Advanced Clinical Research Center
Division of Molecular Therapy

Our division 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. Oncogene inactivation in a mouse model tissue invasion by leukemic cells is stalled by loading them with a designer ribozyme

Tsuyoshi Tanabe, Kenzaburo Tani, et al.

Chronic myelogenous leukemia (CML) is a haematopoietic malignant disease associated with the expression of a chimeric BCR-ABL gene. We have designed an allosterrically controllable ribozyme that specifically cleaves BCR-ABL messenger RNA and induces apoptosis in cultured CML cells, and here we tested it as a possible treatment of CML in a mouse model. We found that this ribozyme completely inhibited tumour-cell infiltration in these mice. To our knowledge, this is the first application of an artificial, allosterically controllable enzyme in animals, opening up the possibility of using ribozyme technology in the treatment of CML.

2.IL-12 Synergized with B7-1 Enhance the Anti-tumor Immunity in C57BL/6 Mice

Zhihua Wang, Kenzaburo Tani, et al.

In this study, we have constructed IL-12, B7-1 expressing vector using retrovirus, which were transfected into EL-4 thymic lymphoma cells respectively and investigated its anti-tumor immunity. The tumorigenicity was decreased significantly in C57BL/6 syngeneic mice after implanted with EL-4/IL-12 transfectant compared with EL-4/Wt or EL-4/IL-12 transfectant compared with EL-4/Wt or EL-4/Neo groups ($p<0.01$). Therapeutic vaccines with EL-4/IL-12 and EL-4/B7-1 cells could retard significantly the growth of established EL-4/Wt tumor compared with those of EL-4/Neo ($p<0.005$). The combination treatment of EL-4/IL-12 and EL-4/B7-1 enhanced the therapeutic effect ($p<0.005$) in this experimental model. These studies suggested that immunogene treatment using IL-12 was effective in hematopoietic malignancy, and combination treatment of IL-12 and B7-1 had as application value in human cancer treatment in the near future.

3. Haematopoietic Progenitor Cells from Common Marmoset as Targets of Gene Transduction by Retroviral and Adenoviral Vectors

Hitoshi Hibino, Kenzaburo Tani, et al.

To establish a new non-human primate model for human cytokine and gene therapy, we characterized lymphocytes and haematopoietic progenitor cells of the small New World monkey, the common marmoset. We first assessed the reactions of marmoset bone marrow (BM) and peripheral blood (PB) cells to mouse anti human monoclonal antibodies (mAbs) for the purpose of isolating marmoset lymphocytes and haematopoietic progenitor cells. Both cell fractions stained with CD4 and CD8 mAbs were identified as lymphocytes by cell proliferation assay and morphological examination. Myeloid specific mAbs such as CD14 and CD33 did not react with marmoset BM and PB cells. No available CD34 and c-kit mAbs could be used to purify the marmoset haematopoietic progenitor cells.

Furthermore, we studied the *in vitro* transduction of the bacterial (β-galactosidase (LacZ) gene into

CFU-GM derived from marmoset BM using retroviral and adenoviral vectors. The transduction efficiency was increased by using a mixed culture system consisting of marmoset BM stromal cells and retroviral producer cells. It was also possible to transduce LacZ gene into marmoset haematopoietic progenitor cells with adenoviral vectors as well as retroviral vectors. The percentage of adenovirally transduced LacZ-positive clusters was 15% at day 4 (multiplicity of infection=200), but only 1-2% at day 14. The differential use of viral vector systems is to be recommended in targeting different diseases. Our results suggested that marmoset BM progenitor cells were available to examine the transduction efficiency of various viral vectors *in vitro*.

4. MHC (Major Histocompatibility Complex) - DRB Genes and Polymorphisms in Common Marmoset

Ming-Shiuan Wu, Kenzaburo Tani, et al.

A New World monkey, the common marmoset (*Callithrix jacchus*), will be used as a preclinical animal model to study the feasibility of cell and gene therapy targeting immunological and hematological disorders. For elucidating the immunogenetic background of common marmoset to further studies, in the present study, polymorphisms of MHC-DRB genes in this species were examined. Twenty-one Caja-DRB exon 2 alleles, including seven new ones, were detected by means of subcloning and the polymerase chain reaction-single strand conformation polymorphism (PCR-SSCP) methods followed by nucleotide sequencing. Based on the alignment of these allele sequences, we designed two pairs of specific primers and established a PCR-SSCP method for DNA-based histocompatibility typing of the common marmoset. According to the family segregation data and phylogenetic analyses, we presumed that Caja-DRB alleles could be classified into five different loci. Southern blotting analysis also supported the existence of multiple DRB loci. The patterns of nucleotide substitutions suggest that positive selection operates in the antigen-recognition sites of Caja-DRB genes.

5. Differential graft-versus-leukemia effect by CD28 and CD40 costimulatory blockade after graft-versus-host disease prophylaxis

Junko Ohata, Kenzaburo Tani, et al.

Costimulatory blockade may be a promising strategy for tolerance induction in transplantation. In allogeneic bone marrow transplantation (BMT) for leukemia treatment, however, preserved graft-versus-leukemia (GVL) effect is another critical requirement for clinical application. In this study, we have compared the GVL effect between CD28 and CD40 costimulatory blockade after graft-versus-host disease (GVHD) prophylaxis in allogeneic BMT model with simultaneous transfer of BCL-1 leukemia. Despite the comparable improvement of GVHD mortality, CD40 blockade clearly accelerated the growth of leukemia, while CD28 blockade preserved the antileukemia responses. In splenocytes at 14 days post BMT, an obvious difference in IFNγ expression by T cells and cytotoxicity against alloantigen was not observed. Depletion of donor natural killer (NK) cells reduced the GVL effect at similar levels by both costimulatory blockade, suggesting the comparable inhibition of effector function by T and NK cells. On the other hands, CD40 blockade preferentially reduced IL-12 mRNA expression and a consequent $CD8^+$ T cell expansion. These results imply the importance of antigen-presenting cell (APC) activation and cytotoxic T lymphocyte generation for eliciting GVL effect and the dominant contribution of CD40 signal in this process. This gives warning against the means of APC inactivation for tolerance induction in leukemia patients undergoing BMT.

