 4C, 20C or 37C after incubation in serum-free medium for 2 hours to clear non-specific receptors. After panning, CD19+ cells were isolated by magnetic bead capture to purify CLL target cells from contaminating non-leukemia cells. Peptide-presenting phage were then amplified from acid eluted fractions of these CD19+ cells or by hypotonic lysis of the target cells

themselves. In some selections, phage were pre-incubated with non-CLL cells to remove promiscuous cell binding peptides from the selection. 60 different 20-mer peptides were obtained which fell into three populations: 1) peptides that bound CLL, B cells, T cells, and monocytes; 2) peptides that bound CLL and B cells, but not T cells or monocytes; and 3) a set of six peptides that bound CLL cells, but did not cross-react with autologous or allogeneic B cells, T cells, or monocytes. One CLL-specific peptide (1-5) and one pan-tropic peptide (4*-5) were tested as synthetic peptides out of the context of phage where they demonstrated binding activities similar to the same peptides on phage. Coupling the 1-5 peptide to an adenoviral vector increased the percentage of of GFP-expressing CLL cells from <1% with the native advector, to 34.8% with peptide-labelled advector, indicating the promise of this approach for gain of function CLL transduction *ex vivo* and targeted transduction *in vivo*. We are now optimizing the affinity of the selected CLL-binding and CLL-specific peptides and incorporating them genetically into adenovirus and other gene therapy vectors.

8. Molecular cloning of common marmoset (CM) CD34 cDNA and the production of anti-CM CD34 monoclonal antibody : Implication for preclinical non-human primate model targeting stem cell gene therapy

K.Izawa, K. Tani, et al.

To establish a new preclinical non-human primate model for human gene therapy targeting hematological disorder, it is very important to purify hematopoietic progenitor cells. Common marmoset (Callithrix jacchus) (CM), the small New World monkey, has been broadly used as preclinical animal models for the development of new drugs. In the field of gene therapy, our previous data (Blood 93: 2839-2848, 1999) suggested that the marmoset's peripheral blood progenitor cells were transduced by human MDR1-retroviral vector because we could detect the human MDR1 gene in multilineage peripheral and bone marrow blood cells of CM for almost 3 years at longest. The transduction efficiency, however , was not high enough to reflect our in vivo results to human clinical gene therapy. To obtain higher transduction efficiency to marmoset's hematopoietic stem cells, the purification of the stem cells were considered to be imminent. For this purpose, we previously assessed the reactions of CM bone marrow (BM) mononuclear cells (MNC) to commercially available anti-human CD34 MoAbs . No available CD34 MoAbs was useful to purify the CM hematopoietic progenitor cells (Eur J Haematol 66:272-280,2001). On this background, we, first of all, have cloned cDNA encoding CM CD34 protein. CM CD34 cDNA was obtained by the reverse transcription polymerase chain reaction (RT-PCR) method followed by 5' and 3' rapid amplification of cDNA ends (5'and 3'RACE method). The open reading frame (ORF) of the cloned CM CD34 cDNAs had 89% homology with human CD34 cDNA sequence. To construct the expression vector, the ORF of CM CD34 cDNAs was amplified by PCR using primers with 3' FLAG tag sequence. The PCR products was cloned into a mammalian expression vector and the vector DNA were transfected into COS7 cells. Western blotting analysis using anti-FLAG tag MoAb showed a band of approximately 120kD. After immunization of mice with the cells expressing CM CD34, we obtained an anti-CD34 MoAb producing hybridoma cell clone, MA10 hybridoma cells. Flow cytometric analysis using the MA10 Ab demonstrated that approximately 4% of MA10(CD34) positive cells were detected in CM BM MNC population. The colony-forming assays showed that the multilineage hematopoietic progenitors including burst-forming units -erythroid (BFU-E) and colony-forming units - granulocyte/macrophage (CFU-GM) were significantly enriched in the MA10(CD34) positive populations. Our results suggested that the purification of CM CD34 positive cell population using our newly produced anti-CM MoAb would be very helpful to assess the *in vivo* effects of newly developed gene transfer vector targeting human hematopoietic progenitor cells. As an example, we are now currently transducing the CM CD34 positive cell population using VSV-G pseudotyped hGFP- lentivirus vector to observe this gene transduced cells *in vitro* and *in vivo*.

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Our major research interest is to elucidate hematopoiesis and its regulatory mechanism for the development of clinical cellular therapy. Currently most of our studies are focused on 1) ontogeny of hematopoiesis, 2) characterization of hematopoietic stem cells, 3) molecular mechanism regulating hematopoiesis and 4) early development of lymphoid cells.

