Advanced Clinical Research Center

Division of Molecular Therapy
分子療法分野

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The main theme of our research is toward the development of novel therapeutic options against intractable hematological disorders including leukemia and lymphoma. For this purpose, we are making every effort to master the mechanisms of normal and neoplastic hematopoiesis on the basis of molecular and cellular biology.

(1) Preclinical study of therapeutic gene transfer mediated by various viral vectors:
We have two main research projects in this field. One is a murine therapeutic model of tumor vaccine secreting GM-CSF (GVAX) in combination with nonmyeloablative allogeneic HSCT. The other is a human experimental model of ribozyme technology for inactivation of leukemogenic fusion mRNA such as BCR-ABL.

(2) Preclinical study of targeted drug delivery using various cell-targeting strategies and novel molecular target agents:
We are developing various cell-targeting strategies using cytokines, adhesion molecules as well as monoclonal antibodies. PEG-liposome has been applied for this purpose. In addition, we have made two types of cytokine derivatives by genetic engineering for preclinical study. We are also studying anti-leukemic effects of a novel signal transduction inhibitor and anti-GvHD effects of a novel cytokine synthesis inhibitor for the future clinical trial.

(3) Analysis of tumor stem cells and search for molecular targets for their elimination:
Cure of malignant tumors requires eradication of tumor stem cells. As a representative model for tumor stem cells, we are studying the identification and characterization of leukemia stem cells using cell tracking strategies and flow cytometry.

(4) Analysis of normal and neoplastic hematopoiesis based on their interaction with microenvironments:
Not only normal but also neoplastic hematopoiesis can be supported by the specific interaction between stem/progenitor cells and bone marrow microenvironments. To simulate this cell to cell contact in vitro, we are using a co-culture system in which stem/progenitor cells are overlaid on the layer of hematopoiesis-supporting stroma cells. This co-culture system is applied for determination of drug sensitivities and gene transfer effects.

1. Comparative Characterization of Two Philadelphia Chromosome-positive Acute Lymphoblastic Leukemia (Ph-ALL) Cell Lines Supported by Adhesive Interaction with a Murine Bone Marrow Stromal Cell

Line

Izawa K, Sekine R, Nagamura T, Kobayashi S, Tojo A

We established two Philadelphia chromosome -positive acute lymphoblastic leukemia (Ph-ALL) cell lines and characterized their unique growth properties supported by adhesive interaction with a murine bone marrow stromal cell line, HESS-5. IMS-PhL1 (L1) cells mainly expressed p 210-type BCR-ABL mRNA with wild type sequences in the ABL kinase domain and were weakly positive for p190-type mRNA. IMS-PhL2 (L2) cells exclusively expressed p190-type transcripts with Y253H mutation and showed much lower sensitivity to imatinib than L1 cells. The growth of L1 cells was autonomous in suspension culture, and their apoptosis was prevented by co-culture with HESS-5 cells. In contrast, the sustained growth and survival of L2 cells was absolutely dependent on direct contact with HESS-5 cells and did not respond to soluble cytokines. Both cell lines adhered to and migrated beneath the HESS-5 cell layer, resulting in the formation of cobblestone areas. This migration was significantly reduced by the pretreatment of L1 and L2 cells with a neutralizing antibody against α4-integrin. After intravenous injection, both cell lines developed leukemia in NOD-SCID mice and concomitantly underwent *in vivo* down-regulation of α5-integrin expression in a reversible manner. The study of L1 and L2 cells will provide further insights into microenvironmental regulation of survival and clonal expansion of Ph-ALL cells.

2. Constitutive expression of Pax5 in cord blood progenitor cells rather perturbs B lymphopoiesis through the exon 9-dependent and independent mechanism.

Sekine R, Tojo A

Pax5 is a key regulator of B cell commitment and is indispensable throughout the early stages of B cell differentiation. Human B lymphopoiesis can be simulated *in vitro* by cultivating cord blood (CB) progenitor cells on the murine bone marrow stromal cell line such as HESS-5. Using this culture system, we have identified two C-terminal splice variants of Pax5; one skips exon 9 without subsequent frameshift (del9), and the other has a frameshift insert between exon 8 and 9, resulting in novel C-terminal sequences (ins 8'). Quantitative RT-PCR analysis revealed that del9 mRNA was barely detected in CB $CD34^+$ cells and thereafter upregulated in progenitor B cells during the culture. In contrast, wild-type Pax5 (wt) and ins8' mRNA could not be detected until 3 weeks of culture. After 4 weeks, wt mRNA became 100-fold or more abundant than del9 and ins8' mRNA. Cotransfection experiments with a reporter plasmid containing high-affinity Pax5 binding sites from the CD19 gene upstream of the basal promoter showed that del9 showed moderately lower but ins8' had markedly lower transcriptional activity than wt Pax5. Furthermore, TLE4, a Pax5 partner of the Groucho co-repressor family, inhibited the transcriptional activity of wt and del9 Pax5 but not ins8' isoform. We next examined the impact of Pax5 on B cell development from CB $CD34^+$ cells by transduction with a retroviral vector encoding each isoform cDNA linked by an IRES element to EGFP. After infection, sorted $EGFP^+$ cells were subjected to the co-culture assay on HESS-5 cells. Although Pax5 isoforms induced ectopic expression of CD19 in immature non-adherent cells in the order of wt>ins8'=del9, the number of $CD19^+$ B lymphoid cells after 6 weeks of culture was substantially suppressed by Pax5 isoforms in the same order as their CD 19 inducibility. These results suggest that constitutive expression of Pax5 isoforms in cord blood progenitor cells rather impairs B lymphopoiesis through the exon 9-dependent and independent mechanism. We also raise the possibility that developmentally regulated concentration of Pax 5 isoforms may be crucial for human B lymphopoiesis.

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

Izawa K, Tojo A.

Philadelphia chromosome-positive acute lymphoblastic leukemia (Ph-ALL) readily acquires resistance to chemotherapeutic drugs including imatinib mesylate. We hypothesize that the adhesive interaction of Ph-ALL cells with bone marrow stromal cells might cause their escape from drug-induced apoptosis and subsequent minimal residual disease, resulting in the generation of a chemoresistant clone. To gain insight into this possibility and a novel strategy against imatinib resistance, we used two Ph-ALL cell lines designated as IMS-PhL1 (L1) and IMS-PhL2 (L2). L1 cells had wild type BCR-ABL, whereas L2 cells had Y253H mutant and revealed 10-fold or more resistance to imatinib, as compared with L1 cells. Both cell lines adhered to and migrated beneath the HESS-5 cell layer, resulting in the formation of cobblestone areas

(CA). While floating L1 cells were eradicated by 1 μM imatinib, a portion of adherent L1 cells could survive even at 10 μM imatinib. Similarly, L2 cells forming CA beneath the HESS-5 cell layer considerably resisted prolonged exposure to 10 μM imatinib. Leptomycin B (LMB), a potent inhibitor of CRM1/exportin-1, can trap BCR -ABL in the nucleus and can aggressively eliminate BCR-ABL$^+$ cells in combination with imatinib. We tested LMB for its ability to eliminate CA or adherent Ph-ALL cells in combination with imatinb. Dramatically, combined use of 10 μM imatinib and 1 nM LMB for 7 days exerted a synergistic effect on reduction in the number of CA. L1 cells were also susceptible to the combination of imatinib and LMB. Our results suggest that nuclear entrapment of BCR-ABL may be a promising strategy for overcoming imatinib resistance mediated by stromal cells as well as a certain BCR-ABL mutant.

