Affiliated Facilities

Laboratory Animal Research Center
実験動物研究施設

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Assistant Professor	Misako Yoneda D.V.M., Ph.D.	助 教	農学博士	米 田 美佐子
Assistant Professor	Hiroki Sato Ph.D.	助 教	理学博士	佐 藤 宏 樹

Our major research interests are to elucidate molecular mechanisms of pathogenicity and species specificity of minus and single strand RNA viruses (Mononegavirales), and to control viral diseases. For these purposes, we are studying virus replication and identifying viral and host factors important for the expression of pathogenicity using a novel reverse genetics technique. We are also developing new virus vaccines and virus vectors by genetic engineering. In the animal research center, more than 30,000 mice, mainly transgenic or knockout, are kept for research of IMSUT, and the technical staff support their breeding, frozen storage of eggs and microbiological cleaning.

A novel monolayer cell line derived from human umbilical cord blood cells shows high sensitivity to measles virus

Fumio Kobune, Yasushi Ami[1], Miki Katayama[1], Motohide Takahashi[1], Renchin Tuul[2], Gulay Korukluoglu[3], Tomoko Kiyohara[1], Ryuichi Miura, Hiroki Sato, Misako Yoneda and Chieko Kai[*]: [1]National Institute of Infectious Diseases, Japan, [2]National Center for Communicable Diseases, Ulaanbaatar, Mongoliaand and [3]National Measles Laboratory, Virology Department, Refik Saydam National Hygiene Center, Ankara, Turkey

Measles virus (MV) research is largely dependent on the B95a cell line that is derived from marmoset B lymphocytes. Since this cell line is persistently infected with Epstein Barr virus (EBV), we established a new cell line, COBL-a, from human umbilical cord blood. COBL-a cells have a significant advantage over B95a cells because they are of human origin, are free from EBV, and have higher sensitivity to wild-type MV. Thus, COBL-a cells should prove very valuable for both epidemiological and basic studies of MV.

Inhibition of host protein synthesis in B95a cells infected with the HL strain of measles virus

Yoshihisa Inoue, Kyoko Tsukiyama-Kohara[1], Misako Yoneda, Hiroki Sato and Chieko Kai: [1]Department of Experimental Phylaxiology, Faculty of Medical and Pharmaceutical Sciences, Kumamoto University, Kumamoto, Japan

The shut-off of host protein synthesis in virus-infected cells is one of the important mechanisms for viral replication. we showed that MV-HL as well as other field isolates, which were isolated from human blood lymphocytes using B 95a cells, induce the shut-off in B95a cells. Since the Edmonston srrain of MV failed to induce the shut-off in B95a cells, the ability to induce the shut-off was considered to be dependent on virus strains. Although, the modification of eukaryotic translation initiation factors (eIF) in-

cluding eIF4G, eIF4E, and 4E-BP1 was reported for shut-off by various viruses, the involvement of these eIFs was not observed in MV-HL-infected B95a cells. Instead, the accumulation of phosphorylated eIF2α was found to coincide to the decrease of host protein synthesis, suggesting the involvement of phosphorylation of eIF2α in inhibition of translation as one of the mechanisms of the shut-off.

Measles virus N protein inhibits host translation by binding to eIF3-p40.

Hiroki Sato, Munemitsu Masuda, Moeko Kanai, Kyoko Tsukiyama-Kohara[1], Misako Yoneda and Chieko Kai: [1]Faculty of Medical and Pharmaceutical Sciences, Kumamoto University, Kumamoto, Japan.

The non-segmented, negative-sense RNA genome of MV is encapsidated by the virus-encoded nucleocapsid protein (N). In this study, we searched for N-binding cellular proteins using MV-N as bait and screening human T-cell cDNA library by yeast two-hybrid, and isolated the p40 subunit of eIF3 (eIF3-p40) as a binding partner. The interaction between MV-N and eIF 3-p40 in mammalian cells was confirmed by co-immunoprecipitation. Since eIF3-p40 is a translation initiation factor, we analyzed the potential inhibitory effect of MV-N on protein synthesis. GST-fused MV-N (GST-N) inhibited translation of reporter mRNAs in rabbit reticulocyte lysate translation system in a dose-dependent manner. Encephalomyocarditis virus internal ribosomal entry site-mediated translation, which requires canonical initiation factors to initiate translation, was also inhibited by GST-N. In contrast, a unique form of translation mediated by the intergenic region of plautia stali intestine virus, which can assemble 80S ribosomes in the absence of canonical initiation factors, was scarcely affected by GST-N. In vivo expression of MV-N induced by the Cre/loxP switching system inhibited the synthesis of a transfected reporter protein, as well as overall protein synthesis. These results suggest that MV-N targets eIF3-p 40 and may be involved in inhibiting MV-induced host translation.