1. Generation of definitive hematopoietic stem cells from murine early yolk sac (YS) and paraaortic splanchnopleura (P-Sp) by aorta-gonad-mesonephros (AGM) region-derived stromal cells

Sahoko Matsuoka, Hiroaki Hisakawa, Ming-jiang Xu, Yasuhiro Ebihara, Daisuke Sugiyama, Kohichiro Tsuji, Shigetaka Asano[1]:[1]Division of Molecular Therapy, Advanced Clinical Research Center, IMSUT

Experiments using chimeric embryos in nonmammalian vertebrates demonstrated that mesodermally derived ventral compartments (YS or its analog) and dorsal compartments (intraembryonic region) contribute to hematopoiesis in a different manner. On the other hand, the ontogenic source of mammalian definitive hematopoiesis has remained controversial, because the *in utero* development of mammals excludes embryo grafting experiments. Results of earlier mouse studies led to the general acceptance of a model that murine definitive hematopoiesis begins in YS, shifts to fetal liver (FL), and finally resides in bone marrow (BM), in contrast to the conclusion derived from nonmammal vertebrates. However, recent studies have shown that early development of murine hematopoiesis is more complex than heretofore considered.

While the developmental relationship between primitive and definitive hematopoiesis in murine embryogenesis remains unanswered, localization and developmental stage are critical factors for embryonic hematopoiesis, hence the importance of the microenvironment surrounding embryonic hematopoietic cells or their precursors. To better understand the roles of the microenvironment in the development of murine embryonic hematopoiesis, we recently established endothelial cell lines, AGM-S3, from the AGM region at 10.5 days postcoitum (dpc), which can support the development of murine and human immature hematopoietic cells including long term-repopulating hematopoietic stem cells (LTR-HSC). In this study, we further asked whether AGM-S3 cells have the potential to support the generation of LTR-HSC capable of reconstituting definitive hematopoiesis from early YS and intraembryonic P-Sp, since it has been shown that LTR-HSC initiate autonomously within 10 dpc AGM region in an *in vitro* organ culture system. YS and P-Sp cells at 8.5 dpc generated no definitive hematopoiesis-derived colony-forming cells in co-cultures with AGM-S3 cells, but spleen colony-forming cells and HSC capable of reconstituting definitive hematopoiesis simultaneously appeared on day 4 of co-culture. Surprisingly, precursors for definitive HSC were also present in YS and P-Sp at 8.0 dpc, a time when YS and embryo were not connected by blood vessels. Based on these results, we propose that precursors with the potential to generate definitive HSC appear independently in YS and intraembryonic P-Sp, and that P-Sp or AGM region affords the microenvironment which facilitates generation of definitive hematopoiesis from precursors.

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

Daisuke Sugiyama, Feng Ma, Kohichiro Tsuji, Toshio Kitamura

Although precursors with the potential to generate definitive HSC appear independently in YS and intraembryonic 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 adress this issue, we developed an embryo-grafting system using WEC. In this system, whole embryos from 8.25 dpc, a time before the formation of omphalomesenteric artery which cennect 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.

3. Megakaryopoiesis of murine primitive hematopoiesis in the early yolk sac

Ming-jiang Xu, Sahoko Matsuoka, Yasuhiro Ebihara, Kohichiro Tsuji

In developing mouse embryo, primitive hematopoiesis yields unique erythrocytes distinguishable from those in definitive hematopoiesis by their morphology and hemoglobins contained. However, there have been no reports on megakaryopoiesis in primitive hematopoiesis. In the present study, we demonstrated the presence of megakaryocytic progenitors in the early YS, which originate from primitive hematopoiesis. The megakaryocytic progenitors immediately differentiated and matured to megakaryocytes with proplatelet formation in the methylcellulose culture, as compared with those in adult BM. The unique megakaryocytic progenitors in YS are first detected at 7.5 dpc and increased at 8.5 dpc, but then gradually decreased. The analysis of globin types in hematopoietic mixed colonies containing megakaryocytes and erythrocytes showed that the megakaryocytic progenitors in the early YS originate from primitive hamatopoiesis. In the presence of fetal bovine serum, the megakaryocytic progenitors were sensitive to various cytokines, such as interleukin (IL)-3, stem cell factor (SCF), thrombopoietin (TPO) and erythropoietin (EPO). The primitive hematopoiesis may generate the unique megakaryocytic progenitors to rapidly give rise to a great amount of platelets for preventing the simultaneously developing blood vessels from bleeding.

4. Developmental change of CD34 expression on murine hematopoietic stem cells

Sohoko Matsuoka, Yasuhiro Ebihara, Kohichiro Tsuji

In spite of clinical importance of CD34 antigen as a marker for primitive hematopoietic cells in HSC transplantation and gene therapy, it has been controversial whether or not HSC express CD34. In human, the engraftment systems using immunodeficient mice and preimmune fetal sheeps, and the clinical transplantation of CD34-enriched cell population indicated the presence of LTR-HSC in $CD34^+$ cells, while recent studies have raised questions whether $CD34^+$ cell fraction includes all of human stem cell activity. The reports regarding CD34 expression on murine adult HSC have also been conflicting. On the other hand, reports on CD34 expression on murine fetal HSC are in agreement, irrespective of the residence or gestational stage, suggesting a possibility that CD34 expression on murine HSC varies during the development from fetus to adult. To test this possibility, we examined CD34 expression on HSC of murine fetus, neonate and various ages of adult, using the competitive long-term reconstitution analysis. We then found the developmental change of CD34 expression on murine HSC. In fetus and neonate, CD34 was expressed on $Lin^-c\text{-}Kit^+$ HSC isolated from BM, liver and spleen. However, the CD34 expression on HSC decreased along with aging, and in over 10 week-old mice marrow, HSC were most enriched in lineage markers-negative (Lin^-)-Kit^+CD34^- cell fraction. The present findings have important implications for further expansion of therapeutic HSC transplantation, especially CD34-enriched cell transplantation and cord blood (CB) transplantation.