4. In vitro validation of bioluminescent monitoring of disease progression and therapeutic response in leukemia model mice

Inoue Y*, Tojo A, Izawa K, Sekine R, Soda Y, Okubo T*: *Department of Radiology, IMSUT Hospital

The application of *in vivo* bioluminescence imaging to noninvasive, quantitative monitoring of tumor models relies on the expected correlation between bioluminescent signal and tumor burden. We investigated the relationship between bioluminescent signal and viable cell number in murine leukemia cells expressing luciferase (Luc). Interleukin-3 (IL-3)-dependent Ba/F3 cells retrovirally transduced with both Luc and p190 BCR-ABL showed autonomous growth as well as stable Luc expression, which made them detectable by bioluminescence imaging when transplanted into nude mice. The intensity of bioluminescent signal in cell culture reflects viable population of Ba/F3p190-Luc cells with or without imatinib mesylate. However, Luc activity per viable cell was influenced by IL-3 concentration in Ba/F3-Luc cells, and by proliferative status and imatinib concentration in Ba/F3p 190-Luc cells, resulting in the impaired proportionality between viable cell number and bioluminescent signal. Luc activity per cell changed in association with the proliferation index in these cells. Although *in vivo* bioluminescence imaging would allow noninvasive monitoring of leukemia cells in animal model, it should be noted that environmental factors and therapeutic interventions may cause some discrepancies between tumor burden and intensity of bioluminescent signal.

5. Investigation of possible involvement of regulatory T cells in chronic graft versus host disease after allogeneic stem cell transplantation

Hirano M, Nagamura T*, Tojo A: *Department of Cell Processing and Transfusion, IMSUT Hospital

CD25+CD4+ regulatory T (Treg) cells play a crucial role in immunological tolerance and their quantitative and/or qualitative abnormalities would result in development and amelioration of autoimmune disorders. Recent reports suggest that decrease in the number of peripheral Treg cells is significantly associated with active chronic graft versus host disease (cGVHD). Ontogeny of Treg cells is finely tuned by a unique transcription factor Foxp3. We compared the absolute number of peripheral Treg cells by enumerating CD25+CD4+ cells in flow cytometry as well as by measuring Foxp3 transcripts in real-time PCR between normal volunteers and patients with cGVHD after allogeneic stem cell transplantation. In normal subjects (n=26), there is a significant correlation between the ratio of Treg versus WBC and Foxp3 transcripts corrected by GAPDH transcripts (correlation coefficient=0.67). There seems to be a tendency that the peripheral Treg number from cGVHD patients (29/μl, n=6) is lower than that from normal subjects (45/μl).

6. Screening and identification of novel 27 base ShRNAs for specifically silencing p 190 BCR-ABL mRNA; a novel therapeutic approach toward Ph-positive acute lymphoblastic leukemia

Hatano T, Futami M, Soda Y, Tojo A

Patients with Ph-ALL have poor prognosis despite intensive treatment including allogeneic stem cell transplantation. We previously demonstrated that an allosterically controllable novel ribozyme, designated as Maxizyme (Mz), specifically induced cell death of Ph-ALL cells expressing p190 BCR-ABL transcripts involved in the pathogenesis of this disease. However, in recent years, RNA interference (RNAi) technology has been recognized as a more powerful and widely applicable tool for suppressing gene expression. To develop a new therapeutic approach based on RNAi, we have designed a series of shRNAs interfering p190 BCR-ABL chimeric mRNA as well as its normal counterparts (BCR and ABL mRNA), respectively. We inserted shRNA sequences under control of U6 promoter in a mammalian expression vector.

293 cells were co-transfected with a series of shRNA expression vectors and a p190 BCR-ABL cDNA expression vector. Screening of shRNAs was performed by real-time PCR analysis of BCR-ABL transcripts as well as by western blot analysis of p190. The influence on endogenous BCR and ABL transcripts/proteins was also monitored. We found that novel 27 base length shRNAs targeting BCR-ABL junctions was most excellent in silencing effect, compared with usual 21 base length shRNAs. This shRNA did not affect either BCR or ABL proteins, indicating its specificity against BCR-ABL mRNA. We are now trying to test these shRNAs in Ph-ALL cell lines and patient samples using a lentiviral vector system.

Publications

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Our major projects are (1) the roles of small GTPases and GAPs in the control of cell division and differentiation, (2) Molecular therapy targeting signal transduction pathways, (3) molecular mechanism regulating hematopoiesis, (4) molecular mechanisms of MLL-fusion protein-induced leukemias, (5) characterization of hematopoietic stem cells, (6) adhesion of mast cells, (7) characterization of new PIR (paired Ig receptors) family proteins and (7) molecular mechanism of leukemogenesis,

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

Toshiyuki Kawashima, Yukinori Minoshima, Ying Chun Bao, Tomonori Hatori, Yasushi Nomura, Takaya Satoh[1], Yoshito Kaziro[2], Hideaki Nakajima[3], Tetsuya Nosaka, David Williams[4] and Toshio Kitamura: [1]Kobe University, [2]Biochemistry and Cell Biology Unit, HMRO, Kyoto University Graduate School of Medicine, [3]Project of mesenchymal stem cells, The 21st century center of excellence program, Institute of Medical Science, The University of Tokyo, [4]Cincinnati Children's Hospital Medical Center, USA.

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

2. Molecular therapy targeting signal transduction pathways using small molecule compounds

Toshiyuki Kawashima, Akiho Tsuchiya, Yukinori Minoshima, Ken Murata and Toshio Kitamura

Internal tandem duplications of the juxtamembrane region of the *Flt-3* (ITD-Flt3) are found in about 30% of the human acute myeloid leukemia patients. We previously identified small molecule compound GTP14565, a specific inhibitor of ITD-Flt3. GTP14564 preferentially inhibited the growth of the Ba/F3 cells transformed by the mutant *Flt-3*, but not Ba/F3 cells driven by the Flt-3 ligand/wild type Flt-3. Based on the in vitro results, we found that ITD-Flt3-induced cell growth was dependent on STAT5 activation while wild-type Flt3-induced cell growth was dependent on ERK and MAPK activation, suggesting the difference in signaling between phathological and physiological conditions. Unfortunately, GTP14564 is unstable and insoluble, and cannot be used for preclinical trials.

STAT3 is frequently activated in many cancers and leukemias, and is required for transformation of NIH3T3 cells. Therefore, we started searching for STAT3 inhibitors. We already established an efficient screening protocol for identification of STAT3 inhibitors, and identified several compounds that inhibit STAT3 activation. We will characterized these molecules.

3. Molecular mechanisms of hematopoietic stem cell-supportive activities of ISF, a subunit of proton pump-associated ATPases.

Hideaki Nakajima[3], Fumi Shibata, Yumi Fukuchi, Yuko Goto-Koshino, Miyuki Ito, Atsushi Urano, Tatsutoshi Nakahata[5], Hiroyuki Aburatani[6], and Toshio Kitamura: [5]Department of Pediatrics, Kyoto University, [6]Research Center for Advanced Science and Technology, The University of Tokyo.