6. Rapid tumor formation and development of neutrophilia and splenomegaly in nude mice transplanted with human cells expressing human T cell leukemia virus type I or Tax1

Yasushi Soda, H Hoshino, et al

Human T cell leukemia virus type I (HTLV-I) or its transcriptional transactivator, Tax1, was introduced into a human osteosarcoma cell line, HOS, and a Moloney murine sarcoma virus-positive HOS cell line, S+L-HOS. These HTLV-I- or Tax1-expressing cells were injected subcutaneously into nude mice to investigate the effects of HTLV-I on their tumorigenicities. HOS cells did not form any tumors even in the presence of HTLV-I or Tax1. S+L-HOS cells did form small tumors in two-thirds of nude mice. Infection of S+L-HOS cells with HTLV-I, or transduction of Tax1 into S+L-HOS cells markedly facilitated the tumor formation, and the tumor-bearing mice showed marked splenomegaly and neutrophilia. Elevated levels of granulocyte colony-stimulating factor (G-CSF) were detected in sera of these mice and also in the culture supernatants of Tax1-expressing human cells, suggesting that G-CSF in the mouse sera was produced by the human cells. In sera of some mice with splenomegaly and neutrophilia, high levels of murine granulocyte-macrophage colony-stimulating factor (mGM-CSF) were observed, suggesting that Tax1 produced by human cells induced mouse cells to produce mGM-CSF. Only S+L-HOS cell lines expressing Tax1 showed high tumorigenicity in nude mice. Thus, this system will be a useful model of tumor formation, splenomegaly and neutrophilia dependent on Tax1.

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Advanced Clinical Research Center
Division of Cellular Therapy

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 and Shigetaka Asano

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, Kazuki Nakao[1], Motoya Katsuki[1], Kohichiro Tsuji and Shigetaka Asano: [1]Laboratory of DNA Biology and Embryo Engineering, Center for Experimental Medicine, IMSUT

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 embryografting 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 and Shigetaka Asano

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 and Shigetaka Asano

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 and Shigetaka Asano

In the present study, we examined the expression of Flk2/Flk3, a tyrosine kinase receptor, on human CB $CD34^+$ hematopoietic progeniotr/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

Hiroaki Hisakawa, Sumiko Watanabe[2], Daisuke Sugiyama, Tatsutoshi Nakahata, Kohichiro Tsuji and Shigetaka Asano:[2]Division of Molecular and Developmental Biology, IMSUT

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. Erythroid progenitors differentiate and mature by self-produced erythropoietin

Takeshi Sato, Taira Maekawa[3], Sumiko Watanabe[2] and Kohichiro Tsuji:[3]Department of Transfusion Medicine, IMSUT

We previously reported that stimulation of glycoprotein (gp) 130 by a combination of human soluble IL-6 receptor (sIL-6R) and IL-6 could support proliferation, differentiation and terminal maturation of erythroid cells in the absence of EPO from human $CD34^+$ cells in culture with SCF. This observation suggested that differentiation of hematopoietic stem/progenitor cells to erythroid cells was progressed along an intrinsic program, and that EPOR could be replaced by other cytokine receptors and was dispensable for erythropoiesis. We then examined the role of EPOR in erythropoiesis stimulated by SCF, sIL-6R and IL-6. Surprisingly, elimination of EPOR expression by antisense oligodeoxynucleotide suppressed erythropoiesis stimulated not only by SCF and EPO but also by SCF, sIL-6R and IL-6. EPO mRNA was detected in the erythroid cells but not myeloid cells cultured in the presence of SCF, sIL-6R and IL-6. Furthermore, high concentrations of anti-EPO neutralizing antibody abrogated erythropoiesis in cultures without extrinsic EPO. Based on these results, we suggest that erythroid progenitors *per se* secrete EPO and have the potential to differentiate and maturate in an autocrine manner by self-produced EPO.

8. Mechanisms regulating the polyploidization and cytokinesis in human megakaryocytes

Ryuhei Tanaka, Sachiyo Hanada, Tetsuya Nosaka[4], Toshio Kitamura[4], Yasuhiro Ebihara, Kohichiro Tsuji and Shigetaka Asano:[4]Division of Hematopoietic Factors, IMSUT

Megakaryocytes are unique cells, developing a polyploid DNA content regularly during the normal life cycle of the cells. Understanding the true nature of megakaryocytic polyploidization and the mechanisms that control polyploidization in megakaryocytes have been hampered partly because there have been no suitable model system for resolving these issues. In the present study, we have established a novel factor-dependent human leukemia cell line YMP91 from peripheral blood of a patient with acute magakaryoblastic leukemia. The

expression of c-Kit and gp130 on the YMP91 cells has been confirmed by flow cytometric analysis, and their proliferation depends on both signals from c-Kit and gp130 by SCF and IL-6/sIL-6R, respectively.

Four independent clones of YMP91, designated YMP91-A, -B, -C, and -D, have been established by limiting dilution methods in the presence of SCF and IL-6/sIL-6R. The cells in the cytospin smear of YMP91-C are heterogeneous in size, and large polyploid cells can be observed in 10 - 20 %, whereas the cells of YMP91-A are homogeneous in size and there are no multinucleated cells when they are cultured with SCF and IL-6/sIL-6R. Platelet peroxidase (PPO) activity examined by electron microscopic analysis of YMP91-A and -C is negative and scantily positive, respectively. The maturation process of YMP91-A and -C as megakaryocyte is promoted when cultured with IL-3 or erythropoietin (EPO). In response to EPO, YMP91-A and -C exhibit significant increment in PPO activity. DNA content analysis of these sublines showed emergence and increment of cells in high ploidy class (i.e., 8N or more) in the presence of EPO or IL-3. These data indicate that the megakaryocytic leukemia cell line YMP91 is able to traverse maturational processes of megakaryocytes in response to cytokines and its sublines show distinct phenotypes in respect of megakaryocytic maturation. YMP91 and its sublines may thus be expected to serve a model system for clarifying the mechanisms regulating the megakaryocytepoiesis particularly the relationship between poloploidization and cytokinesis in megakaryocytes.