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

Yasuhiro Ebihara, Mika Wada, Takahiro Ueda, Ming-jiang Xu, 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), SCF, 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.

6. Human granulocyte-macrophage colony-stimulating factor receptor (hGM-CSFR) stimulates both primitive and definitive erythropoiesis *in vitro* in EPOR-deficient mice

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GM-CSF induces biological effects, such as proliferation induction in early progenitors and stimulation of maturation along various myeloid lineages. We recently produced transgenic mice (Tg-mice) that constitutively express hGM-CSFR, composed of two distinct subunits designated as α and β, at almost all stages of hematopoietic cell development, and indicated that hGM-CSF supported erythropoiesis in the abscence of EPO in the Tg-mice. However, there is a possibility that the hGM-CSF-dependent erythropoiesis is caused by the interaction between GM-CSFR and EPOR signals or EPO self-produced by erythroid cells. On the other hand, homozygous EPOR-/- embryos died *in utero* between days 13 and 15 of gestation due to failure of definitive FL erythropoiesis. We then generated hGM-CSFR-expressing EPOR-/- embryos to examine whether hGM-CSFR signal completely substitutes EPOR signal in erythropoiesis. In response to hGM-CSF, hGM-CSFR was efficient in supporting differentiation of the EpoR-/- FL progenitors into hemoglobinized erythroid colonies *in vitro*. Reverse transcriptase polymerase chain reaction (RT-PCR) with single colonies revealed that expression of adult-type globins, such as α- and β-major globins, was detected in all of rescued erythroid colonies, but embryonic βH1-globin was diminished in some colonies, indicating that hGM-CSF can support both primitive and definitive erythropoiesis in FL of hGM-CSFR-expressing EPOR-/- embryo. Thus, this study demonstrated that a signal transmitted through hGM-CSFR stimulates proliferation and terminal differentiation of hGM-CSFR-expressing EPOR-/- FL progenitors *in vitro* and that EPOR signaling has no instructive role in red blood cell differentiation.

7. Effect of IL-3 on early development of human B-cells

Koichiro Miyamoto, Kohichiro Tsuji, Tatsutoshi Nakahata[3]

Although IL-3 is known as a stimulator of proliferation and differentiation of various hematopoietic cells, the effect of IL-3 on the development of lymphoid cells is unclear. We recently established a co-culture system with murine stromal cells, MS-5, for the generation of B-cells from CB $CD34^+$ cells. Using the culture system, we examined the effect of IL-3 on the development of $CD19^+$ B-cells from $CD34^+$ cells. When added to the co-culture, IL-3 suppressed the generation of B-cells in a dose-dependent manner. This inhibitory effect of IL-3 on B-cell development was effective only when IL-3 was added in early stage of the culture. To examine whether IL-3 directly acts on B-lymphoid cells, we cultured IL-$3R\alpha^+$ and IL-$3R\alpha^-$ cells sorted from CB $CD34^+$ cells. B-cells were mainly generated from IL-3Rα-expressing cells, but flow cytometric analysis showed that the IL-3Rα expression gradually diminished during the culture, coinsident with the observation that the inhibitory effect of IL-3 was efficient only on early stage of B-cells. These results indicate that IL-3 suppresses early development of human B-cells directly through IL-3Rα on B-cell progenitors.

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Advanced Clinical Research Center
Division of Infectious Diseases

Main subjects of the Division of Infectious Diseases are human immunodeficiency virus 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[1], Naotoshi Kaji[1,3], Takashi Odawara[1], Joe Chiba[3], Aikichi Iwamoto[1,2], and Yoshihiro Kitamura[1]:[1]Division of Infectious Diseases, Advanced Clinical Research Center, [2]Department of Infectious Disease and Applied Immunology, Institute of Medical Science, The University of Tokyo, 4-6-1 Shirokanedai, Minato-ku, Tokyo 108-8639, Japan, [3]Department of Biological Science and Technology Science University of Tokyo 2641 Yamazaki Noda, Chiba 278-8510, Japan

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

Takahashi, T., Endo, T., Nakamura, T., Sakashita, H., Kimura, K., Ohnishi, K., Kitamura, Y., and Iwamoto, A.

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 dihydrofolate reductase (DHFR) gene of *P. carinii* isolated from 27 patients with *P. carinii* pneumonia (PCP) in Japan. We found 4 substitution sites with 2 synonymous and 2 non-synonymous changes. Two synonymous substitutions at nucleotide positions 540 and 312 were identified in one and thirteen patients, respectively. Two amino acid substitutions (Ala67Val, Cys166Tyr) 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 inhibi-

tors before they developed PCP and were successfully treated with co-trimoxazole.

