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Division of Clinical and Experimental Oncology Department of Molecular Oncology & leukemia Program Project

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The main purpose of our department is to elucidate the molecular mechanisms of leukemogenesis. We are trying to identify key factors that contribute to leukemogenesis from clinical samples, and also from basic research that is focused on the cytokine-dependent and ER-stress induced cell death. The latter work includes analysis of glaucoma in molecular level and application of apoptosis regulatory genes to bioengineering. The final goal of our study is to obtain basic data for development of new therapeutical approaches for leukemia by using molecular and cellular biology, as well as by establishing and analyzing gene-targeted mice, which has been performed based on a collaboration with Department of Developmental Oncology.

In order to complete these missions, we started Leukemia Program Project, as a project center in Hiroshima University in collaboration with Departments of Hematology and Oncology, Pediatrics as well as Biochemistry. Our work is also deeply involved in the COE project, which is driven mainly by RIRBM.

The research projects in this laboratory, which were carried out in 2003 and are being planned for the following years are summarized as follows.

1. Analysis of expression control of the *Bim* gene.

Matsui, T., Ohama, K., Inaba, T.

Purpose: Identification of molecular mechanisms that regulate the expression of *Bim* gene, which encodes a BH3-only cell death activator and plays critical roles in hematopoiesis.

Methods & Results: Expression control systems were analyzed by DNase hypersensitivity assay, reporter analysis, gelshift assay, nuclear run off assay, and experiments monitoring the half life of mRNA. By reporter assay, we isolated *cis*-elements,

which upregulate (several hundreds base pairs upstream of the transcriptional initiation site) or downregulate (in the first intron) the transcriptional efficiencies. Unexpectedly, the silencer sequence in the first intron is cytokine independent. Moreover, nuclear run off assay suggested that the expression of Bim is not controlled by transcriptional mechanisms, but by the regulation of the half-life of its message. Therefore, Bim mRNA is likely regulated by modification of mRNA degradation. In vitro assay, the half-life of Bim mRNA was elongated by cytokines. We found that this is mediated by heat shock cognate protein (Hsc70), which binds and stabilizes the mRNA. The binding potential of Hsc70 to mRNA is turned out to be regulated by cochaperons such as Hsp40, Hip, and Bag proteins.

2. Implications of FOXO3a in leukemogenesis.

Imagawa, J., Inaba T.

Purpose: FOXO3a (also FKHRL1) transcription factor on the chromosome band 6q21 is phosphorylated by cytokine-initiated signals and regulates the expression of p27, a CDK inhibitor, or Bim. FOXO3a is also involved in the leukemia associated with the t (6;11) (q21;q23). We tested whether FOXO3a is involved in leukemia with 6q-.
Methods & Results: We determined the consensus binding site of FOXO3a. We are trying to establish gelshift assay system using probes containing this sequence to test the expression or abnormality of FOXO3a in human leukemias.

3. Regulation of cell survival by neurotrophic factors and by ER-stress in retinal ganglion neurons (RGCs)

Yamazaki, M., Inaba, T., Murata K., Mishima, H. (Dept. Ophthal., Faculty of Medicine)

Purpose: Recent studies indicate that apoptosis of RGCs due to loss of survival signals from neurotrophic factors and/or ER-stress induced apoptosis may be causes of glaucoma. We planned to elucidate molecular mechanisms through which deprivation of neurotrophic factors and/or ER-stress induce apoptosis and effectiveness of erythropoietin to protect RGCs from ER-stress.
Methods & Results: Primary culture systems of RGCs from newborn rat eyes to obtain more than one million of retinal ganglion cells with more than 90% purity was established. RGC cells are cultured with or without neurotrophic factors such as BDNF and erythropoietin (Epo) and critical factors in the signal transduction pathways that support the survival of the neurons are identified. We found that Epo receptors are expressed in RGCs and Epo inhibits apoptosis of RGCs caused by glutamate and nitric oxicide, although Epo has no neurotrophic effects for RGCs. Because Epo has been widely used as a drug, it would be useful for the treatment of ocular diseases.

4. Development of a biological photo-switch in animal cells.

Inaba, T., Mabuchi, K. (Univ. of Tokyo)

Purpose: Development of photo-induction system of apoptosis, to obtain technology that enables to make microstructure of tissues in process of making artificial organs.
Methods & Results: Using photo-dependent binding system in plant cells such as PhytochromeB (phyB) and PIF3, we planned to develop a photo-reactive switch in animal cells. We established a method for purify phycocyanorubilin (PCB), which is essential for this system. We successfully translocated phyB-GFP protein from cytosol to nucleus by addition of PCB and red light (660nm). By replacing GFP with transcription factor, we are now trying to establish a photo-inducing molecular switch by establishing cell lines constitutively expressing these proteins.