9. Cytokine requirement for the development of T-lymphoid lineage potential in clonal lymphohematopoietic progenitors *in vitro*

Feng Ma, Mika Wada, Kohichiro Tsuji and Shigetaka Asano

The early process of T cell development prior to thymic colonization has been poorly investigated because of a lack of a sensitive assay. In this study, we developed a two-step *in vitro* culture system combining a clonal culture with FTOC. Immature colonies cultured from BM cells of 5-fluorouracil (5FU)-treated mice by various combinations of early-acting cytokines were transferred into FTOC. All the combinations among SCF, IL-3 and IL-6 capable of inducing the colony formation supported T cell generation. IL-11 and Flt3 ligand possessed similar activities to IL-6 and SCF, respectively. Thus, there seemed to be no specificity in cytokines required for the generation of T lineage potential from lymphohematopoietic progenitors, if their mitosis was induced, suggesting that their differentiation to T lineage may be an event determined intrinsically rather than induced by specific stimuli. Furtheremore, we examined T lineage potential in the colonies derived from $Lin^-c\text{-}Kit^+Sca^-1^+$ cells clone-sorted from post-5FU marrow cells. While no colonies containing only myelocytic progenitors revealed T lineage potential, 19% of colonies with a hematopoietic multipotentiality did. Therefore, the branching of T lineage potential may occur in early stage of the hierarchy of hematopoiesis. The developed method should prove valuable for the exploration of molecular and cellular changes occuring during early T cell development before thymic colonization.

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

Koichiro Miyamoto, Kohichiro Tsuji and Tatsutoshi Nakahata

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-3Rα^+ and IL-3Rα^- 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. Studies on HIV-1 infected lon-term non-progressors: Comparison of proviral accessory genes between long-term nonprogressors and progressors of human immunodeficiency virus type 1 infection

Takeshi Yamada and Aikichi Iwamoto.

We compared proviral accessory gene sequences (*nef, vpu, vpr, vif, rev, tat*) of human immunodeficiency virus type 1 (HIV-1) in the peripheral blood mononuclear cells (PBMC) between seven long-term non-progressors (LTNP) and seven progressors. LTNP frequently harbored proviruses with mutated accessory genes, while almost all accessory genes were intact in progressors. Presence of quasispecies in attenuated viruses suggests that they were not just a fossil record of nonreplicating proviruses. These attenuated viruses in LTNP may be the source of their strong immune response against HIV-1. Our result might have relevance to a design of potent therapeutic vaccines.

2. Typing Pneumocystis carinii f. sp. hominis isolates in Japan

Hosoya, N., Takahashi, T., Wada, M., Endo, T., Nakamura, T., Sakashita, H., Kimura, K., Ohnishi, K., Nakamura, Y., Mizuochi, T., and Iwamoto, A.

Pneumocystis carinii (*P. carinii*) , a fungus, causes opportunistic pneumonia (PCP) frequent in immunocompromised patients, especially those with HIV-1 infection.

Genotyping of *P. carinii* isolated from 24 bronchoalveolar lavage (BAL) fluid obtained from patients with PCP in Japan was carried out based on nucleotide sequence variations in internal transcribed spacer regions 1 and 2 (ITS1 and ITS2, respectively) of rRNA genes. We found 11 ITS1 genotypes including 2 novel ones and 11 ITS2 genotypes including 3 new ones. Combination of ITS1 and ITS2 genotypes resulted in 30 ITS genotypes of which 10 were newly described in this report. Two or more genotypes in ITS regions in a specimen were observed in 16 of 24 patients. Our results will be of help for epidemiological investigation of *P. carinii* infection.

We next examined mutations in the dihydropteroate synthase (DHPS) gene of *P. carinii* isolated from 24 patients with PCP in Japan. DHPS mutations were identified at amino acid positions 55 and/or 57 in 6 (25.0 %) of 24 patients, who had the underlying diseases of 4 human immunodeficiency virus-1 infections and 2 malignant lymphomas. This frequency was almost same with those reported from Denmark and USA. None of six patients with DHPS mutations were recently exposed to sulfa drugs before they developed the current PCP, suggesting that DHPS mutations are not only selected by the pressure of sulfa agents, but may be incidentally acquired. Co-trimoxazole treatment failed more frequently in patients with DHPS mutations than in those who had wild-type DHPS [n=4 (100%) versus n=2 (11.1%), p=0.002]. Our results thus suggest that DHPS mutations may contribute to failures of co-trimoxazole treatment for PCP.

3. Polymorphism in IL-4 promoter and CCR5 coding region

Emi E. Nakayama[1], Yoshihiko Hoshino[1], Xiaomi Xin[1,2], Huanliang Liu[1], Mieko Goto[1], Nobukazu Watanabe[1], Hitomi Taguchi[1], Akihiro Hitani[1], Ai Kawana-Tachikawa[1], Masao Fukushima[2], Kaneo Yamada[3], Wataru Sugiura[4], Shin-Ichi Oka[5], Atsushi Ajisawa[6], Hironori Sato[4], Yutaka Takebe[4], Tetsuya Nakamura[1], Yoshiyuki Nagai[2,4], Aikichi Iwamoto[1], and Tatsuo Shioda[1]:[1]Division of Infectious Diseases, Advanced Clinical Research Center and [2]Division of Viral Infection, [3]Institute of Medical Science, University of Tokyo, St. Marianna University School of Medicine, [4]AIDS Research Center, National Institute of Infectious Diseases, [5]AIDS Clinical Center, International Medical Center of Japan, and Department of Infectious Diseases and [6]Tokyo Metropolitan Komagome Hospital, Tokyo, Japan

The emergence of syncytium-inducing (SI) variants of human immunodeficiency virus type 1 (HIV-1) in infected individuals is an indicator of poor prognosis and is often correlated with faster CD4+ cell depletion and rapid disease progression. Interleukin-4 (IL-4) is a pleiotropic cytokine with various immune-modulating functions including induction of immunoglobulin E (IgE) production in B cells, down-regulation of CCR5 (a coreceptor for HIV-1 non-SI [NSI] strains), and up-regulation of CXCR4 (a coreceptor for HIV-1 SI variants). Here we show that homozygosity of a polymorphism in the IL-4 promoter region, IL-4 589T, is correlated with increased rates of SI variant acquisition in HIV-1-infected individuals in Japan. This mutation was also shown to be associated with elevated serum IgE levels in HIV-1-infected individuals, especially in those at advanced stages of disease. In contrast, neither a triallele polymorphism in IL-10, another Th2 cytokine, nor a biallele polymorphism in the RANTES promoter affected acquisition of the SI phenotype. This finding suggested that IL-4-589T increases IL-4 production in the human body and thus accelerates the phenotypic switch of HIV-1 from NSI to SI and possibly disease progression of AIDS.