3. Novel polymorphisms in human macrophage inflammatory protein-1 alpha (MIP-1alpha) gene

Xin X, Nakamura K, Liu H, Nakayama EE, Goto M, Nagai Y, Kitamura Y, Shioda T, Iwamoto A.:Division of Infectious Diseases, Advanced Clinical Research Center, Institute of Medical Science, University of Tokyo, Tokyo, Japan

Human macrophage inflammatory protein-1 alpha (MIP-1alpha) is a chemotactic cytokine, which binds to macrophages, T cells, and B cells affecting their activation. We found novel polymorphisms at four sites within MIP-1alpha gene in Japanese population: C to T in exon 2; A to G in intron 2; C to G and A to G in exon 3. They occurred on the same allele. Although MIP-1alpha effectively suppresses the replication of HIV-1 in vitro, we observed no statistically significant difference in the allele frequency of this polymorphism between HIV-1-infected and uninfected individuals in Japanese population. Since an increased transcription level of MIP-1alpha has been reported to be associated with inflammatory diseases such as atopic dermatitis, we also investigated the frequency of these polymorphisms among patients with atopic dermatitis, HIV-1-infected individuals (with a normal IgE level), and healthy donors. A small increase in ratio of homozygotes to other genotypes was observed in patients with atopic dermatitis ($P = 0.04$).

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Advanced Clinical Research Center
Division of Bioengineering

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

1. Development of innovative cancer therapy

a. Cancer immunotherapy using dendritic cells with systemic administration of cytokines

Yong-kook Kim, Naoya Ichikawa, Hideaki Tahara

Dendritic cells (DCs) are highly potent antigen-presentation cells capable of inducing a primary T lymphocyte response. To examine whether DC therapy combined with cytokines can elicit synergic antitumor effects *in vivo* and *in vitro*, we performed intratumoral DC injection with systemic administration of interleukin (IL)-2 using tumor bearing mice.

Systemic administration of IL-2 has been used for the treatment of the patients with metastatic renal carcinoma and melanoma. Although high dose IL-2 treatment mediates potent antitumor effects, it is associated with severe adverse effects in the clinical situation. It is suggested that direct activation of NK cells by IL-2 has a key role in the toxicity. Here, we have evaluated whether the systemic administration of IL-2 can enhance the antitumor effects when combined with intratumoral (i.t.) injection of dendritic cells (DCs) in mouse tumor models.

C57BL/6 female mice (8 to 12 wk old) received intradermal inoculation of MCA205 fibrosarcoma cells, and 7 days later they received i.t. injection of bone marrow derived (BM)-DCs or HBSS. HBSS as a control, or low (20,000IU) or high (100,000IU) dose of IL-2 were given to mice intraperitoneally.

The treatments IL-2 alone were associated with the same extent of dose-dependent suppression of tumor growth. Although the treatment with high dose IL-2 alone showed distinct antitumor effects without obvious adverse effects. Therapy using DCs i.t. injection in combination with IL-2 administration was associated with the most significant suppression of tumor growth. In addition, tumors in 60% of mice completely vanished when they were treated with DC and high dose IL-2.

These results in murine tumor models suggest that this therapeutic approach is promising as an immunotherapy in humans. Further clinical development of this combination therapy is warranted for patients with cancer.

b. Immunomonitering for cancer vaccine using HLA- tetramer

Toshiyuki Baba, Takuya Tsunoda, Hiroyuki Mushiake, Hideaki Tahara

The HLA tetramer is a powerful tool to quantitatively 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 line using CE3 that was nonamer peptide encoded in CEA and prepared A24/CE3 tetramer, and succeeded in evaluations the frequency of CE3 specific CTL using A24/CE3 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 not 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 the 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 for novel chemoimmunotherapy using S-1 and Lentinan

Hiroyuki Mushiake, Takuya Tsunoda, Hedeaki 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 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. Mobilization and maturation of dendritic cells *in vivo* using Flt3-Ligand gene transfer with *in vivo* electroporation

Katsuhisa Enomoto, Takuya Takayama, Hideaki Tahara

Dendritic cell (DC) is one of the most potent antigen-presenting cells (APCs), and can play pivotal role in immunological response. DCs appears to stimulate T-lymphocytes and can regulate immune responses. Flt3-Ligand (Flt3L), a recently reported cytokine, is a stimulator for proliferation and differentiation of DCs *in vivo* and *in vitro*. In this study, we analyzed the effect of Flt3L *in vivo* electroporation (IVE) on DC mobilization, proliferation, and maturation *in vivo*. After Flt3L transfection with IVE, Flt3L was detected in the serum for 10 days after IVE at significant levels. The peak concentration of 5100pg/ml was observed 4 days after IVE. To investigate the effects of Flt3L expression using IVE, we examined the frequency of DCs in spleen and bone marrow 7 days after IVE by using flowcytometry. The frequency of DCs are significantly increased in both spleen and bone marrow after Flt3L IVE transfection when compared to those of control group. Furthermore, the weight of spleen and the number of splenocytes increased after Flt3L transfection using IVE. These results implied that Flt3L gene transfer using IVE mobilize, proliferate, and promote maturation of DC *in vivo*. This method could be utilized for various types of immunoregulation.