5. Isolation of tumor suppressor genes from micro-deleted regions of the chromosome in hematological malignancies by LA-PCR assisted array-CGH.

Hiroya Asou, Toshiya Inaba

Purpose: Isolation of tumor-suppressor genes from deleted region in the chromosome-arm 7q in *de novo* AML, MDS and therapy-related AML.

Methods & Results: To isolate tumor suppressor genes in chromosome band 7q, we extracted DNA from leukemia samples stocked in RIRBM, and made probes for array-CGH by long-distance PCR technique. These probes with about 5kb-length sequences are spanning in chromosome 7q21.3-7q31.1.3 and do not contain repetitive sequences. We performed pilot experiments using 20 probes and solved a number of technical difficulties to detect haplo-deficiency and are now expanding study to make more than 100 probes.

6. Characterization of leukemia cell lines for developing molecular target therapy.

Hiroya Asou, Toshiya Inaba

Purpose: Characterization of newly established leukemia cell lines to establish experimental models for developing molecular target therapy.

Methods & Results: 1) Kasumi-3, which is sensitive for imatinib (gleevec) harbors internal tandem duplication (ITD) in the juxtamembrane domain of PDGFRα. This suggests there is a group of leukemias other than Ph^1-positive leukemia that could be treated by imatinib. 2) Kasumi-5 is a T-ALL cell line with t(4;11)(q21;p15) resulting in NUP98- GAP1GDS1 fusion gene. The role of activated GAP1GDS1, which is known as a guanidine exchange factor, is investigated whether constitutive activation of RhoA is induced by activated GAP1GDS1 in Kasumi-5 cells. Proliferation of Kasumi-5 cells is inhibited specifically by a RhoA kinase inhibitor. 3) Kasumi-6 is an AML M2 cell line with mutated C/EBPα gene. Effects of over-expression of normal C/EBPα gene is now investigating by enforcedly expressing the normal C/EBPα in Kasumi-6 cells.

7. Roles of normal and pathological forms of AML1 transcription factor in hematopoiesis and leukemogenesis.

Harada, H., Harada, Y. (Div. of Blood Transfusion Service, Univ. Hosp.), Honda, H. (Department of Developmental Oncology), Kimura A. (Dept. of Hematology/Oncology), Inaba, T.

Purpose: Elucidation of the biological roles of AML1 in the development of normal hematopoiesis and the function of AML1 mutants in leukemogenesis.

Methods & Results: 1) Isolation of AML1 mutations in AML and MDS patients. We previously isolated several novel point mutations in MDS patients among atomic bomb survivors of Hiroshima at unexpectedly high frequency and reported these data in *Blood*. We also identified many mutants in sporadic MDS patients in C-terminal region of AML1, where no mutations were reported so far. The overall *AML1* gene mutations in RAEB, RAEBt and AML following MDS reached as much as 25%, thus AML1 point mutation turned out to be one of the major cause of these low blast percentage myeloid leukemia with myelodysplasia. 2) Analysis of ART-1, a member of the tetraspan superfamily, as a direct downstream target of AML1. We established monoclonal antibody against ART-1 and found analyzing its expression in normal hematopoietic progenitors, as well as leukemia cell lines. 3) Development of mice carrying one allele that contains conditional knocked-in AML1 mutated genes. We are also generating conditional knocked-in mouse with AML1 mutants and AML1 fusion genes (AML1/ETO, AML1/Evi1)

8. Roles of mutated SHIP2 phosphatase in leukemogenesis.

Harada, H., Harada, Y. (Div. of Blood Transfusion Service, Univ. Hosp.), Niimi Hiromasa, Akiro Kimura (Dept. of Hematology/Oncology), Inaba, T.

Purpose: Elucidation of the biological roles of mutated and constitutively active SHIP2 phosphatase in leukemogenesis.

Methods & Results: We found point mutation of SHIP2 in some of MDS patients. Since the same mutant is reported as a cause of Noonan syndrome or juvenile myelomonocytic leukemia, we are planning to elucidate its role in leukemogenesis.