In the search for stromal-derived growth factors, we have identified a novel secreted short form of immune suppressor factor (ISF) using a combination of a genetic approach and retrovirus-mediated functional screening. This protein was isolated based on its ability to support proliferation of a mutant clone S21, which was established from Ba/F3 cells that are usually interleukin-3-dependent but became dependent on a stroma cell line ST2 after chemical mutagenesis. ISF is a membrane protein harboring six transmembrane domains, and turned out to be a subunit of vacuolar H (+)-ATPase proton pumps. When overexpressed in bone marrow stroma cells, ISF conferred the cells with an ability to support the growth of S21 cells as well as hematopoietic stem cells (HSCs). To elucidate the molecular mechanisms, we analyzed the expression profiles using cDNA microarrays, and found that ISF overexpression resulted in the up-regulation of MMP3, and down-regulation of TIMP3 and SFRP-1. We also demonstrated that down-regulation of TIMP3 and SFRP-1 could lead to maintainance of HSCs.

4. Functional characterization of the septin family genes that are fused to MLL in infant leukemias with chromosomal translocations, and molecular analysis on the mechanism of leukemogenesis mediated by MLL-SEPT6.

Ryoichi Ono, Hideaki Nakajima[3], Yasuhide Hayashi[7], Katsutoshi Ozaki, Hidetoshi Kumagai, Toshiyuki Kawashima, Tomohiko Taki[8], Toshio Kitamura, and Tetsuya Nosaka: [7]Gunma Children's Medical Center, [8]Kytoto Prefectural University of Medicine Graduate School of Medical Science.

We identified a human ortholog of mouse Septin6 as a fusion partner of MLL in three cases of de novo infant acute myeloid leukemia with complex chromosomal abnormalities involving 11q23 and Xq22-24 (Ono, R. et al., Cancer Res. 62, 333-337, 2002). Septins comprise a eukaryotic GTPase subfamily and are involved in cytokinesis. Among septin family genes, nearly complete ORFs of SEPT5, SEPT6, SEPT9, and SEPT11 were fused to 5' half of MLL in leukemic patients, resulting in formation of chimeric proteins. It was controversial whether the MLL fusion protein is sufficient to induce acute leukemia without additional genetic alterations. We demonstrated that the fusion partner-mediated homo-oligomerization of MLL-SEPT6 is essential to immortalize hematopoietic progenitors in vitro. MLL-SEPT6 induced myeloproliferative disease with long latency in mice, but not acute leukemia, implying that secondary genotoxic events are required to develop leukemia. We developed in vitro and in vivo model systems of leukemogenesis by MLL fusion proteins, where activated FMS-like receptor tyrosine kinase 3 (FLT3) together with MLL-SEPT6 not only transformed hematopoietic progenitors in vitro but also induced acute biphenotypic or myeloid leukemia with short latency in vivo. In these systems, MLL-ENL, another type of the fusion product that seems to act as a monomer, also induced the transformation in vi-

tro and leukemogenesis in vivo in concert with activated FLT3. These findings show direct evidence for a multistep leukemogenesis mediated by MLL fusion proteins and may be applicable to development of direct MLL fusion-targeted therapy.

5. Disruption of sept6, a fusion partner gene of MLL, does not affect ontogeny, leukemogenesis induced by MLL-SEPT6, or phenotype induced by the loss of sept4.

Ryoichi Ono, Masafumi Ihara[2], Hideaki Nakajima[3], Katsutoshi Ozaki, Yuki Kataoka-Fujiwara[9], Tomohiko Taki[8], Koh-ichi Nagata[10], Masaki Inagaki[11], Nobuaki Yoshida[9], Toshio Kitamura, Yasuhide Hayashi[7], Makoto Kinoshita[2], and Tetsuya Nosaka: [9]Laboratory of Gene Expression and Regulation, Center for Experimental Medicine, Institute of Medical Science, The University of Tokyo, [10]Department of Molecular Neurobiology, Institute for Developmental Research, Aichi Human Service Center, [11]Divisions of Biochemistry and Virology, Aichi Cancer Center Research Institute.

Septins are evolutionarily conserved GTP-binding proteins that can heteropolymerize into filaments. Recent studies have revealed that septins are involved in not only diverse normal cellular processes but also the pathogenesis of various diseases, including cancer. SEPT6 is ubiquitously expressed in tissues and one of the fusion partner genes of MLL in the 11q23 trunslocations implicated in acute leukemia. However, the roles of Sept6 in vivo remain elusive. We have developed Sept6-deficient mice that exhibited neither gross abnormalities, changes in cytokinesis, nor spontaneous malignancy. Sept6 deficiency did not cause any quantitative changes in any of the septins evaluated in this study, nor did it cause any additional changes in the Sept4-deficient mice. Even the depletion of Sept11, a close homolog of Sept6, did not affect the Sept6-null cells in vitro, thus implying a high degree of redundancy in the septin system. Furthermore, a loss of Sept6 did not alter the phenotype of myeloproliferative disease induced by MLL-SEPT6, thus suggesting that Sept6 does not function as a tumor suppressor. To our knowledge, this is the first report demonstrating that a disruption of the translocation partner gene of MLL in 11q23 translocation does not contribute to leukemogenesis by the MLL fusion gene.

6. Functional characterization of the AF5q31 gene that is fused to MLL in infant acute lymphoblastic leukemia by chromosomal translocation.

Atsushi Urano, Masaki Endoh[12], Tadashi Wada[12], Yoshihiro Morikawa[13], Miyuki Itoh, Yuki Kataoka[9], Tomohiko Taki[8], Hideaki Nakajima[3], Nobuaki Yoshida[9], Yasuhide Hayashi[7], Hiroshi Handa[12], Toshio Kitamura, and Tetsuya Nosaka: [12]Graduate School of Bioscience and Biotechnology, Tokyo Institute of Technology, [13]Department of Anatomy and Neurobiology, Wakayama Medical University.

We identified a gene AF5q31 from an infant early pre-B acute lymphoblastic leukemia (ALL) with ins(5;11)(q31;q13q23) as a fusion partner of MLL. The AF5q31 gene encodes a protein of 1163 amino acids, and is homologous to AF4-related genes, including AF4, LAF4, and FMR2 (Taki T et al., PNAS 96, 14535, 1999). The AF4 is directly involved in infant ALL with t(4;11)(q21; q23) of poor prognosis, and AF4 knockout mice display altered lymphoid development. The LAF4, a lymphoid-restricted nuclear protein, is expressed at the highest level in pre-B cells and is suggested to play a regulatory role in early lymphoid development. On the other hand, FRM2 was identified as a gene associated with FRAXE mental retardation. Although MLL is known to play an essential role in leukemogenesis by regulating HOX family gene expression, the fusion partner of MLL also appears to be important to modify the MLL function, thereby determining the phenotype of the leukemia. To clarify the role in leukemogenesis and the biological function of AF5q31, we have generated the AF5q31 knockout mice and found that AF5q 31 is essential for spermatogenesis. Although most AF5q31-deficient mice died in utero and neonatally, some AF5q31-deficient mice survived. The survived male mice were sterile with azoospermia. Histological examinations revealed the arrest of germ cell development at the stage of spermiogenesis, and virtually no spermatozoa were seen in the epididymis. AF5q31 was preferentially expressed in Sertoli cells. Furthermore, mutant mice displayed severely impaired expression of protamine 1, protamine 2, and transition protein 2, which are indispensable to compact the haploid genome within the sperm head, and an increase of apoptotic cells in seminiferous tubules. Thus, AF5q31 seems to function as a transcriptional regulator in testicular somatic cells and is essential for male germ cell differentiation and survival. These results may have clinical implications in the understanding of human male infertility. Clarification of the AF5q31-mediated gene regulation in testes will also help us to elucidate the molecular mechanism by which the fusion converts normal MLL into the

leukemogenic form.