2. Mechanistic study on transplantation immunology

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

Kaname Yamamoto, Yoshifumi beck, Yuichi Ando, Hideaki Tahara

We analysed 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 $CD11^+CD86^-$ 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 sepalated 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. The enhancement of anti Tumor effect after Mini-transplant by DLI and DC injection without GVHD

Kaname Yamamoto, Yoshifumi beck, Yuichi Ando, Hideaki Tahara

Using the murine mini-transplant model, we tried to evaluate the anti tumor effect by DLI and tumor antigen pulsed DC injection. At first, we analysed donor chimerism after minor mismatched BMT(BALB/c → DBA/2) with non-lethal irradiation (550R). Mixed chimerism of T lymphocyte has been observed at 2 weeks after minor mismatched BMT and donor chimerism resulted over 90% at 8 weeks. Next, we analysed tumor growth after innoculation into BALB/c and DBA/2. BALB/c rejected P815 (DBA/2 derived mastocytoma)$5x10^5$ cells within 10 days, but DBA/2 died within 4 weeks. We evaluate the effect of both anti tumor effect and the sevearity of acute GVHD by the injection of BALB/c donor splenocytes after bone marrow transplantation into P815 innoculated DBA/2 mice.

c. 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

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.

We plan to clarify the putative tolerogenic capability of DC involved with HLA molecules based on this HLA TGM heart transplant model. Application of syngeneic (recipient:C3H.B51) DC exposed to allo-antigen (HLA-B*3501 derived peptides) with some modification is expected to establish donor(C3H.B35) specific tolerance. In brief, application of syngeneic (recipient:C3H.B51) DC exposed to allo-antigen (HLA-B*3501 derived peptides) with an addition of immunosuppresive cytokines, i.e. vIL-10, TGF, and/or co-stimulatory blockade by genetic manipulation is expected to establish donor(C3H.B35) specific tolerance. Some of these strategies have already been utilized in fully allogeneic rodent combinations. While simultaneous blockade of the co-stimulatory pathway and/or sustained immaturity of DC seem to be important in order to elicit allo-specific tolerance in these models, none so far have shown an induction of fully convincing, reproducible allo-specific tolerance with DC alone. Little has been investigated in the context of combining modified syngeneic DC and allogeneic epitope peptide.

C3H.B35 to C3H.B51 skin graft and heart graft were both rejected within an acceptable period. We are currently repeating these in vivo studies together with in vitro studies in order to gain concrete evidence that the C3H.B35 to C3H.B51 TGM transplant model is truly reliable.

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Advanced Clinical Research Center
Division of Clinical Immunology

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. Role of β1 integrins and association molecules in T cell functions in normal and disease conditions

Satoshi Iwata, Hiroshi Kobayashi, Rikako Nishijima, Akiko Souta-Kuribara, Takahiro Sasaki, Seiji Kobayashi, 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. We have shown 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 have shown that the ligation of β1 integrins induces protein tyrosine phosphorylation of pp125FAK (focal adhesion kinase), paxillin, and pp105 in H9 cells or peripheral T cells. Pp105 was first identified 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 designated Cas-L.

Currently, we are focusing on the biological significance of Cas-L and downstream signaling events of Cas-L as well as the functions of β1 integrin-associated molecules such as CD82 and CD9 in human T cells as well as in cancer cells. Both CD9 and CD82 belong to transmembrane 4 superfamily (TM4SF), which share common structural features. Moreover, we are investigating the clinical significance of those molecules in various immunological disorders.

a. Cloning and characterization of binding molecules for Cas-L

Cas family proteins (p130Cas, Cas-L/HEF-1, Efs/Sin) have common structural motifs such as N-terminal SH3 domain, followed by a cluster of YXXP motifs and a YDYVHL motif. Based on the structural analysis, we have shown that FAK binds SH3 domain of Cas-L, and that Crk, Nck, SHP-2, and Src family PTKs such as fyn, lck binds YXXP motifs and YDYVHL motif of Cas-L, respectively.

Although our recent analysis revealed that Cas-L plays a crucial role in T cell costimulation and migration through the engagement of β1 integrins and TCR/CD3, biological significance of their Cas-L-associated molecules has not fully understood. To further gain an insight for the link between Cas-L

and biological outcome through β1 integrins such as cell motility and cytokine gene expression, we have screened cDNA libraries from a HTLV-I transformed human T cell line and human fetal brain by Two-Hybrid system in search for Cas-L binding molecules. Accordingly, we have isolated some known clones and some uncharacterized clones. The known clones contain FAK and vimentin, which have been already reported as Cas-L binding proteins. We are now confirming the association of those clones with Cas-L by gene transfer and immunoprecipitation analysis using mammalian cells.

b. Overexpression of Cas-L by HTLV-I tax, which is also observed in HTLV-I-related diseases

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.

Clinically, we have shown that leukemic cells from ATL patients express significantly higher amount of Cas-L compared to the healthy individuals. Furthermore, it has been revealed that tyrosine phosphorylation of Cas-L is spontaneously elevated in the case with ATL patients, suggesting that extremely motile behavior of ATL cells may be attributed to the enhanced expression and phosphorylation of Cas-L.

c. Distinctive signaling pathways through CD82 and β1 integrins in human T cells

CD82, a member of TM4SF, is a multifunctional molecule that is involved in cell activation, costimulation and cell spreading of T cells. We have shown that immobilized anti-CD82 mAbs as well as anti-α4β1 integrin mAbs induced tyrosine phosphorylation of Cas-L in human peripheral T cells and H9 cells. Furthermore, one of anti-CD82 mAbs (8E4), which induces homotypic aggregation of T cells and H9 cells but has no costimulatory activity, partially inhibited VLA-4 integrin ligand-mediated costimulation of T cells, whereas it failed to inhibit VLA-5 integrin ligand-mediated costimulation.