A. Original Papers

1. Asou, H., Gombart, A.F.[*1], Takeuchi, S.[*2], Tanaka, H.[*3], Tanioka, M.[*3], Matsui, T., Kimura, A.[*3], Inaba, T., Koeffler, H.P.[*1] ([*1]Dept. of Hematology/Oncology, Cedars-Sinai Medical Center, [*2]Dept. of Internal Med., Kochi Medial School, [*3]Dept. of Hematol./Oncol.) Establishment of the acute myeloid leukemia cell line Kasumi-6 from a patient with a dominant-negative mutation in the DNA-binding region of the C/EBPalpha gene. **Genes Chromosomes and Cancer** 36:167-174, 2003.(I)

2. Harada H., Harada Y.[*1], Tanaka H.[*2], Kimura A.[*2], Inaba T.([*1]Div. of Blood Transfusion Service, Univ. Hosp. [*2]Dept. of Hematol./Oncol.)Implications of somatic mutations in the AML1 gene in radiation-associated and therapy-related myelodysplastic syndrome/acute myeloid leukemia. **Blood** 101: 673-680, 2003.(I)

3. Yamaguchi T.[*1], Okada T.[*1], Takeuchi T.[*1], Tonda T.[*2], Ohtaki M.[*2], Shinoda S.[*1], Masuzawa T.[*1], Ozawa K.[*1], Inaba T. ([*1]Jichi Med. School, [*2]Dept. of Environmetrics and Biometrics)Enhancement of thymidine kinase-mediated killing of malignant glioma by BimS, a BH3-only cell death activator. **Gene Therapy**, 10: 375-385, 2003.(I)

4. Takeshita A.[*1], Naito K.[*1], Shinjo K.[*1], Nakamura S.[*1], Sahara N.[*1], Matsui H., Ohnishi K.[*1], Beppu H.[*1], Ohtsubo K.[*1], Hisatomi H.[*1], Horii T.[*1], Maekawa M.[*1], Inaba T., Ohno R.[*2] ([*1]Hamamatsu Univ. School of Med. [*2]Aichi Cancer Center) Del(6p23) and der(11p15) including NUP98 translocation in a case of secondary myeloproliferative disorder which eventually underwent clonal evolution and transformed to acute myeloid leukemia. **Cancer Genet. Cytogenet**., *in press*(I)

5. Matsunaga T.[*1], Inaba T., Matsui H., Okuya M.[*1], Kinoshita T.[*2], Miyajima A.[*2], Funabiki T.[*3], Endo M.[*4], Inukai T.[*5], Look AT.[*6], Kurosawa H.[*1]([*1]Dokkyo Univ. School of Med., [*2]Inst. of Molecular and Cellular Bioscience, Tokyo Univ.,[*3] Yokohama City Univ.,[*4]Iwate Med. Univ., [*5]Yamanashi Univ. School of Med., [*6]Dana-Faber Cancer Inst., Boston) Regulation of annexin II by cytokine-initiated signaling pathways and E2A-HLF oncoprotein. **Blood**, *in press*(I)

6. Akiyama T.[*1], Bouillet P.[*1], Miyazaki T.[*2], Kadono Y.[*2], Chikuda H.[*2], Chug U.[*2], Fukuda A.[*2], Hikita A.[*2], Seto H.[*2], Okada T.[*3], Inaba T., Sanjay A.[*5], Baron R.[*5], Kawaguchi, H.[*1], Oda H.[*1], Nakamura K.[*1], Strasser A.[*1], Tanaka S.[*1]([*1]Univ. of Tokyo, [*2]Juntendo Univ., [*3]Jichi Med. School, [*5]Yale Univ. School of Med.) Regulation of osteoclast apoptosis by ubiqutination of proapoptotic BH3-only Bcl-2 family member Bim. **EMBO J**., *in press*(I)

7. Harada H., Harada Y.[*1], Niimi H.[*2], Kyo T.[*3], Kimura A.[*2], Inaba T. ([*1]Div. of Blood Transfusion Service, Univ. Hosp., [*2]Dept. of Hematol./Oncol., [*3]Hiroshima Red Cross/A-Bomb Survivors Hosp.) High incidence of somatic mutations in the AML1/RUNX1 gene in myelodysplastic syndrome and low blast percentage myeloid leukemia with myelodysplasia. **Blood**, *in press*(I)

8. Kuribara R.[*1], Honda H.[*2], Matsui H., Shinjyo T.[*3], Inukai T.[*4], Sugita K.[*4],Nakazawa S.[*4], Hirai H.[*5], Ozawa K.[*1], Inaba T. ([*1]Jichi Med. School, [*2]Dept of Developmental Biol.,[*3]Ryukyu Univ.,[*4]Yamanashi Univ. School of Med.,[*5]Univ. of Tokyo) Implications of Bim, a BH3-only containing cell death activator, in the leukemogenesis of chronic myeloid leukemia. **Mol. Cell. Biol**., *in press*(I)