7. Integrin αIIbβ3 induces the adhesion and activation of mast cells through interaction with fibrinogen.

Toshihiko Oki, Jiro Kitaura, Koji Eto[14], Yang Lu, Yoshinori Yamanishi, Hideaki Nakajima[3], Hidetoshi Kumagai, and Toshio Kitamura: [14]Laboratory of stem Cell Therapy, The Institute of Medical Science, University of Tokyo

Integrin αIIb, a well-known marker of megakaryocyte-platelet lineage, has been recently recognized on hemopoietic progenitors. We demonstrated that integrin αIIbβ3 is highly expressed on mouse and human mast cells and that mast cells, with exposure to various stimuli, adhere to extracellular matrix proteins such as fibrinogen and von Willebrand factor in an integrin αIIβ3-dependent manner. In addition, the binding of mast cells to fibrinogen enhanced proliferation, cytokine production and migration and induced the uptake of soluble fibrinogen, implicating integrin αIIbβ3 in a variety of mast cell functions. Our goal is to delineate the biological significance of integrin αIIbβ3 on mast cells by in vivo allergy and inflammation models using integrin αIIb knockout mice.

8. Identification and characterization of a new pair of immunoglobulin-like receptors, leukocyte mono-Ig-like receptors (LMIRs).

Yoshinori Yamanishi, Jiro Kitaura, Kumi Izawa, Toshihiko Oki, Fumi Shibata, Kaori Tamitsu, Si-Zhou Feng, Hideaki Nakajima[3], Hidetoshi Kumagai, and Toshio Kitamura

We originally identified and characterized two mouse cDNAs in a mouse bone marrow-derived mast cell cDNA library. They encode type I transmembrane proteins including a single variable immunoglobulin (Ig) motif in the extracellular domain with about 90% identity of amino acids. LMIR1 contains immunoreceptor tyrosine-based inhibition motif (ITIM) in the intracellular domain, while LMIR2 harbors a short cytoplasmic tail which could associate with immunoreceptor tyrosine-based activation motif (ITAM)-bearing molecules such as DAP12. In addition to LMIR1/2, related genes were identified by homology search in the close proximity on the same chromosome 11: LMIR3 is an inhibitory type receptor like LMIR1, and LMIR4, 5, 6 are activation type receptors like LMIR2. We are now trying to outline the biological role of LMIRs on immune cells by finding the ligands and analyzing deficient mice of LMIRs.

9. Molecular basis of acute leukemia, myelodysplastic syndromes (MDS), MDS overt leukemia, and myeloproliferative disorder (MPD).

Naoko Watanabe, Toshihiko Oki, Koichiro Yuji, Hironori Harada[15], Toshiya Inaba[15], Hideaki Nakajima[3], Tetsuya Nosaka, Jiro Kitaura, and Toshio Kitamura: [15]Department of Molecular Oncology, Research Institute for Radiation Biology and Medicine, Hiroshima University.

To elucidate the molecular mechanisms of leukemia, MDS, and MPD, we attempted to establish mouse model using bone marrow transplant (BMT); we transduce mouse bone marrow cells with genes of leukemogenic mutations such as MLL-fusions using retroviruses. In the result, we are now able to reproduce acute leukemia, or MPD-like symptoms in mice, and are now in the process of characterizing these mouse models. We also establish bone marrow-derived immature cell lines transduced with MLL fusions and AML-1 with mutations. While the differentiation of these cell lines are blocked probably through the dominant positive and negative effects of MLL-fusions and the mutated AML-1, respectively, they still remain dependent on cytokines including SCF, IL-3, and Flt-3 ligand. In the mouse BMT model, we are beginning to understand that leukemogenesis (acute leukemia as well as MDS overt leukemia) require multiple mutations; mutations that block differentiation, and mutations that block apoptosis or induce factor-independent proliferation. Based on the mouse BMT model, we assume that there are the second hit mutations in addition to mutations such as MLL-fusions and AML-1 in patients' leukemic cells. To identify such mutations, we use retrovirus-mediated expression cloning method. To this end, we make cDNA libraries of patients' leukemic cells, and attempt to isolate cDNAs that give rise to the autonomous growth of the cytokine-dependent cell lines established as stated above. In this way, we isolated ITD-Flt3 (constitutively activated Flt 3 mutant found in 30% of patients with acute myeloid leukemia) and some proteins that activate Ras pathways.

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Advanced Clinical Research Center

Division of Infectious Diseases
感染症分野

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

1. Analysis of HIV-1 specific CD8＋T cell response.

A. Kawana-Tachikawa et al.

MHC class I tetramer is a powerful tool for detection and analysis of epitope-specific CD8＋ T cells. In the case of HIV infection, however, viruses escape easily from CD8＋ T cell recognition. MHC class I tetramers which have been used were made with peptide which had original amino acid sequence. Here, we made tetramers which presented original peptide or mutant peptide, and examined staining pattern of HIV patients' PBMCs with these tetramers.

We have found escape mutant of HLA-A*2402 restricted CTL epitope Nef 138 (RYLPTFGWCF). Escape occurred through a substitution of Y with F at position 2 of the epitope (2F) in more than 80% of HLA-A*2402＋ patients. We made APC-labeled A24/Nef138 (wt) tetramer, and PE-labeled A24/Nef138 (2F) tetramer. At first, we stained two Nef138-specific CTL clones with both tetramers simultaneously. One clone which recognized Nef138 (wt) and Nef138 (2F) equally was stained with both tetramers. In the other hand, the other clone which recognize Nef138 (wt) better than Nef138(2F) was stained with only A24/Nef138 (wt) tetramer.

Then, we stained HIV patients' PBMC which have been cultured for 2 weeks under the specific stimulation. The staining pattern was quite different from each patients. There were CD8＋ T cells which could be detected by only A24/Nef138 (2F) in some patients. We also found there were some patients who had three different populations, which stained with either A24/Nef138 (wt) or A24/Nef138 (2F), and both. This dual-tetramer analysis is considered to be useful for analysis of CD8＋ T cells specific for epitopes in which escape mutation occur frequently.

2. Gene Transfer to Dendritic Cells

N. Hosoya et al.

Antigen-presented dendritic cells (DCs) are used as natural adjuvants for vaccination and considered to be adequate to immunotherapy against HIV-1 infection. We investigated immu-

nogenecity of gene-transduced DCs with Sendaivirus (SeV) and Adenovirus (AdV) vectors.

To compare gene transfer efficiencies of these vectors to DCs, human monocyte-derived DCs were infected with SeV and AdV expressing GFP. DCs infected with GFP/SeV or GFP/AdV showed maximum expression at moi of 2 or 1000, respectively. Both SeV and AdV infections upregulated expression of surface proteins, such as CD83, CD86, MHC class I, MHC class II. To determine immunogenecity of gene-transduced DCs by these vectors in HIV-1 patients, we used cultured imDCs from four HIV-1 infected patients infected with SeV or AdV expressing HIV-1 proteins as APCs. Autologus PBMCs were co-cultured with the imDCs and T cell activation was determined by ELISPOT assay. HIV-specific immune responses were induced by DCs infected with both viruses, and SeV an AdV-specific immune responses were also induced in some patients.