4. Polymorphisms in MIP1-α gene

Xiaomi Xin[7], Koichiro Nakamura[8], Huanliang Liu[9], Emi, E. Nakayama[9], Mieko Goto[7], Yoshiyuki Nagai[10], Yoshihiro Kitamura[7]*, Tatsuo Shioda[9], and Aikichi Iwamoto[7]:[7]Division of Infectious Diseases, Advanced Clinical Research Center, Institute of Medical Science, and [8]Department of Dermatology, Faculty of Medicine, University of Tokyo, Tokyo, Japan, [9]Department of Immune and Living Body Defense, Institute of Microbal Disease, Osaka University and [10]National Institute of Infectious Disease, Tokyo, Japan

Human macrophage inflammatory protein-1 alpha (MIP-1 α) is a chemotactic cytokine, which binds to macrophages, T cells, and B cells affecting their activation. We found novel polymorphisms at four sites of MIP-1 α 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-1 α 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-1 α 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). These data may prove useful for further investigation of a potential role of the MIP-1 α in atopic dermatitis.

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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 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. Functional analysis of Dendritic cells for antitumor immunology

Saori Nakahara, Takuya Tsunoda, Hideaki Tahara

a-1) Antitumor immunity induced by the streptococcal preparation OK432 activated DC

Recently dendritic cells (DC) are revealed that they are potent antigen presenting cells and initiate immune responses. But in human anticancer immunotherapy using DC, clinical response is not still satisfactory. The purpose of our study is to investigate the effect of OK-432 (Streptococcal preparation, clinical grade in Japan) on DC, and to evaluate that OK-432 is a potent adjuvant for DC therapy or not. We examined the effect of OK-432 by follows; 1. The change of phenotype of DC with flow cytometry (FACS) 2. The change of endocytosis capability of DC 3. Cytokine inducing capacity. We report OK-432 efficiently leads DC maturation and induce IFN-γ production. Peripheral blood was obtained from healthy donors. After separation with Ficoll-Paque, mononuclear cells were adhered on 6-well plates for 2 hours. Adherent cells were cultured in AIM-V medium with 1000U/ml GM-CSF and 1000U/ml IL-4 for 6 days. Non-adherent cells were cultured in AIM-V medium without cytokines for 6 or 8 days and used as a responder for IFN-γ induction assay. The immunophenotype of Day 6 DC was CD1a-, CD14-, CD40+, CD80-, CD83-, CD86+, HLA-ABC+, HLA-DR+ by FACS, and it was consistent with immature DC. On day 6, DC were stained with red fluorochrome, and added FITC labeled dextran (FITC-DX) at 37℃ and 0℃. After endocytosis of dextran, DCs were detected as double positive cells by FACS analysis. More than 85% of DCs showed the ability to endocytose at 37℃, and no more than 10% of DCs were double stained at 0℃. On day 6, the DCs were divided into 2 groups; A. cultured with TNF-γ at 10 ng/ml. B. cultured with OK-432 at 10μg/ml without additional cytokines. On day 8, the cells were harvested for further analyses. Both Group A and B showed the feature of mature DC: CD80+, CD86+, CD83+, HLA-ABC++, HLA-DR++. Group B showed a slightly higher expression of CD83 than Group A. On day 8, DC lost the ability to endocytose of dextran in both conditions. The capacity of IFN-γ induction was tested by mixed lymphocyte reaction. DC stimulated with OK-432 showed the high ability to induce IFN-γ, whereas neither day 6 DC nor DC stimulated with TNF-α induce IFN-γ at all.

We conclude that streptococcal preparation OK-432 can lead DC maturation and stimulate Th1 response. OK-432 will be one of good candidates of adjuvant for DC therapy, especially in the case of mature DC is preferred, like peptide pulsed DC. The analyses of the effect of OK-432 on DC migration and CTL induction are now ongoing.

a-2) Development for new immunogenic compounds that activate DC

The polysaccharides from Lentinus edodes, Lentinan (LNT), were found in 1969. LNT has the multiple functions to augment the immunological response *in vivo* and *in vitro*. However, to our knowledge, there was no report to investigate the function of DC. Interestingly, it is easy to make the conjugates with LNT and tumor antigens i.e. CEA. Our goal of

this study is, at first, that LNT demonstrates the function of DC, and that LNT conjugated CEA shows the strong immunostimulants to augment the antitumor effects *in vitro* and *in vivo*. As results, LNT does not change the maturation of DC by analyses of cell surface makers, CD80, CD83, CD86. On the other hands, LNT strongly augments the endocytosis of DC *in vitro*. The analysis of the effect of LNT on CTL induction is now ongoing. Furthermore, CEA transgenic mouse will be utilized to investigate the LNT conjugates *in vivo*.

b. Mapping the p53 epitope peptide restricted HLA-A*2402 and development of strong immunodominant mutated peptide

Saori Nakahara[1], Hiroyuki Baba[1], Hiroyuki Mushiake[1], Takuya Tsunoda[1], Hideaki Tahara[1] and Yasukazu Umano[2]:[1]Department of Surgery and Division of Bioengineering (IMSUT) and [2]Second Department of Surgery, Wakayama Medical School

The p53 protein is an attractive target for immunotherapy. Because mutations in the p53 gene are the most common genetic alterations found in human tumor, these mutations result in high levels of p53 protein in the tumor cell. Whereas the expression level of wild type p53 in non-malignant tissue is usually much lower. We tested p53 encoded HLA-A24 binding peptides for their capacity to elicit anti tumor cytotoxic T lymphocytes (CTL) *in vitro*. These peptides were predicted from murine p53-derived cytotoxic peptides which being presented to CTL by H-2K^d and H-2K^b molecules. Because the HLA-A24 peptide binding motifs are similar to the H-2K^d and H-2K^b one. For CTL induction, we used CD8$^+$ T lymphocytes from peripheral blood mononuclear cells (PBMC) of a healthy donor and peptide pulsed autologous dendritic cells (DC) as antigen presenting cells (APC). We can identify a peptide, PU161 (AIYKQSQHM), which was capable of eliciting CTL lines that lysed tumor cells expressing HLA-A24 and p53. The effectors lysed C1RA24 cells (p53$^+$, HLA-A*2402 transfectant), but not its parent cell lines C1R (p53$^+$, HLA-A, B, C null cell). These results indicate that the cytotoxic activity of the CTL showed HLA-A24 restricted manner. In addition, to potentially increase binding affinity and immunogenicity while retaining p53 specificity, we investigated a new synthetic peptide (PU161Y2L9: AYYKQSQHL) modified at anchor residues to enhance binding to HLA-A24.