Immune responses against measles virus in cynomolgus monkeys

Hiroki Sato, Fumio Kobune[1], Yasushi Ami[2], Misako Yoneda, and Chieko Kai: [1]Department of Viral Disease and Vaccine Control and [2]Division of Experimental Animal Research, National Institute of Infectious Disease, Japan.

MV induces profound suppression of the immune response during and for weeks after acute infection. On the other hand, virus-specific immune responses that mediate viral clearance and confer long-lasting immunity are efficiently generated. To investigate this paradox we studied the immune responses to MV using a monkey model of acute measles. Cynomolgus monkeys were experimentally infected with wild-type MV (MV-HL) and showed marked leukopenia associated with a steady reduction in CD4+ T cell numbers for 18 days post inoculation. Transient expression of interferon and IL-6 were observed in the serum between four and six days post inoculation, and IL-10 levels increased after 11 days post inoculation. Interestingly, IL-8 showed a three-peak increase that correlated with an increase in neutrophils. A nonhuman primate model of measles allows the early immune response against MV to be studied in more detail.

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Affiliated Facilities

Amami Laboratory of Injurious Animals
奄美病害動物研究施設

Professor	Chieko Kai, D.V.M., Ph.D.	教　授　農学博士	甲　斐　知恵子
Associate Professor	Shosaku Hattori, D.V.M., Ph.D.	准教授　農学博士	服　部　正　策

The Amami Laboratory of Injurious Animals was established in 1965 at Setouchi-cho in Amami-oshima Island in order to study on endemic diseases involving parasite, arthropods, and venomous snakes in the tropics or subtropics.
The Amami-oshima Island belongs to the Nansei (Southwest) Islands and the fauna is quite different from that in other islands of Japan. Since establishment of the laboratory, trials have been carried out to utilize small mammals found unique in the Amami islands as experimental animals in addition to studies on prevention of Habu bites. As well known, successful eradication of filariasis from this island is one of the monumental works of the laboratory. Our present works are as follows:

1. Research on the Habu control

Shosaku Hattori, Hiroshi Kihara[1], Motonori Ohno[2], Naoko Ueda[2], Shigenari Terada[3], Hiro Yonezawa[4], Yoshihiro Hayashi[5], Michihisa Toriba[6] and Tomohisa Ogawa[7]: [1]Bioscience research Institute, Takara Shuzo Co., Ltd., [2]Departmen of Applied Life Science, Faculty of Bioscience, Sojo University, [3]Department of Biochemistry, Faculty of Science, Fukuoka University, [4]Department of biochemistry, Faculty of Science, Kagoshima University, [5]Department of Veterinary Anatomy, Faculty of Agriculture, University of Tokyo, [6]The Japan Snake Institute, [7]Faculty of Agriculture, Tohoku university

Snake bites by the venomous snake Habu, *Protobothrops flavoviridis*, have been reported annually about 80 cases in the population of 100,000 in the Amami Islands. Moreover, there is no indication that the population of the Habu itself has decreased, despite a campaign for capture of snakes by the Kagoshima Prefectural Government. Rat-baited box traps have been introduced to catch the snakes and found to be quite effective. However, maintenance of live rats requires man power and its cost is expensive. Therefore, our effort has been focused on the development of attractant for Habu. The attractant extracted from rats seems ineffective if compared with use of live rats.

It was known that the Habu survived the injection of the Habu venom since early times, because some proteins in the serum of the Habu blood combine to the elements of the Habu venom. The research of these binding proteins has been initiated with an objective of clinical trials. Phospholipase A_2 and its isozymes isolated from Habu venom have myonecrotic activity and hemorrhagic activity, and metal protease has hemorahagic activity. The binding proteins isolated from serum of Habu inhibit myonecrotic activity of phospholipase A_2 and its isozymes. We found that protein-HSF and peptide-AHP isolated from the Habu serum effectively control the hemorrhage caused by venom of the Habu, *Ovophis okinavensis*, *Agkistrodon blomhoffi brevicaudus*, *Calloselasma rhodostoma*, *Bitis arietans*, *Bothrops asper*, and, *Trimeresurus stejnegeri*.