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

I. Miyata and Matsumura et al.

Although many pathogens such as influenzavirus cause acute respiratory infections and pneumonia, few of those infections have been diagnosed for their causative pathogens. To develop a rapid and specific method for identifying pathogens causing acute respiratory infections, we employed Loop-Mediated Isothermal Amplification (LAMP™, Eiken Chemical), a method for amplifying DNA at 60-65°C only for 5-30 min. For RNA viruses, we converted RNA to DNA with reverse transcriptase and amplified it by LAMP™ (RT-LAMP™) in a tube simultaneously. Our current target pathogens include SARS coronavirus, influenzaviruses, mycoplasmas, and so forth. As LAMP™ requires four primers as a set for amplifying one specific region of DNA, we prepared several sets of primers for several target regions in target pathogen genomes, which were chosen in a bioinfomatic fashion. Among those primer sets, we found some sets were eligible for RT-LAMP/LAMP™ and others were not. So far, we have obtained five sets, one each primer set for SARS CoV, Type-A influenzavirus, Type B-influenzavirus, human coronavirus, and mycoplasma. We found that they amplified corresponding target sequences as quickly as within ten minutes. We also found that they are specific to corresponding target pathogen sequences: they did not amplify unrelated pathogen sequences even in the presence of more than 10^6 copies of genome. To compare the sensitivity of each primer set with those of the others, we constructed chimeric RNA by connecting two sequences: the reference sequence (i.e., an HIV sequence) and one of the target pathogen sequences. We did limiting-dilution of these chimeric RNA molecules, and determined the discrepancy of detection limit of dilution between the reference primer set (i.e., the primer set for HIV) and a primer set for a pathogen sequence on the chimeric RNA molecules. We found that the five sets were up to 10^2 -fold more sensitive than the reference HIV primer set.

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Advanced Clinical Research Center

The Advanced Clinical Research Center Division of Bioengineering

臓器細胞工学分野

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Our department has been focusing on the development of innovative cancer therapy using immunologic approaches and gene therapy strategies.

Development of innovative cancer therapy using gene therapy strategies

a. In vivo electroporation of human Flt3-Lig- and plasmid DNA induce effectively mobilize and activate dendritic cells in situ

Takuya Takayama, Tetsuya Saito, Shigenori Nagai, Hideaki Tahara

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

transfer using in vivo electroporation could mobilize DCs into tumor site. Additional means to induce maturation of these DCs could have positive impact on anti-tumor effects of this strategy.

b. Potent and systemic anti-tumor immunity enhanced by intra-tumoral gene delivery of IL-18 in combination with dendritic cells mobilization in situ by Flt3 ligand therapy.

Tetsuya Saito, Takuya Takayama, Shigenori Nagai, Hideaki Tahara

Interleukin-18 (IL-18), which induces IFN-α production and enhances the cytolytic activity of NK cells, is considered to be a good candidate to be used for cancer immunotherapy. However, we have already found that treatment with systemic or local administration of IL-18 alone was not enough to induce a potent systemic anti-tumor immunity. On the other hand, we also have reported that dendritic cells (DCs) can capture tumor antigens from tumor cells killed by NK cells activated with IL-18 and efficiently induce tumor-specific CTL in vitro. In order to enhance the systemic anti-tumor response induced by local administration of IL-18 in vivo, we examined the effects of the combination therapy with IL-18 and Flt3 ligand (Flt3L). The Flt3L is a hematopoietic cytokine which increases and mobilizes both DCs and NK cells in vivo.

The C57BL/6 female mice received intra-dermal inoculation of MCA205 fibrosarcoma on day 0 in the bilateral flanks. On day 5 and 12, mice were treated with in vivo electroporation (IVE) with DNA plasmids carrying cDNA of human Flt3L or EGFP to bilateral hind legs. As the combination therapy, some of the mice were also treated with intra-tumoral injection of adenoviral vector carrying IL-18 gene (Ad.IL-18) or EGFP gene (Ad.EGFP) on day 9 and 16. In the treated tumors, significant anti-tumor effect was observed in mice treated with Ad.IL-18 alone and the ones treated with combination therapy of Flt3L-IVE and Ad.IL-18 when compared to those mice with control ($p<0.01$). The combination treatment with Ad.IL-18 resulted in the more potent anti-tumor response when compared to Flt3L-IVE treatment alone ($p<0.01$), and the complete eradication was observed more frequently (100% vs 33%: $p<0.05$) in mice treated with the combination therapy when compared to ones with Ad.IL-18 treatment alone. In the un-injected tumors, only the combination therapy showed significant anti-tumor. Lymphoid cells in regional lymph nodes of the mice treated with the combination therapy showed a significant cytolytic activity against MCA205. Moreover, cytolytic activity of the combination therapy against YAC-1 (NK target) was significantly higher than that of Ad.IL-18 treatment alone ($p<0.05$). Flow cytometric analysis about tumor infiltrating cells showed that the combination therapy induced a significant increase in number of both NK cells and DCs compared to the control ($p<0.01$). These results suggested that local gene transfer of IL-18 combined with DCs mobilization in situ with Flt3L may enhance the anti-tumor effect and induce a potent systemic anti-tumor immunity.

c. CCL21 rapidly promotes the generation and expansion of tumor-specific cytotoxic T cell in local tumor site

Shigenori Nagai, Takuya Takayama, Tetsuya Saito, Hideaki Tahara

Chemokines and receptors are controlling chemotaxis. CCL21, which is a group of CC chemokine, controls the recruiting of naïve T cells and mature DCs by corresponding receptor CCR7 to secondary lymphoid tissue. Several in vivo studies have demonstrated that local elaboration of CCL21 induced anti-tumor responses accompanied by the infiltration of dendritic cells (DCs) and T cells into local tumor site. However, it has not been fully understood about the functional roles of CCL21 on T cell immunity. In this study, we examined the role of CCL21 on T cell immunity using in vitro co-culture system. We found that CCL21 induced T-helper type 1 (Th1) skewing capacity and promoted the rapid generation of tumor-specific CTLs. These results indicated that T cells stimulated by CCL21 play a significant role in inducing tumor specific immunity through promoting Th1 skewing and CTL generation.

Development of innovative cancer therapy using immunologic approaches

a. Development of anti-angiogenic cancer therapy with vaccination using epitope peptides derived from human vascular endothelial growth factor receptor 2 (VEGFR2)

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

Angiogenesis has been shown to be a critical mechanism for tumor progression. Multiple studies have suggested that tumor growth can be suppressed if tumor angiogenesis can be inhibited using various types of anti-angiogenic agents. Recent studies in mouse systems have shown

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

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

Takako Suda, Takuya Tsunoda, Takeshi Watanabe, Naotaka Uchida, Satoshi Wada, Yoichi Furukawa, Yusuke Nakamura, Hideaki Tahara

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

We previously performed gene expression profile analyses of 20 intestinal-type gastric cancers, and identified a set of genes whose expression levels were elevated in the cancer tissues compared to their corresponding non-cancerous tissues. Among them, we in this study focused on *Immunoglobulin superfamily 11 (IGSF11)*. Its expression was also elevated in colorectal cancers and hepatocellular carcinomas as well as intestinal-type gastric cancers. Northern blot analysis showed that it showed abundant expression in testis and ovary. These data suggest that IGSF11 is a good candidate of cancer-testis antigen (CTA). Furthermore, suppression of *IGSF11* by siRNA retarded the growth of gastric cancer cells. To investigate the possibility of clinical application of peptide vaccine to IGSF11, we synthesized candidate epitope peptides for IGSF11 and tested whether the peptides elicit IGSF11-specific CTLs. As a result, we successfully established oligo-clonal CTLs by the stimulation with IGSF11-9-207. In addition, we also established additional CTLs by IGSF-9V, anchor-modified peptides of IGSF11-9-207. These peptides showed IGSF11-specific cytotoxic activity in an HLA-A*0201-restricted fashion, suggesting that these peptides may be applicable for cancer immunotherapy. These findings have brought novel insight into carcinogenesis of stomach, colon, and liver, and will be helpful for the development of novel therapeutic strategies to a wide range of human cancers.