The identification of this novel p53 epitope for CTL offers the possibility to design and develop epitope based immunotherapeutic approaches for treating HLA-A24 positive patients with tumors that express p53.

2. Mechanistic study on transplantation immunology

Evaluation of *in vivo* tolerogenicity of genetically engineered recipient dendritic cells pulsed with immunogenic peptides derived from donor HLA molecule in HLA class I transgenic mouse.

Sumito Tamura, Yoshifumi Beck, Hideaki Tahara

Dendritic cells (DC), a potent antigen presenting cell (APC), not only activates lymphocytes but also tolerizes T cell to specific antigens under certain conditions. Genetic engineering of DC to express immunosuppressive molecules is known to potentiate its tolerogenic capacity. We earlier created two strains of transgenic mouse (TGM), HLA-B35TGM and HLA-B51TGM, both with C3H back ground which are genetically identical except for HLA class I. Using these strains, we demonstrated intrathymic administration of donor derived immunogenic HLA peptides induce donor specific tolerance *in vivo*. We will evaluate the tolerogenic capacity of genetically modified DC by using this TGM pair, an established *in vivo* model.

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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.

I. Role of β1 integrin molecule and its association molecules in T cell signaling and function in normal and disease conditions.

Satoshi Iwata, Hiroshi Kobayashi, Rikako Nishijima, Mamoru Nori, Akiko Souta-Kurihara, Osamu Hosono, Hiroshi Kawasaki, Hirotoshi Tanaka, and Chikao Morimoto

The β1 integrins are the major adhesion molecules in T cells and are involved in the trafficking of T cells. Recent studies have clearly shown that, in addition to their role in cell adhesion, β1 integrins transduce signals into the interior of cells and induce various biological events such as cell differentiation, proliferation, survival, migration, and cytoskeleton organization. The binding of T cells to components of the extracellular matrix (ECM) through β1 integrins provides costimulatory signals to CD3-dependent T cell proliferation and IL-2 production. One of the major biochemical signals induced by β1 integrin stimulation is tyrosine phosphorylation of various proteins, including phospholipase C (PLC)-γ, focal adhesion kinase (pp125FAK), paxillin, p59Fyn/p56Lck, mitogen-activated protein (MAP) kinase, and pp105/Cas-L in T cells. Therefore, tyrosine phosphorylation of these proteins appears to be involved in β1 integrin-mediated costimulation in T cells.

Recently, we cloned a p130Cas (Crk-associated substrate)-related protein, pp105/Cas-L, and demonstrated that Cas-L is expressed abundantly in lymphocytes. Cas family proteins (p130Cas, Cas-L/Hef-1) share the common structural characteristics of a N-terminal SH3 domain, followed by multiple YXXP motifs and a YDYVHL motif, which are potential binding sites for SH2 domains. These structural analyses indicate that Cas family proteins are docking proteins that participate in tyrosine phosphorylation-mediated signaling pathways; however, their biological significance remains unclear. More recently, we found that pp125FAK phosphorylates tyrosine residues of Cas-L and p130Cas following β1 integrin stimulation. Since p130Cas is not detected in lymphocytes, Cas-L might be a major Cas family protein involved in generation of the immune response. We have reported that tyrosine-phosphorylated Cas-L binds to the SH2 domain of Crk, Nck, and SHP2 following β1 integrin stimulation in T cells. These biochemical findings suggest the involvement of Cas-L in β1 integrin-mediated signaling in T cells. Currently, we are focusing on the biological significance of Cas-L and downstream signaling events of Cas-L as well as the

function of β1 integrin associated molecules such as CD82 and CD9 in human T cells as well as in cancer cells. Moreover, we are focusing on the clinical significance of the above molecules on the various immune-mediated disorders.

a. Role of pp105/Cas-L in pathogenesis of arthritis in HTLV-I tax transgenic mouse

Satoshi Iwata, Rikako Nishijima, Shinobu Saijo[1], Hiroshi Kobayashi, Akiko Souta-Kurihara, Osamu Hosono, Hiroshi Kawasaki, Hirotoshi Tanaka, Youichiro Iwakura [1], and Chikao Morimoto: [1]Laboratory of Cell Biology, Center for Experimental medicine, Institute of Medical Science, University of Tokyo

Crk-associated substrate lymphocyte-type (Cas-L) is a 105kDa docking protein that is heavily tyrosine phosphorylated upon the engagement of β1 integrins in T cells. Accumulating evidences have suggested that Cas-L plays curtail roles in β1 integrin-mediated costimulation of IL-2 production and cell migration in T cells. Cas-L is overexpressed in various HTLV-I-transformed T cell lines. Furthermore, we have found that induction of tax gene, HTLV-I-encoded transactivator, caused marked increase in Cas-L proteins in JPX-9 cells. To further investigate the role of Cas-L in HTLV-I-mediated diseases, we first utilized HTLV-I tax transgenic mouse, which has been shown to develop RA like arthritis. Transendothelial migration assay revealed that migratory activity of spleen cells from tax transgenic mice with arthritis (Atg) was much higher than that of those without arthritis (Ntg) and control mice. Immunoprecipitation analysis using the lysate of spleen cells has shown that heavily tyrosine-phosphorylated 78kD protein was precipitated by both anti-Cas-L and anti-phosphotyrosine Ab (4G10) in Atg and Ntg mice, of which amount was much greater in the case of Atg mice compared to that of Ntg mice. Furthermore, major phosphorylated protein was 78kD and 105kD/Cas-L, although 78kD band was more strongly phosphorylated than 105kD/Cas-L.

Finally, the expression of 78kD protein was higher in the pre- or early stage of RA (4 weeks and 8 weeks) mice compared to the late stage of RA (16 weeks) mice.