To further elucidate the relationship between CD82- and VLA-4-mediated signaling pathways, we defined the IL-2 production by the costimulation of Jurkat T cells with marginal amount of Cas-L, and subsequently found that mAbs against CD82 had strong costimulatory activity to CD3/TCR, whereas mAbs engagement of β1 failed to do so in those cells. We have further demonstrated that this discrepancy between β1 integrin- and CD82-mediated costimulation partly lies in differential activation in NF-AT, AP-1, and NF-κB in Jurkat T cells. These results present an evidence for distinctive signaling of CD82- and β1 integrin-mediated costimulation at the transcriptional level of IL-2 gene.

d. The $CD9^+CD45RA^+$ naive human T cell subset involved in autoreactivity

In the previous report, we found that human CD9 was preferentially expressed on $CD45RA^+$ naive T cell subset in the peripheral blood. Functional study revealed that CD9 was a costimulatory molecule and crosslinking of anti-CD9 mAb could trigger apoptosis. To clarify the significance of preferential expression of CD9 on $CD45RA^+$ naive T cell subset, we investigated the responsiveness of $CD9^+$ T cells to autoantigen, since autoreactive T cells had been reported to be within $CD45RA^+$ T cell subset. We have observed that $CD45RA^+$ T cells responded to the autoantigen, β2 glycoprotein I, meanwhile $CD45RO^+$ T cells respond to the memory antigen, tetanus toxoid. Furthermore, when CD45RA+ T cells separated into CD9+ and CD9- subsets by cell sorting, $CD9^+CD45RA^+$ T cell population maximally responded to β2 glycoprotein I compared to $CD9^-CD45RA^+$ T cell population. These results suggest that $CD9^+CD45RA^+$T cells contain autoreactive T cells and CD9 regulates the nature of the function of this subset via costimulation and/or apoptosis. Further work will also have to determine whether $CD9^+$ T cell plays a role in autoimmune disease.

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

Chikao Morimoto, Kei Ohnuma, Akikazu Murakami, Hiroshi Kobayashi, Satoshi Iwata, Osamu Hosono, Hiroshi Kawasaki, and Hirotoshi Tanaka(in collaboration with Nam H Dang, MD Anderson Cancer Center, USA, and with Fafeal Fraonco, University of Barcelona, Spain)

CD26 is a 110kDa cell surface glycoprotein that possesses dipeptidyl peptidase IV (DPPIV; EC 3.4.14.5)[3] activity in its extracellular domain, and is known to bind adenocine deaminases and plays an important role in T-cell activation. It has been reported several chemokines such as RANTES and SDF-1α was the substrates for this DPPIV enzyme. CD26 expression level is tightly regulated on T cells, and its density is markedly enhanced after T cell activation. In the resting state of T cells, CD26 is expressed on a subset of $CD4^+$ memory T cells, and this $CD4^+CD26^{high}$ T cell population has been shown to

respond maximally to recall antigens. In fact, CD26 itself is involved in the signal transduction process of T cells. Cross-linking of CD26 and CD3 with immobilized monoclonal antibodies (mAbs) can induce T cell activation and IL-2 production. Moreover, anti-CD26 antibody treatment of T cells leads to a decrease in the surface expression of CD26 via its internalization, and this modulation of CD26 on T cells results in an enhanced proliferative response to anti-CD3 or anti-CD2 stimulation. Currently, we are focusing on molecular basis for CD26-mediated T cell costimulation, enhancement of memory T cell responses by soluble CD26. Moreover, we are focusing on utilization of anti-CD26 monoclonal antibody and soluble CD26 molecule for treatment of CD26 positive and negative malignant tumors as well as immune-mediated diseases.

a. CD26-mediated signaling for T cell activation occurs in lipid rafts through its association with CD45RO

CD26 is a T cell activation antigen that contains dipeptidyl peptidase IV activity and is known to bind adenosine deaminase. The mechanism by which CD26 costimulation potentiates T cell receptor-mediated T cell activation, leading to subsequent exertion of T cell effector function, is still not clearly defined. In this article, we demonstrate 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. Moreover, we show that CD26 directly binds to the cytoplasmic domain of CD45. Our results therefore indicate a mechanism whereby CD26 engagement promotes aggregation of lipid rafts and facilitates colocalization of CD45 to T cell receptor signaling molecules p56(Lck), ZAP-70, and TCRzeta, thereby enhancing protein tyrosine phosphorylation of various signaling molecules and subsequent interleukin-2 production.

b. Expression of CD26 and its associated dipeptidyl peptidase IV enzyme activity enhances sensitivity to doxorubicin-induced cell cycle arrest at the G(2)/M checkpoint