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

Hiroyuki Mushiake, Takuya Tsunoda, Hideaki Tahara

Except in the early stages of and in certain

types of cancer, we have not been satisfied with the outcome of anticancer therapy. To overcome this situation, new strategies for cancer treatment are required. One of them, chemoimmunotherapy has been advocated as a modality of combination chemotherapy and immunotherapy since the 1970's. Recent attention has been paid to their combinatorial effect on the host immunological response. Actually, some cytokines (IL-2, GM-CSF, IFN-α and IFN-β, etc) or biochemical response modifiers (BRM; BCG, LNT, etc.) have been used clinically, and these have advantages in eliciting antitumor-immunological response. Especially, the elucidation of immunological molecular mechanism has provided us strategies to utilize them as a weapon to treat cancer patients. When addressing the priming immune response, we could not disregard the role of dendritic cells (DCs), professional antigen presenting cells (APC). In immune response to tumors, immature DCs acquire tumor antigens in tumor tissues, process the antigens and then migrate into regional lymph nodes. They differentiate into mature DCs and present the tumor antigens on their surface MHC class I and class II molecules to prime naive T cells[5]. The activated T cells show specific cytotoxic activity to the target cells and mediate tumor destruction.

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

Publications

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

I. β1 integrins and Cas-L (Crk-associated substrate lymphocyte type)

Satoshi Iwata, Takahiro Sasaki, Teruo Inamoto, Sakiko Inamoto, Yasuyo Urasaki, Yutaka Hashizume, Koji Yo, Yukiko Nakamura, Akiko Souta-Kuribara, Hiroyuki Kayo, Hiroto Yamasaki, Osamu Hosono, Hiroshi Kawasaki, Hirotoshi Tanaka and Chikao Morimoto

β1 integrins play crucial roles in a variety of cell processes such as adhesion, migration, proliferation, and differentiation of lymphocytes. Previously we showed that co-immobilized anti-β1 integrin mAbs or its ligand with a submitogenic dose of anti-CD3 mAb induced a marked increase of IL-2 secretion and proliferative response of T cells, indicating the role of β1 integrins in T cell activation. Furthermore, we showed that the ligation of β1 integrins induced protein tyrosine phosphorylation of FAK (focal adhesion kinase), paxillin, and pp105 in H9 T cell line and peripheral T cells. Pp105 was first described in our laboratory as a protein that is predominantly tyrosine phosphorylated by the ligation of β1 integrins in H9 cells. Next we demonstrated that pp105 is a hematopoietic variant of p130Cas (Crk-associated substrate) by cDNA cloning, and designated Cas-L (Cas lymphocyte type).

We have found that transfection of Cas-L cDNA into Jurkat T cell line restored β1 integrin -mediated co-stimulation as well as cell migration, indicating that Cas-L plays a key role in the β1 integrin-mediated T cell functions.

Our present projects aim at identifying associating molecules of Cas-L, investigating its biological significance in vitro, and evaluating the clinical relevance of Cas-L-mediated signaling

pathways in inflammatory diseases and malignancies.

a. Cas-L and TGF-β (transforming growth factor-β) pathway

To identify the Cas-L binding proteins, we performed yeast two-hybrid screening of cDNA library from a HTLV-I (human T-lymphotropic virus type I)-transformed T cell line (SLB-I). Subsequently, we isolated putative Cas-L binding clones, which turned out to be HTLV-I Tax, Smad7, and so on.

Smad7 is an inhibitory Smad (I-Smads) that interferes signaling by cytokines of TGF-β superfamily. Co-precipitation experiments revealed that Smad2, Smad3, Smad6, Smad7 associated with Cas-L, whereas Smad4 failed to do so. It was shown that the Cas-L binding sites of Smad 6 and Smad7 are located both in N-terminal and C-terminal domain. On the other hands, domain deletion of SH3, SD (substrate domain), SR (serine-rich region) or c-terminal half of Cas-L abrogated the binding ability with Smad6 and Smad7, suggesting that tertiary structure of Cas-L might be important for the association. Co-localization study showed that Smad6 and Smad 7 partly merged with Cas-L in the cytoplasm of 293T cells. Functionally, we found that exogenous Cas-L restored Smad6 and Smad7-mediated transcriptional inhibition of SBE (Smad binding element)-Luc, and p3TP-Lux, both of which are under the control of the complex of common mediator Smads (e.g. Smad4) and receptor-regulated Smads (e.g. Smad2 and Smad3). Furthermore, introduction of Cas-L RNAi in human hepatoma cell line (Huh7) partially inhibited growth arrest induced by TGF-β. Taken together, Cas-L may regulate TGF-β signaling pathway through the interaction with I-Smads, Smad6 and Smad7.

b. HTLV-I Tax and with Cas-L

Cas-L cDNA was originally cloned from an ATL (adult T cell leukemia)-derived T cell line, H9. We previously showed that Cas-L is highly expressed in ATL-derived and HTLV-I-transformed T cell lines. In the present study, we attempted to evaluate the role of Cas-L in the pathophysiology of ATL. Examination of PBMCs from ATL patients as well as ATL-derived T cell lines showed an elevation of Cas-L in these cells. We showed that tyrosine phosphorylation as well as expression of Cas-L was markedly elevated through the induction of HTLV-I Tax in JPX-9 cells, with these cells showing marked motile behavior on the ligands for integrins. We next performed Yeast Two-Hybrid screening of cDNA library from an HTLV-I-transformed T cell line, which resulted in the identification of Tax as a putative binding partner for Cas-L. Co-precipitation experiments revealed that the serine-rich region of Cas-L might serve as the binding site with the highest affinity for Tax. Co-localization study showed that Tax and Cas-L partly merged in the cytoplasm. Finally, we showed that exogenous Cas-L inhibited Tax-mediated transactivation of NF-κB (nuclear factor κB), while Tax-independent activation of NF-κB remained intact, hence indicating that Cas-L might specifically regulate Tax -NF-κB pathway.

c. NEDD9 (neural precursor cell expressed, developmentally down-regulated 9) a Cas-L homologue, is upregulated and involved in the differentiation after transient global ischemia in rats.

Some proteins involved in self-repair after stroke in adult brain are primarily expressed during embryonic development and strongly downregulated during the early postnatal phase. Nedd9 was recognized to be identical to Cas-L, a docking protein that associates with a variety of signaling molecules such as FAK, Pyk2 (proline-rich tyrosine kinase 2), and Crk. We investigated the involvement of these proteins in the pathophysiology of global cerebral ischemia. We found that Nedd9 was a splicing variant of Cas-L and selectively induced in neurons of cerebral cortex and hippocampus 1 to 14 days after the ischemia. Induced Nedd9 protein was tyrosine phosphorylated and was bound to FAK in dendrite and soma of neurons after the ischemia. Finally, it was demonstrated that Nedd9 promoted neurite outgrowth of PC-12 cells. Our study may support the potential of Nedd9 for participation in the differentiation of neurons after global ischemia in rats.

d. Roxithromycin specifically inhibits development of collagen-induced arthritis and production of proinflammatory cytokines by human T cells and macrophages

Roxithromycin (RXM) is a macrolide antibiotic that is effective in the treatment of chronic lower respiratory tract diseases including diffuse panbronchiolitis and bronchial asthma. However, its mechanism of action apart from its antibacterial action remains unclear. To further determine the mechanism of action of RXM, we attempted to evaluate the effect of RXM on T cell functions and the inflammatory responses in mice with collagen-induced arthritis.