9. Miyanishi, S.[*1], Asou, H., Kawano, S.[*2], Okumura, A.[*1], Hayashida, M.[*1]([*1]Dept. of Biomedical Informatics, Tenri Inst. of Med. Res., [*2]Dept. of Clinical Pathology/Immunology, Kobe Univ.) Genetic characterization of newly established acute myeloid leukemia cell line having t(8;21)(q22;q22) and t(9;22)(q34;q11). **Clin. Pathol.** 50: 253, 2002.(I)

10. Ikezoe, T.[*1], Asou, H., Kyo, T.[*2], Taguchi, H.[*1]([*1]Dept. of Internal Med., Kochi Medial School, [*2]Dept. of Internal Med.,

Hiroshima Red Cross and Atomic Bomb Survivor's Hospital) PC-SPES decreases proliferation and induces differentiation and apoptosis of human acute myeloid leukemia cells. **Clinical Hematol.** 43: 115, 2002.

11. Ikezoe, T.[*1], Chen, S.[*2], Saito, T.[*2], Asou, H., Kyo, T.[*3], Tanosaki, S.[*2], Heber, D.[*2], Taguchi, H.[*1], Koeffler, H.P.[*2]([*1]Dept. of Internal Med., Kochi Medial School, [*2]Dept. of Hematology/Oncology, Cedars-Sinai Medical Center, [*3]Dept. of Internal Med., Hiroshima Red Cross and Atomic Bomb Survivor's Hospital) PC-SPES decreases proliferation and induces differentiation and apoptosis of human acute myeloid leukemia cells. **Int. J. Oncol.** 23: 1203-1211, 2003.(I)

B. Meeting Presentations

1. Harada, Y.[*1], Harada, H., Niimi, H.[*2], Kimura, A.[*2], Inaba, T.([*1]Div. of Blood Transfusion Service, Univ. Hosp.,[*2]Dept. of Hematol./Oncol.): A hematopoietic-specific transmembrane protein, ART-1, is directly regulated by AML1. The 65th General meeting of Japanese Society of Hematology. The 45th Annual meeting of Japanese Society of Clinical Hematology, Osaka, August 28-31, 2003.

2. Harada, H., Harada, Y.[*1], Niimi, M.[*2], Kimura, A.[*2], Inaba T.([*1]Div. of Blood Transfusion Service, Univ. Hosp., [*2]Dept of Hematol./Oncol.): Implication of somatic mutation in AML1 gene in myelodysplastic syndrome. The 65th General meeting of Japanese Society of Hematology. The 45th Annual meeting of Japanese Society of Clinical Hematology, Osaka, August 28-31, 2003.

3. Asou, H., Miyanishi, S.[*1], Kawano, S.[*2], Inaba, T.([*1]Dept. of Biomedical Informatics, Tenri Inst. of Med. Res., [*2]Dept. of Clinical Pathology/Immunology, Kobe Univ.)A novel AML cell line carrying both AML1-MTG8 and BCR-ABL fusion genes for molecular target therapy. The 65th General meeting of Japanese Society of Hematology・The 45th Annual meeting of Japanese Society of Clinical Hematology, Osaka, August 28-31, 2003.

4. Matsui, H., Asou, H., Inaba, T. EVI1 gene related leukemias highly differentiate toward dendritic cells. The 65th General meeting of Japanese Society of Hematology. The 45th Annual meeting of Japanese Society of Clinical Hematology, Osaka, August 28-31, 2003.

5. Niimi, H.[*1], Imagawa, J.[*1], Harada, H., Harada, Y.[*2], Kimura, A.[*1] ([*1]Dept. of Hematol./Oncol., [*2]Div. of Blood Transfusion Service, Univ. Hosp.) Acute leukemia with AML1 point mutation derived from chronic myeloproliferative disorders. The 65th General meeting of Japanese Society of Hematology. The 45th Annual meeting of Japanese Society of Clinical Hematology, Osaka, August 28-31, 2003.

6. Asou, H., Tanaka, H.[*1], Kimura, A.[*1], Inaba, T.([*1]Dept. of Hematol./Oncol.)Inhibitory effect of Rho kinase inhibitor against T-cell acute lymphoblastic leukemia cells having NUP98-RAP1GDS1 fusion gene. The 62nd Annual Meeting of the Japanese Cancer Association. 9.25-27, 2003, Nagoya.