These results suggest that spontaneous hyperphosphorylation of Cas-L-related pp78 might be involved in the development of arthritis in HTLV-1 tax transgenic mice. Since β1 integrins are reported to play a key role in triggering and maintaining inflammation of RA, the present findings strongly suggest that Cas-L protein appears to play an important role in the pathogenetic mechanism of RA.

b. Molecular cloning and functional analysis of 5H9 antigen, CD9 in human T cells

Hiroshi Kobayashi, Mamoru Nori, Akiko Souta-Kurihara, Satoshi Iwata, Rikako Nishijima, Osamu Hosono, Hiroshi Kawasaki, Hirotoshi Tanaka, and Chikao Morimoto

We have established monoclonal antibody 5H9 that preferentially reacted CD45RA positive human T cells. This antigen was up regulated on both CD45RA and CD45RO+ T cells upon stimulation with mitogen, suggesting that it is an activation antigen. Functionally, immobilized 5H9 mAb was shown to induce costimulatory effect with submitogenic doses of anti-CD3 mAb. These findings suggested that 5H9 antigen might be a novel antigen preferentially expressed on naïve T cells, and prompted us to identify the antigen by cDNA cloning. By expression cloning with retrovirus gene transfer system, we have isolated a cDNA clone that has 687 bp open reading frame. It has been shown that the clone coding for 5H9 antigen is identical to CD9, which belongs to Transmembrane 4 superfamily (TM4SF).

To investigate the biological role of CD9/5H9 antigen on CD45RA positive T cells, we first established stable transformant of CD9 molecule of Jurkat T cells that has a CD45RA+ phenotype. Surprisingly, liquid phase crosslinking of CD9 molecule alone significantly caused apoptosis of Jurkat cells, whereas that of CD82, another TM4SF molecule, CD28, and β1 integrins failed to do so. Time course experiment showed that apoptosis caused by CD9 begins 6 hrs after ligation, which is later than Fas-mediated one, suggesting that apoptosis caused by CD9 might be mediated by distinctive mechanism to Fas-mediated apoptosis. Next, we analyzed the effect of CD9 molecules on autologous MLR and autoantigen (we employed β2-GPI)-specific proliferative response, since autoreactive T cells have been reported to reside within the CD45RA+ T cell population. As a result, soluble 5H9 mAb significantly blocked autologous MLR. Furthermore, when CD45RA+ T cell population was separated into CD9+ and CD9- subsets by cell sorting, we have found that CD9+ CD45RA+ T cells show greater proliferative response against β2-GPI than CD9- CD45RA+ T cells. Together with these findings, CD9 might be involved in autoantigen responding population within CD45RA+ T cells.

c. Functional dissection of CD82 and VLA-4 in human T cells

Satoshi Iwata, Hiroshi Kobayashi, Rikako Nishijima, Mamoru Nori, Akiko Souta-Kurihara, Osamu Hosono, Hiroshi Kawasaki, Hirotoshi Tanaka, and Chikao Morimoto

Transmembrane 4 superfamily is an emerging group of cell surface molecules involved in cell mo-

tility and cell adhesion. A growing body of evidence has shown that molecules belong to TM4SF associate with a variety of integrins. CD82, a member of TM4SF, is a multifunctional molecule that is involved in cell activation, costimulation and cell spreading of T cells, HTLV-I-mediated cell fusion. Here we show that immobilized anti-CD82 mAbs as well as anti-α4β1 integrin mAbs induced tyrosine phosphorylation of pp105/Cas-L in peripheral T cells and HTLV-I-infected H9 cells. Furthermore, one of anti-CD82 mAbs, which induces homotypic aggregation of T cells and H9 cells, partially inhibited α4β1 integrin-mediated costimulation of T cells, whereas it failed to inhibit α5β1 integrin-mediated costimulation. In Jurkat T cells, in which Cas-L is marginally expressed, coligation of CD3 and β1 integrin failed to induce IL-2 production, whereas that of CD3 and CD82 markedly induce IL-2, suggesting the functional difference between CD82 and β1 integrin. Transcriptional analysis on enhancer elements in IL-2 gene revealed that activation of AP1 and NF-AT was selectively impaired in Jurkat T cells, when compared to the engagement of CD82. Among three anti-CD82 mAbs, stimulation with 8E4 selectively failed to induce AP1 element-mediated transcription, indicating that its insufficient activation on IL-2 promoter may attribute to its defect on the costimulatory activity in peripheral T cells.

In this study, we present supportive evidences of the involvement of TM4SF in β1 integrin-associated signaling and costimulation of T cells, and functional dissection of CD82- and β1-mediated signaling pathway.

d. Novel *in vitro* effect of Terfenadine in relation with T cell migration

Mamoru Nori, Satoshi Iwata, Yasuhiko Munakata[3], Tomonori Ishii[2], Rikako Nishijima, Hiroshi Kobayashi, Osamu Hosono, Hiroshi Kawasaki, Hirotoshi Tanaka, and Chikao Morimoto:[2]Division of Tumor Immunology, Dana-Farber Cancer Institute, Boston, MA, USA

Chemotaxis in response to antigenic (allergenic) stimulation is important process in allergic diseases, especially in the late phase responses. The effect of anti-allergic drugs on immune cell migration is not yet fully understood. To study the effect of terfenadine, a prototype non-sedating H1 receptor antagonist, on T cell function concerning the transendothelial migration. PHA-activated T cells were cultured on Transwells covered with HUVEC in the presence of terfenadine, and T cells migrating through the HUVEC monolayer and T cells attached on the HUVEC were counted. Immunofluorescence study was performed to analyze the effect of terfenadine on intracellular filamentation of actin fiber. Tyrosine phosphorylation of Crk-associated substrate lymphocyte-type (Cas-L), which is required for β1-integrin mediated signaling pathway, was also examined in the presence of terfenadine in relation to T cell motility. Terfenadine inhibited both transendothelial T cell migration and T cell adhesion to HUVEC in a dose dependent fashion. Furthermore, it inhibited the formation of filamentous actin and tyrosine phosphorylation of Cas-L in T cells. Terfenadine has the novel inhibitory effect on T cell adhesion and migration. This inhibitory effect may partly be attributed to the inhibition of cytoskeletal reorganization and the β1-integrin mediated signaling pathway, tyrosine phosphorylation of Cas-L.