CD26, a M(r) 110,000 surface-bound ectopeptidase with dipeptidyl peptidase IV (DPPIV) activity, has an array of diverse functional properties, with a role in T-cell physiology and the development of certain human cancers. In this study, we report that surface expression of CD26, through its associated DPPIV enzyme activity, enhanced sensitivity of Jurkat T-cell transfectants to G(2)-M arrest induced by the chemotherapeutic drug, doxorubicin. This was associated with disruption of cell cycle-related events, including hyperphosphorylation and inhibition of p34(cdc2) kinase activity, phosphorylation of cdc25C, and alteration in cyclin B1 expression. In addition, we demonstrate that the addition of exogenous soluble DPPIV enhanced sensitivity of lymphoid tumor cell lines to doxorubicin, suggesting a potentially useful clinical role for CD26/DPPIV in the treatment of selected human hematological malignancies.

c. Comodulation of CXCR4 and CD26 in human lymphocytes

We provide convergent and multiple evidence for a CD26/CXCR4 interaction. Thus, CD26 codistributes with CXCR4, and both coimmunoprecipitate from membranes of T (CD4(+)) and B (CD4(-)) cell lines. Upon induction with stromal cell-derived factor 1alpha (SDF-1alpha), CD26 is cointernalized with CXCR4. CXCR4-mediated down-regulation of CD26 is not induced by antagonists or human immunodeficiency virus (HIV)-1 gp120. SDF-1alpha-mediated down-regulation of CD26 is not blocked by pertussis toxin but does not occur in cells expressing mutant CXCR4 receptors unable to internalize. Codistribution and cointernalization also occurs in peripheral blood lymphocytes. Since CD26 is a cell surface endopeptidase that has the capacity to cleave SDF-1alpha, the CXCR4.CD26 complex is likely a functional unit in which CD26 may directly modulate SDF-1alpha-induced chemotaxis and antiviral capacity. CD26 anchors adenosine deaminase (ADA) to the lymphocyte cell surface, and this interaction is blocked by HIV-1 gp120. Here we demonstrate that gp120 interacts with CD26 and that gp120-mediated disruption of ADA/CD26 interaction is a consequence of a first interaction of gp120 with a domain different from the ADA binding site. SDF-1alpha and gp120 induce the appearance of pseudopodia in which CD26 and CXCR4 colocalize and in which ADA is not present. The physical association of CXCR4 and CD26, direct or part of a supramolecular structure, suggests a role on the function of the immune system and the pathophysiology of HIV infection.

3. Study on regulation of eukaryotic gene expression

Hirotoshi Tanaka, Yuichi Makino, Noritada Yoshikawa, Tetsuya Nakamura[1], Tsunenori Kodama, Rika Ouchida, Hiroshi Nakamura, Chikao Morimoto, Tetsuya Hisada, and Hirosi Handa[2] (in colaboration with Lorenz Poellinger Lab, Karolinska Institute, Sweden)

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

(i) Redox Regulation of the Glucocorticoid Receptor

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

(ii) Development of Dissociating Ligand for the Glucocorticoid Receptor

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

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

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

(i) Recruitment of the Transcriptional Coactivators CBP and SRC-1 to the Hypoxia-inducible Factor-1α

HIF-1α functions as a transcription factor that is activated by decreased cellular oxygen concentrations to induce expression of a network of genes involved in angiogenesis, erythropoiesis, and glucose homeostasis. Here we demonstrate that two members of the SRC-1/p160 family of transcriptional coactivators harboring histone acetyltransferase activity, SRC-1 and TIF2, are able to interact with HIF-1α and enhance its transactivation potential in a hypoxia-dependent manner. HIF-1α contains within its C-terminus two transactivation domains. The hypoxia-inducible activity of both these domains was enhanced by either SRC-1 or the CBP/p300 coactivator. Moreover, SRC-1 produced this effect in synergy with CBP. Interestingly, this effect was strongly potentiated by the redox regulatory protein Ref-1, a dual function protein harboring DNA repair endonuclease and cysteine reducing activities. These data indicate that all three proteins, CBP, SRC-1, and Ref-1, are important components of the hypoxia signaling pathway and have a common function in regulation of HIF-1α function in hypoxic cells.

(ii) Transcriptional Network Controlling Angiogenesis in Health and Diseases

Angiogenesis is regulated by a combination of variety factors including transcription factors. Recently, we have isolated cDNA encoding the novel protein IPAS which can squelch HIF-1α. Moreover, its tissue-specific expression argues the physiological role of transcriptional network for orchestrated regulation of angiogenesis. We are currently studying the molecular mechanism of the interaction between HIF-1α and IPAS. This negative regulatpr may also therapeutically applicable for treating a number of angiogenic disorders including cancer, diabetic retinopathy, and rheumatoid arthritis. On the other hand, we have recently identified that HIF-1α function is regulated in a various fashion in certain physiological settings, which may be important of homeostatic control of tissue function. In this line, we are now identifying the molecular mechanism for such regulation of HIF-1α.