7. Harada, H., Harada, Y.[*1], Niimi, H.[*1], Kimura, A.[*1]([*1]Dept. of Hematol./Oncol.) Implications of somatic mutations in the C-terminal region of AML1(Runx1)gene in myelodysplastic syndrome. The 62ndAnnual meeting of the Japanese Cancer Association. 9.25-27, 2003, Nagoya.

8. Matsui, H., Ohama, K., Imagawa, J.[*1], Inaba T.([*1]Dept. of Hematol./Oncol.) Regulation of apoptosis by cytokines through posttranscriptional control: identification of proteins interacting with Bim mRNA3'untranslated region. The 62ndAnnual

meeting of the Japanese Cancer Association. September25-27, 2003, Nagoya.

9. Asou, H., Imagawa, J., Harada, K., Harada, H., Harada, Y.[*1], Kyo, T.[*2], Inaba, T.([*1]Div. of Blood Transfusion Service, Univ. Hosp. [*2]4th Internal Medicine, Hiroshima Red Cross & Atomic Bomb Survivor's Hospital) Acute myeloid leukemia with either mutant KIT or PDGFRA-ITD is a novel therapeutic target of imatinib mesylate. The 45th Annual meeting of the American Society of Hematology, December 6-9, 2003, San Diego.

10. Harada, H., Harada, Y.[*1], Niimi, H.[*2], Kimura, A.[*2], Inaba, T.([*1] Div. of Blood Transfusion Service, Univ. Hosp. [*2]Dept. of Hematol./Oncol.) Point mutations in the carboxy-terminal region of the *AML1/RUNX1* gene associated with myelodysplastic syndrome. The 45th Annual meeting of the American Society of Hematology, December 6-9, 2003, San Diego.

11. Sato, H.[*1], Goi, K.[*1], Sugita, K.[*1], Inukai, T.[*1], Takahashi, K.[*1], Nemoto, A.[*1], Akahane, K.[*1], Hirose, K.[*1], Inaba, T., Nakazawa, S.[*1].(Pediatrics, Faculty of medicine, Yamanashi University) The histone-deacetylase inhibitor trichostatin A effectively induces p21-mediated cell cycle arrest and Caspase-dependent apoptosis in B-precursor leukemia cells. The 45th Annual meeting of the American Society of Hematology, December 6-9, 2003, San Diego.

C. Others

1. Toshiya Inaba, AML1/RUNX1 point mutation in low blast percentage myeloid leukemia with myelodysplasia. Hokkaido University, Sapporo 2003.5.23

2. Toshiya Inaba: Gene monitoring system for survivors of nuclear disaster.
The 44th Annual Meeting of Late Effects of Atomic Bomb, Hiroshima(2003.
6.1)

3. Inaba T.,: Radiation and leukemia. Hiroshima University Open Class, Hiroshima(2003.6.11)

4. Inaba, T. : AML1/RUNX1 point mutations in low blast percentage myeloid leukemia with myelodysplasia. Annual Meeting of the Late Effects for Atomic Bomb Research Committee, the Ministry of Health, Labor and Welfare of Japan, Hiroshima, 2002. (9.4.2003)(R, G)

5. Toshiya Inaba: Regulation of mRNA half-life by heat shock cognate protein(Hsc70). Annual meeting of Yamanouchi foundation for research on metabolic disorders. Tokyo (2003.10.25)

6. Toshiya Inaba: 'Post-chimera' research for leukemia/lymphoma. Yamaguchi University, Ube(2003.10.29)

7. Toshiya Inaba: Point mutations in the AML1/RUNX1 gene in radiation-related MDS/AML. Kyoto University, Osaka (2003.11.7)

8. Toshiya Inaba: 'Post-chimera' research for leukemia/lymphoma. Chugai Asian Conference on Modern Therapies in Hematology Osaka (2003.11.8)

9. Inaba, T. : AML1/RUNX1 point mutations in low blast percentage myeloid leukemia with myelodysplasia Annual Meeting

of the Translocation-related Genes in Hematological Malignancies Research Committee, the Ministry of Health, Labor and Welfare of Japan, Tokyo, 2002. (11.14.2002) (R, G)

10. Inaba, T. : AML1/RUNX1 point mutations and abnormal activation of receptor tyrosine kinase. Annual Meeting of the Late Effects for Atomic Bomb Research Committee, the Ministry of Health, Labor and Welfare of Japan, Hiroshima, 2002. (2.24.2004) (R, G)