T cell proliferation, cytokine production by T

cells stimulated through CD28, CD26, or PMA with or without anti-CD3 mAb, cytokine production by macrophages stimulated with LPS, and transendothelial migration of T cells were analyzed in the presence or absence of various concentrations of RXM. Moreover, we evaluated the effect of RXM treatment of collagen-induced arthritis mice.

RXM did not affect the production of Th1-type and Th2-type cytokines, whereas it specifically inhibited production of such proinflammatory cytokines as TNF-α and IL-6 by T cells as well as macrophages. In addition, RXM inhibited T cell migration. Moreover, it was shown that RXM treatment of collagen-induced arthritis mice reduced the severity of arthritis and serum level of IL-6, as well as leukocyte migration into the affected joints and destruction of bones and cartilages.

Our findings strongly suggest that RXM may be useful for the therapy of rheumatoid arthritis as well as other inflammatory diseases such as Crohn's disease.

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

Kei Ohnuma, Masahiko Uchiyama, Tadanori Yamochi, Shinichiro Kina, Yoshiaki Ito, Satoshi Iwata, Osamu Hosono, Hiroshi Kawasaki, Hirotoshi Tanaka and Chikao Morimoto (in collaboration with Nam H Dang, Nevada Cancer Institute, USA).

CD26 is a 110-kDa cell surface glycoprotein that posseses dipeptidyl peptidase IV(DPPIV) (EC. 3.4.14.5) activity in its extracellular domain and a primary marker of activated T cells. In the resting state, CD26 is preferentially expressed on a subset of CD4 memory T cells where they account for the majority of IL-2 secretory capabilities and help for B cell Ig production and are the primary responders to recall antigen such as tetanus toxoid. CD26 is also capable of providing a potent costimulatory or "second" signal which can augment other activation pathways leading to proliferation, cytokine production and effector functions. The mechanism of costimulation remains unclear since the cytoplasmic domain consists of only 6 amino acid and lacks a phosphorylation site, leading to the conclusion that CD26 interacts with other cell surface molecules. We have already shown that CD26 may interact with CD45RO which modulates TcR/CD3 activity through its intracellular tyrosine phosphatase domain. Recently, we have detected another CD26 binding protein, the mannose-6-phosphate/insulin-like growth factor II receptor (M6P/IGFIIR) as being critical for this interaction for CD26 mediated T cell costimulation in addition to adenosine deaminase (ADA). More recently, we have shown that CD26 localizes into lipid rafts, and targeting of CD26 to rafts is necessary for signaling events through CD26. Importantly, aggregation of CD 26 by anti-CD26 mAb crosslinking also causes coaggregation of CD45 into rafts. In addition, we have demonstrated that recombinant soluble CD26 (sCD26) has an enhancing effect on T cell proliferation in the presence of the recall antigen, tetanus toxoid. This enhancement resulted in an increase in the surface expression of the costimulatory molecule CD86 on monocytes following uptake of sCD26.

Currently we are focusing on the molecular and structural basis for CD26-mediated T cell activation signaling and are searching for its ligand directly involved in CD26-mediated T cell costimulation. Furthermore we are focusing on the translational research of anti-CD26 mAb as well as recombinant soluble CD26 for treatment of malignant tumors, immune-mediated disorders and immune deficiency diseases. Hopefully we will perform phase I clinical trial utilizing humanized CD26 antibody for the treatment of the above diseases, this year.

a. CD26 mediates dissociation of Tollip and IRAK-1 from caveolin-1 and induces upregulation of CD86 on antigen-presenting cells.

CD26 is a T-cell costimulatory molecule with dipeptidyl peptidase IV enzyme activity in its extracellular region. We have previously reported that the addition of recombinant soluble CD26 resulted in enhanced proliferation of human T lymphocytes induced by the recall antigen tetanus toxoid (TT) via upregulation of CD 86 on monocytes and that caveolin-1 was a binding protein of CD26, and the CD26-caveolin -1 interaction resulted in caveolin-1 phosphorylation (p-cav-1) as well as TT-mediated T-cell proliferation. However, the mechanism involved in this immune enhancement has not yet been elucidated. In the present work, we perform experiments to identify the molecular mechanisms by which p-cav-1 leads directly to the upregulation of CD86. Through proteomic analysis, we identify Tollip (Toll-interacting protein) and IRAK-1 (interleukin-1 receptor-associated serine /threonine kinase 1) as caveolin-1-interacting proteins in monocytes. We also demonstrate that following stimulation by exogenous CD26, Tollip and IRAK-1 dissociate from caveolin-1, and IRAK-1 is then phosphorylated in the cytosol, leading to the upregulation of CD86 via activa-

tion of NF-kappaB. Binding of CD26 to caveolin-1 therefore regulates signaling pathways in antigen-presenting cells to induce antigen-specific T-cell proliferation.

b. CD26 regulates p38 mitogen-activated protein kinase-dependent phosphorylation of integrin beta1, adhesion to extracellular matrix, and tumorigenicity of T-anaplastic large cell lymphoma Karpas 299.

CD26 is an antigen with key role in T-cell biology and is expressed on selected subsets of aggressive T-cell malignancies. To elucidate the role of CD26 in tumor behavior, we examine the effect of CD26 depletion by small interfering RNA transfection of T-anaplastic large cell lymphoma Karpas 299. We show that the resultant CD26-depleted clones lose the ability to adhere to fibronectin and collagen I. Because anti-integrin beta1 blocking antibodies also prevent binding of Karpas 299 to fibronectin and collagen I, we then evaluate the CD26-integrin beta1 association. CD26 depletion does not decrease integrin beta1 expression but leads to dephosphorylation of both integrin beta1 and p38 mitogen-activated protein kinase (MAPK). Moreover, our data showing that the p38MAPK inhibitor SB203580 dephosphorylates integrin beta1 and that binding of the anti-CD26 antibody 202.36 dephosphorylates both p38MAPK and integrin beta1 on Karpas 299, leading to loss of cell adhesion to the extracellular matrix, indicate that CD26 mediates cell adhesion through p38MAPK-dependent phosphorylation of integrin beta1. Finally, in vivo experiments show that depletion of CD26 is associated with loss of tumorigenicity and greater survival. Our findings hence suggest that CD26 plays an important role in tumor development and may be a novel therapeutic target for selected neoplasms.