4. Immunobiology of chemokines and chemokine receptors

Hiroshi Kawasaki and Chikao Morimoto (in collaboration with Takahashi TA, Sato K, Matsuyama T, and Hirai K)

We have been pursuing the structure and functional analysis of human chemokine/chemokine receptor system in order to clealy address their roles in innate and acquired immunity. Since the elucidation of the CXCR-4 and CCR-5 as the coreceptors for T-tropic HIV and M-tropic HIV,respectively, this area of immunemediators has drawn tremendous attention.

a. Signaling events following chemokine receptor ligation in human dendritic cells at different developmental stages

We analyzed the mechanism underlying these events. Cell-surface expression of CC chemokine receptor (CCR)-1, -3 and -5 was increased during differentiation of immature DC (iDC) from monocytes. In contrast, these expressions were decreased during development of iDC into mature DC (mDC) to levels similar to those of monocytes. Transcriptional expression of CCR-1, -3 and -5 was increased during differentiation of iDC from monocytes, while the expression was decreased during development of iDC into mDC. Expression of CCR-7 transcript was detected in mDC, but not in monocytes or iDC. Both monocytes and iDC, but not mDC, migrated in response to inflammatory CC chemokines such as regulated on activation normal T cell expressed and secreted (RANTES)/CCL5, whereas mDC, but not monocytes or iDC, migrated to macrophage inflammatory protein (MIP)-3ss/CCL19. Receptor engagement of monocytes or iDC by RANTES (for CCR-1, -3 and -5) resulted in protein tyrosine phosphorylation events including activation of focal adhesion kinase as well as mitogen-activated protein kinase, whereas this stimulation induced little activation of these molecular events in mDC when compared with monocytes or iDC. On the other hand, stimulation with MIP-3ss (for CCR-7) induced tyrosine phosphorylation events in mDC, but not in monocytes or iDC. These results suggest that the down-regulation of cell-surface expression of CCR and of their downstream signaling events may be involved in the reduced chemotaxis of DC to inflammatory CC chemokines during their maturation.

b. Molecular mechanism of the hyporesponsiveness of human naïve CD4$^+$T cells to CCL5

Human memory CD4$^+$T cells exhibit more potent responsiveness to inflammatory CCLs, CCL3 and CCL5, than naive CD4$^+$T cells. We analyzed the molecular mechanism underlying this event. Memory and naive CD4$^+$T cells expressed similar high level of CC chemokine receptor (CCR)1 while CCR5 was only expressed in memory CD4$^+$T cells at low level. Blocking experiments with monoclonal antibodies (mAbs) to several chemokine receptors revealed that CCR1 functioned a major receptor for the binding of CCL5 as well as ligand-induced chemotaxis in both cell types. Stimulation of memory CD4$^+$T cells with CCL5 activated protein tyrosine kinase (PTK)-dependent cascades, which was 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 (RGS) 3 and RGS4 were only detected in naive CD4$^+$T cells. Thus, abortive CCR1-mediated PTK-dependent downstream signaling event as well as the deficiency of CCR5 expression may be involved in the hyporesponsiveness of naive CD4$^+$T cells to CCL3 and CCL5.

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

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.

d. Chemokine receptors in human basophils: inducible expression of functional CXCR4

We examined the expression profile of chemokine receptors in human basophils and their regulation by cytokines. Basophils expressed transcripts of CC chemokine receptors (CCR)1, CCR2, CCR3, and CCR5 and CXC chemokine receptors (CXCR)1, CXCR2, and CXCR4. In contrast to the other receptors, surface-CXCR4 expression was not detected in fresh- and whole-blood basophils, but it became apparent gradually during incubation. Among 16 chemokines tested, eotaxin induced the most potent basophil migration. SDF-1 also induced a strong, mi-

gratory response comparable with that induced by eotaxin in 24-h, cultured basophils, but it failed to induce degranulation. IL-3 abrogated CXCR4 expression completely, and it only down-regulated CCR2 and CCR3 expression slightly. IL-5, GM-CSF, and IL-4 also down-regulated CXCR4 expression. Thus, expression of CXCR4 was the most strongly affected by cytokines, and this may represent an alternative mechanism for control of cell-specific, biological responses to SDF-1.

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Advanced Clinical Research Center

Division of Medical Data Processing Network System

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. Some conceptual research programs and targets of our division are described.

1. 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 need EBM (Evidence Based Medicine) and require to research and develop the most appropriated clinical database and their processing system. Basic clinical database must 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 processed by the appropriate statistical method give us valuable information to find out the most appropriate clinical protocol. The research hospital has introduced clinical order entry system. New EBM-oriented sever computer system is going to operate in the background. Because the Research Hospital has many experiences in blood and immunity disease, EBM database will be expected to give us much useful information about the mechanism of these diseases.

2. 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 automati cDNA injection machine to human cell nucleus. This machine is expected also to be useful to inject variable drugs in human cell organs.

At first, the computational pattern recognition method of human cell organs must be develop. We are successful to recognize the fluorescence pattern in cell organ over about 90% efficiency. This result will be applied to the automatic DNA 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.

3. Computer Simulation System of Blood Differentiation and Immunity Mechanism

Many research projects started to study genomic diseases 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 bio-informatics technology under developed 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 kinds of blood and immunity diseases will be analized. This system will be hoped to contribute much to national health program of Japan.