Further, a statistics analysis and the simulation were done with the snakes captured by the

Government, and the analysis of population dynamics of Habu was attempted. As a result of investigating the individual measurement data of the captured Habu over 9 years, we were able to obtain the generous age composition of the Habu. From analyzing of the age pyramid of the Habu and the result of questionnaire surveys for the inhabitant in the Amami-oshima Island, the total population of the Habu which lives in this island was estimated at about 80,000. By the analysis of the measured data of last nine years, the snake sizes were miniaturized, and the population of young snakes decreased. According to these investigations, the population of the Habu is expected to decrease in the near future.

These studies are supported by grants from the Ministry of Land, Infrastructure and Transport and the Kagoshima Prefectural Government.

2. Chimeric VEGF-E_{NZ7}/PIGF specifically binding to VEGFR-2 accelerates skin wound healing via enhancement of neovascularization

Yujuan Zheng[8], Makoto Watanabe[8], Takeshi Kuraishi, Shosaku Hattori, Chieko Kai and Masabumi Shibuya[8]: [8]Division of Genetics

Objective-$VEGF_{NZ7}$/PIGF molecules composed of Orf virus-derived VEGF-E_{NZ7} and human PIGF1 were previously proven to be potent angiogenic factors stimulating angiogenesis without significant enhancement of vascular leakage and inflammation in vivo. For its future clinical application, there is a pressing need to better understand the beneficial effects of VEGF-E_{NZ7}/PIGF during wound healing in adulthood. *Methods and Results*-In this study, several angiogenic factors were administrated to skin punched wounds of both wild-type and diabetic mice. The treatment with VEGF-E_{NZ7}/PIGF accelerated wound closure accompanied with enhanced angiogenesis, the process was occurring slightly faster than that in VEGF-A_{164} group. Moreover, the macrophage infiltration and lymphangiogenesis level in healed wounds were strikingly lower in VEGF-E_{NZ7}/PIGF group than VEGF-A_{164} group, suggesting that the increased inflammation was the key issue preventing speedy wound healing of VEGF-A_{164}-treated skin. Considering clinical safety, we further examined the antigenicity of chimeric VEGF-E_{NZ7}/PIGF. Compared with the original $VEGF_{NZ7}$, the immunogenicity of VEGF-E_{NZ7}/PIGF molecules was markedly decreased in mice and squirrel monkeys with the increase of PIGF1 humanized ratio. *Conclusion*-These results indicate that VEGF-E_{NZ7}/PIGF molecules are superior to VEGF-A for the acceleration of either normal of delayed skin wound healing and might be regarded as potential drugs in therapeutic angiogenesis.

3. Reproduction of squirrel monkeys.

Shosaku Hattori, Takeshi Kuraishi, Kumiko Ikeda, Hazuki Yoshimura and Chieko Kai

The squirrel monkey, *Saimiri sciurea*, is widely distributed in the tropical rainforest in Central and South America between 10 degrees N and 17 degrees S of latitudes. The advantage of using this species for medical researches resides in its small size and gentle behavior. In this laboratory, about 3 newborns are given annually by 25 adult females.

The aim is to optimize the use of the non-human primate model in future the Amami Laboratory research activities. The laboratory newly established experimental infection systems which require or can be adapted to the squirrel monkey model, particularly the study of human falciparum malaria. Development of parasites, immune response to malaria parasites and pathological changes were investigated in in-vivo condition, further more, in vitro analysis of cell and molecular level was performed. It is also investigating the mechanisms of infection in immunology, vector development, a vaccine production program, and a clinical trials program.

4. The diet of dogs in the Amami-Oshima Island forest, with special attention to predation on endangered animals

Yuya Watari[9], Yumiko Nagai[10], Fumio Yamada[11], Taku Sakoda[10], Takeshi Kuraishi, Shintaro Abe[10] and Yoshimi Satomura[12]: [9]Graduate school of Agricultural and life science, The University of Tokyo, [10]Amami wildlife conservation center, [11]Forestry and forest product research institute, [12]Amami mammalogical society

We analyzed 135 dog fecal pellets sampled in a forest on Amami-Oshima Island, Japan. Rare mammals, including the Amami rabbit (*Pentalagus furnessi*; 45.2%), Amami spinous rat (*Tokudaia osimensis*; 23.7%), and long-haired rat (*Diprothrix legata*; 20.0%), occurred in fecal pellets at high frequencies, indicating that these species are highly vulnerable to dog predation. Dog reproduction in the forest area has not been confirmed. However, many pets or hunting dogs appear to be abandoned or unsupervised, indicating that the morals of pet owners may

largely influence the dog population and, hence, its impact on native species. We propose the following management strategy. 1) Long term: preventing dogs from entering the forest. Enforcing current laws regarding supervision of dogs and educating pet owners is required. These are fundamental actions that will reduce the abundance of dogs in the forest; thus, this should be given high priority. 2) Short term: rapid action against detected dogs. A system to report dog sightings is necessary, together with a system to quickly catch reported dogs.