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

CD26 is a Mr 110,000 surface-bound glycoprotein with diverse functional properties, including having a key role in normal T-cell physiology and the development of certain cancers. In the current study, we show that surface expression of CD26, especially its intrinsic dipeptidyl peptidase IV (DPPIV) enzyme activity, results in enhanced topoisomerase II alpha level in the B-cell line Jiyoye and subsequent in vitro sensitivity to doxorubicin-induced apoptosis. In addition, we show that expression of CD26/DPPIV is associated with increased phosphorylation of p38 and its upstream regulators mitogen-activated protein kinase kinase 3/6 and apoptosis signal-regulating kinase 1 and that p38 signaling pathway plays a role in the regulation of topoisomerase II alpha expression. Besides demonstrating that CD26 effect on topoisomerase II alpha and doxorubicin sensitivity is applicable to cell lines of both B-cell and T-cell lineages, the potential clinical implication of our work lies with the fact that we now show for the first time that our in vitro results can be extended to a severe combined immunodeficient mouse model. Our findings that CD26 expression can be an in vivo marker of tumor sensitivity to doxorubicin treatment may lead to future treatment strategies targeting CD26/DPPIV for selected human cancers in the clinical setting. Our article thus characterizes the biochemical linkage among CD26, p38, and topoisomerase II alpha while providing evidence that CD26-associated topoisomerase II alpha expression results in greater in vitro and in vivo tumor sensitivity to the antineoplastic agent doxorubicin.

d. Anti-CD26 monoclonal antibody mediates in vitro and in vivo growth inhibition of human renal cell carcinoma via Akt and p27 kipl signaling

CD26 is a 110kDa cell surface glycoprotein with diverse functional properties, including a potentially significant role in tumor development, with antibodies to CD26 mediating pleomorphic cellular functions. In this report, we show that binding of soluble anti-CD26 monoclonal antibody inhibits the growth of the human renal carcinoma cell lines Caki-2 and VMRC-RCW in both in vitro and in vivo experiments. In vitro experiments show that anti-CD26 mAb induces cell cycle arrest at the G1/S check point, associated with enhanced p27kip1 expression that is dependent on the attenuation of Akt activity. Furthermore, experiments with a mouse xenograft model bearing human renal carcinoma cells demonstrate that anti-CD26mAb treatment significantly enhances survival of tumor-bearing mice by inhibiting tumor growth. Taken together, our data strongly suggest that anti-CD26 monoclonal antibody treatment may have potential clinical use for CD26-positive renal cell carcinomas.

III. Therapeutically targetting transcription factors

Hirotoshi Tanaka, Noritada Yoshikawa, Nori-

aki Shimizu, Hiroshi Nakamura, Tetsuya Hisada, Kensaku Okamoto, Yuichi Makino, Chikao Morimoto (Division of Clinical Immunology)

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

a. Glucocorticoid receptor project

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

(i) Development of Dissociating Ligand for the Glucocorticoid Receptor

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

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

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

Transcription is a complex process compsed of preinitiation, initiation, promoter clearance, elongation, termination. Major efforts have been put on the initiation process including regulation of transcription factor function. However recent studies have clarified the modulatory role of elongation process in gene expression. Discovery of P-TEFb accelerated the understanding of molecular nature of elongation. It recently is shown that HEXIM1 binds 7SK snRNA and inhibits P-TEFb-mediated transcriptional elongation process. We have found that HEXIM1 directly associates with the GR in the absence of 7 SK and represses GR-mediated transcription. That is, HEXIM1 has a dual role in regulation of gene expression via connecting initiation and elongation. We are currently working on regulation of HEXIM1 expression, physiological role of HEXIM1 in GR action.

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

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

Peripheral T cells encounter rapid decrease in oxygen tension as they are activated by antigen recognition and migrate into inflammatory sites or tumors. Activated T cells, therefore, are thought to have such machineries that enable them to adapt to hypoxic conditions and execute immune regulation in situ. We have recently shown that survival of CD3-engaged human peripheral blood T cells is prolonged under hypoxic conditions and HIF-1 and its target gene product adrenomedullin play a critical role for the process. It is also shown that hypoxia alone is not sufficient but TCR-mediated signal is required for accumulation of HIF-1α in human peripheral T cells. In the present study, we showed that TCR-engagement does not influence hypoxia-dependent stabilization but stimulates protein synthesis of HIF-1α, most possibly via PI3K/mTOR system, and that expression of HIF-1α and its target gene is blocked by treat-

ment with rapamycin. Since some of those gene products, e.g., glucose transporters and phosphoglycerate kinase-1, are considered to be essential for glycolysis and energy production under hypoxic conditions and adequate immune reaction in T cells, this TCR-mediated synthesis of HIF-1α may play a pivotal role in peripheral immune response. Taken together, our results may highlight a novel aspect of downstream signal from antigen recognition by TCR with giving insight of a unique pharmacological role of rapamycin. We are currently working with the mechanism of translational regulation of HIF -1α.

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

a. The Role of TRAIL in the prevention of Acute Graft-Versus-Host Disease.

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

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

b. The control amd trans-activation of Chemokine Receptor Expression by β1 Integrin down stream signaling

Hiroshi Kawasaki, Satoshi Iwata, and Chikao Morimoto

Our laboratory has been showing that β1 integrins and their associating molecules, play crucial roles in the activation of lymphocytes. One of the cruicial elements of β1 Integrin-mediated lymphocyte activation is the tyrosine phosphorylation of Cas-L by Src-family kinase and FAK. The phosphorylated Cas-L ultimately enhances a panel of transcriptional activity through MAP kinase pathway. The ligation of chemokine receptors by respective ligands also activates MAP kinase pathway via G-protein coupled Rac/Ras/Rho, resulting in the upregulation of cell migration, motility, and proliferation. We have already shown that overexpression of CaS-L induced the enhancement of T lymphocyte migration in vivo and in vitro. We are investigating the functional linkage of Cas-L and signaling pathway of chemokine receptors. The manipulation and utilization of Cas-L function might contribute to the elucidation of not only lymphocyte activation but also the development of inflammation.

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Advanced Clinical Research Center

Division of Medical Data Processing Network System

ゲノム医療情報ネットワーク分野

Professor Tetsuo Shimizu

教 授 清 水 哲 男

The purpose of Division of Medical Data Processing Network System is to research and develop advanced system engineering methodology and computer technology suitable for the 21-th century type research hospital. The system is called "Infrastructure for Translational Research of Genome Based Medicine", which is expected to strongly support bi-directional translation between genome based life-science and clinical medicine. Our main research objectives are as follows;

-Construction of knowledge database system for translational research between genomic science and clinical medicine,

-Development of clinical protocol management system using computer technologies,

-Development of living cell-based high-throughput screening systems for translational research between genomic science and medical science,

-Research and development of agent based simulation for epidemiological and biological analysis and application of "Logical Atomism" to systems medical sciences.

1. Integrated knowledge database system (E-pathfinder)

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

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

thermore, we plan to incorporate E-pathfinder with other systems useful for translational research, including clinical protocol management system described as below.

In this year we developed the system for blood and immunological diseases. We believe that many clinicians and basic scientists might have great insights from this knowledge database system in the future.

2. Clinical protocol management system for translational research

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

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

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

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

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

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

4. Research and development of agent based simulation for epidemiological and biological analysis and application of "Logical Atomism" to systems medical sciences

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

demiological and biological issues.

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

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

Also, in biological view, a human body consists of various organs, and the organs consist of numerous cells. The ordinary biological analyses handle the cells by quantitative methods mainly using differential equation, but the analyses of some diseases caused by functional abnormality of cells need representation of qualitative changes and may properly described by the method of agent based simulation. We are also developing hybrid simulation platform of continuous and discrete agent based system using SOARS. In this year we have developed prototype simulation system especially for blood cell interaction taking Hyper-cycle theory into consideration.

"Logical Atomism" is the basic language system proposed by Bertrand Russell for the foundation of all physical sciences. We are planning and preparing its application to genome based medical sciences through constructing "e-pathfinder" system.

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