II. 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

T cell activation antigen CD26 is the multifunctional 110kDa cell surface glycoprotein. Although the molecule is constitutively expressed in the liver, intestine and kidney, the expression level of CD26 is tightly regulated on T cells. The expression of the molecule is enhanced after activation of T cells, although it is expressed on a subset of CD4+ memory T cells in a resting state. In addition, $CD4^{+}$ $CD26^{high}$ T cells respond maximally to recall antigens.

CD26 has a dipeptidyl peptidase IV (DPPIV) activity in its extracellular domain. The enzyme can cleave amino-terminal dipeptides with either proline or alanine in the penultimate position. Recently, it has been reported that an amino-terminal truncation of RANTES by CD26/DPPIV provides a mechanism for regulation of its activity and the target cell specificity. On the other hand, CD26 interacts presumably in its extracellular domain with CD45, a protein tyrosine-phosphatase. In addition, the extracellular domain of CD26 on T cell surface forms a complex with adenosine deaminase (ADA), which reduces the immune suppression of local adenosine by its catalytic removal. The most striking evidence for the importance of ADA for immune system is that ADA deficiency results in severe combined immunodeficiency disease (SCID) in human.

CD26 is not only highly expressed on activated T cells, but itself is involved in the signal transducing process of T cells. Cross-linking of CD26 and CD3 with the immobilized monoclonal antibodies 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 molecule via the internalization, and such modulation results in an enhanced proliferative response to anti-CD3 or anti-CD2 stimulation, as well as an en-

hanced tyrosine phosphorylation of signaling molecules such as CD3ζ and $p56^{lck}$. From these observations, it is suggested that internalization of CD26 molecule plays an important role in T cell activation.

Currently, we are focusing on molecular basis for CD26 mediated T cell costimulation and internalization of CD26, molecular basis for enhancement of antigen-specific T cell responses by soluble CD26, utilization of anti-CD26 antibody for treatment of malignant tumor and immune-mediated diseases.

a. Internalization of CD26 by mannose 6-phosphate/insulin-like growth factor II receptor contributes to T cell activation

Hideto Ikushima, Yasuhiko Munakata[2], Tomonori Ishii, Satoshi Iwata, Hirotoshi Tanaka, and Chikao Morimoto

CD26 is the T cell activation antigen and also known to bind the adenosine deaminase (ADA) and have a dipeptidyl peptidase IV (DPPIV) activity. Cross-linking of CD26 and CD3 with immobilized monoclonal antibodies can deliver a second costimulatory signal and contribute to T cell activation. Our earlier studies revealed that the cross-linking of CD26 induced internalization of the molecules, subsequent tyrosine phosphorylation of signaling molecules and resulted in an enhanced proliferating responses of T cells. Although these findings suggests the importance of internalization in the function of CD26, CD26 has only 6 amino acid residues in its cytoplasmic region with no known motif for endocytosis. In this study, we have identified the mannose 6-phosphate/insulin-like growth factor II receptor (M6P/IGFIIR) as a binding protein for CD26, and that mannose 6-phosphate (M6P) residues in the carbohydrate moiety of CD26 are critical for the binding between CD26 and M6P/IGFIIR. In human peripheral blood lymphocytes, T cell activation resulted in the mannose 6-phosphorylation of CD26. In addition, the cross-linking of CD26 with an anti-CD26 antibody induced not only capping and internalization of CD26 but also colocalization of CD26 with M6P/IGFIIR. Finally, internalization of CD26 by cross-linking and T cell proliferation induced by CD26 mediated T cell costimulation were inhibited by the addition of M6P, but not by the glucose 6-phosophate or mannose 1-phosphate. These results indicate that the interaction between mannose 6-phosphorylated CD26 and M6P/IGFIIR may play an important role in CD26-mediated T cell costimulatory signaling.

b. *In vitro* and *in vivo* antitumor effect of the anti-CD26 monoclonal antibody 1F7 on human CD30+ anaplastic large cell lymphoma karpas 299

Linus Ho[3], Ugur Aytac[3], Chikao Morimoto, and Nam H.Dang[3] and [3]Department of Lymphoma/Myeloma, MD Anderson Cancer Center, The University of Texas, TX, USA

CD26 is a 110-kd surface glycoprotein with diverse functional propertiers, including having a potentially significant role in tumor development, and antibodies to CD26 mediate pleomorphic cellular functions. In this study, we report that binding of soluble anti-CD26 monoclonal Ab 1F7 inhibits the growth of the human CD30+ anaplastic large cell T cell lymphoma cell line Karpas 299 in both *in vitro* and *in vivo* experiments. *In vitro* experiments show that 1F7 induces cell cycle arrest at the G1/S checkpoint, associated with enhanced p21 expression that is dependent on de novo protein synthesis. Furthermore, experiments with a SCID mouse tumor model demonstrate that 1F7 treatment significantly enhances survival of tumor bearing mice by inhibiting tumor formation. Our data suggest that anti-CD26 treatment may have potential clinical use for CD26+ hematologic malignancies.

c. Soluble CD26/dipeptidyl peptidase IV enhances Tetanus Toxoid(TT) induced T cell proliferation through CD86 upregulation on Antigen presenting cells, monocytes

Kei Ohnuma, Yasuhiko Munakata, Tomonori Ishii[2], Satoshi Iwata, Akikazu Murakami, Osamu Hosono, Hiroshi Kawasaki, Hirotoshi Tanaka, and Chikao Morimoto

CD26 is T cell costimuratory molecule with dipeptidyl peptidase IV (DPPIV) enzyme activity in its extacellular region. The expression of CD26 is enhanced after activation of T cells, though it is expressed on a subset of CD4+ memory T cells in a resting state. We have previously reported that the addition of soluble CD26 (sCD26) results in enhancing proliferation of peripheral blood lymphocytes induced by the recall antigen, tetanus toxoid (TT). The preise mechanism of this immune enhancement was not known as yet. Here we demonstrate that the enhancing effect of sCD26 on TT-induced T cell proliferation was shown in the early stage of immune responses. The target cells of sCD26 was CD14 positive monocytes in the peripheral blood cells. Mannose-6-phospahte (M6P) interfered the uptake of sCD26 into monocytes, which suggests that M6P/insulin-like growth factor II receptor plays a role of transportation of sCD26 into monocytes. sCD26 dose not alter the threshold of tetanus toxoid antigen concentration which is required for TT-induced T cell proliferation. However when sCD26 is added after antigen presentation, enhancement of TT-induced T cell proliferation is not seen. More importantly we found that sCD26 can upregulate the expression of

the costimulatory molecule, CD86 but not CD80 or DR antigen on monocytes through its dipeptidyl peptidase IV enzyme activity by using FACS analysis. Our findings strongly suggest that sCD26 molecule appears to be useful for the immuno adjuvants of cancer immunotherapy or virus vaccination.

III. Study on regulation of eukaryotic gene expression

Hirotoshi Tanaka, Yuichi Makino, Noritada Yoshikawa, Tetsuya Nakamura, Tsunenori Kodama, Rika Ouchida, and Chikao Morimoto

We are interested in the mechanism of eukaryotic gene expression and develop 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-steradal 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 a novel strategy for identification of novel therapeutic strategy, especially from the viewpoint of redox regulation.

(i) Redox Regulation of the Glucocorticoid Receptor

Hirotoshi Tanaka, Yuichi Makino, and Chikao Morimoto

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

Hirotoshi Tanaka, Chikao Morimoto, and Noritada Yoshikawa

The GR function could be differencially regulated by ligands. In this line, we are colaborating with European venture company 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

Hirotoshi Tanaka, Yuichi Makino, and Kensaku Okamoto(in colaboration with Lorenz Poellinger Lab, Karolinska Institute, Sweden)

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 endo-

nuclease 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

Yuichi Makino and Hirotoshi Tanaka (in colaboration with Lorenz Poellinger Lab, Karolinska Institute, Sweden)

Angiogenesis is regulated by a combination of variety factors including transcription factors. Recently, we have isolated cDNA encoding the novel protein 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 our novel negative regulator. This negative regulater may also therapeutically be applicable for treating a number of angiogenic disorders including cancer, diabetic retinopathy, and rheumatoid arthritis.

IV. Immunobiology of human chemokine receptors

Hiroshi Kawasaki, and Chikao Morimoto (in collaboration with Sato K[4], Takahashi TA[4], Hirai K[5]):[4]Dep. Cell Processing and [5]Dep. Host Defense, University of Tokyo

We have been interested in a panel of inflammatory and immunostimulatory molecules. In particular, chemokines and Interleukin 12 have drawn our attention. In order to pursue the biological functions of those molecules, we produced monoclonal antibodies against the receptors for them, which resulted in the following unexpected obsevations.

a. TGF-beta mediated regulation of chemokine receptor expression

We examined the effect of TGF-beta 1 on the chemotactic migratory ability of human monocyte-derived dendritic cells (DCs). Treatment of immature DCs with TGF-beta 1 resulted in increased expressions of CCR-1, CCR-3, CCR-5, CCR-6, and CXC chemokine receptor-4 (CXCR-4), which were concomitant with enhanced chemotactic migratory responses to their ligands, RANTES (for CCR-1, CCR-3, and CCR-5), macrophage-inflammatory protein-3 alpha (MIP-3 alpha) (for CCR-6), or stromal cell-derived growth factor-1 alpha (for CXCR-4). Ligation by TNF-alpha resulted in down-modulation of cell surface expressions of CCR-1, CCR-3, CCR-5, CCR-6, and CXCR-4, and the chemotaxis for RANTES, MIP-3 alpha, and stromal cell-derived growth factor-1 alpha, whereas this stimulation up-regulated the expression of CCR-7 and the chemotactic ability for MIP-3beta. Stimulation of mature DCs with TGF-beta 1 also enhanced TNF-alpha-induced down-regulation of the expressions of CCR-1, CCR-3, CCR-5, CCR-6, and CXCR-4, and chemotaxis to their respective ligands, while this stimulation suppressed TNF-alpha-induced expression of CCR-7 and chemotactic migratory ability to MIP-3 beta. Our findings suggest that TGF-beta 1 reversibly regulates chemotaxis of DCs via regulation of chemokine receptor expression.

b. IL-12 mediated regulatoin of cellular signalling

We analyzed the expression of IL-12Rbeta1 and IL-12Rbeta2 and the role of IL-12 in the activation of monocyte-derived dendritic cells (DCs) via IL-12Rbeta1-mediated signaling events. Flow cytometric analysis revealed that IL-12Rbeta1 was expressed in T cells, Con A blasts, and monocyte-derived DCs, but not in monocytes, while its transcript was detected in all of these cell types. Transcriptional expression of IL-12Rbeta2 was observed in T cells, Con A blasts, and monocyte-derived DCs, but not monocytes. The ligation of DCs as well as Con A blasts by IL-12 induced the production of GM-CSF, IL-1beta, IL-6, TNF-alpha, and IFN-gamma at the transcription levels. Furthermore, stimulation of DCs with IL-12 induced IL-12p40 transcript, but not IL-12p35 transcript, whereas this stimulation caused the expressions of both transcripts in Con A blasts. Stimulation of DCs with IL-12 caused a tyrosine phosphorylation of several intracellular proteins, and the pattern of these events were distinct from those of IL-12-stimulated Con A blasts. IL-12 also induced tyrosine phosphorylation of IL-12Rbeta1 as well as recruitment of several tyrosine-phosphorylated proteins to IL-12Rbeta1 in DCs and Con A blasts. Receptor engagement of DCs as well as Con A blasts by IL-12 resulted in activation of Janus kinase 2 and Tyk2 kinases and Stat3 and Stat4 transcription factors and the association of these proteins to IL-12Rbeta1. Stimulation with IL-12 caused a tyrosine phosphorylation and enzymatic activity of a family of mitogen-activated protein kinases, p38mapk. These results suggest that IL-12 acts directly on DCs to induce their functional activation via IL-12Rbeta1-mediated signaling events.

c. Conclusion and future perspectives

Chemokine receptors and IL 12 receptors are intricatedly involved in the regulation of cellular immune responses. Further research amd manipulation of these receptor system should contribute to the clinical application of lymphocytes toward regulation of autoimmunity and allergy, and enhancement of cancer immunity.

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