Publications

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Affiliated Facilities

Laboratory of Molecular Genetics
遺伝子解析施設

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Assistant Professor	Yumi Kanegae, D.M.Sc.	助　教　医学博士	鐘ヶ江　裕　美
Research Associate	Saki Kondo, D.M.Sc.	助　手　医学博士	近　藤　小　貴

This laboratory has two main activities, development of efficient expression vectors for gene therapy, especially for anti-cancer, and supporting the researchers by advising on recombinant DNA technology and on biohazards under the safety guidelines.

The purposes of our laboratory are concerned about not only research but also support for all researchers in this institute. Our supporting activity is involved in advising service on gene-manipulation experiments and on biohazards under the safety guidelines. For the research part, we intend to develop novel methods or new experimental systems leading in the field of gene expression and its regulation. We are concentrating mainly on developing efficient adenovirus expression vectors aiming at gene therapy. We are maintaining more than 20 collaborations within and outside of this institute. In these collaborations, we offer and supply our efficient method to construct recombinant adenovirus (rAd) expressing various genes efficiently. And recently we developed the new cosmid cassette for rAd construction which can choose not only very efficient COS-TPC method (Miyake *et al*., PNAS 93: 1320-1324, 1996) but also an easier method using a full-length viral genome with intact viral termini (Fukuda *.et al*., Microbiol. Immunol., 50: 643-654, 2006). This new cassette is available from Takara Bio and Nippon Gene. We have also developed a method for ON/OFF switching of gene expression in mammalian cells using a combination of adenovirus vector and Cre / *loxP* system (Kanegae *et al*., Nucleic Acids Res. 23: 3816-3821, 1995; Kanegae *et al*., Gene 181: 207-212, 1996) as well as FLP/FRT system (Nakano *et al*., Nucleic Acids Res. 29:e40, 2001; Kondo et al., Nucleic Acids Res. 31:e76, 2003; Kondo et al., Microbiol. Immunol., 50: 831-843, 2006). The methods will promote studies of various fields of molecular biology and medicine and may open a new field of "intracellular gene manipulation". The research activities in 2007 were shown below.

1. Expression of pIX gene induced by transgene promoter: possible cause of host immune response in first-generation adenovirus vectors

Michio Nakai, Kazuo Komiya[1], Masashi Murata[1], Toru Kimura[1], Masaharu Kanaoka[1], Yumi Kanegae and Izumu Saito: [1]Drug Research Division, Dainippon Sumitomo Pharma

First-generation (FG) adenovirus vectors (AdVs) have been widely used not only for basic studies but also gene therapy. Because vectors of this type lack the E1A gene that is essen-

tial for the expression of other viral genes, their expression levels in target cells have been considered low. Nevertheless, significant cellular immune response resulting in transient transgene expression and long-term toxicity are observed when AdV is administered to animals; this problem is an obstacle to the use of FG AdV in preclinical and clinical studies on gene therapy. Thus, in attempts to obtain further long -term expression, AdVs containing attenuated or deleted E2A and E4 genes (also called second- or third-generation AdV) have been constructed. However, the benefits of these deletions remain controversial because although these deletions do indeed minimize AdV late gene expression, they do not drastically improve the duration of transgene expression. Thus, in addition to E2A and E4 genes, another viral gene that is expressed in the absence of E1A gene expression may cause cellular immune responses, hampering the long-term expression of transgenes in FG AdVs. Therefore, the identification of such a viral gene(s) and the development of FG AdVs that do not trigger a host immune response have long been desired.

During our attempt to find another viral gene being expressed in the absence of E1A gene during infection with a FG AdV, we found that the viral pIX gene, located immediately downstream of the inserted expression unit of the transgene, was significantly co-expressed with transgene in cells infected with FG AdV. While CAG and SRα promoters considerably activated the pIX promoter through their enhancer effects, the EF1α promoter hardly did. Previous reports hypothesized that AdV immune responses might arise because of a spontaneous "leak" in the expression of the E2A or E4 gene in the absence of E1A gene expression. Here, we showed that a viral gene expression can occur through a completely different mechanism, where an enhancer used for the expression of the transgene activates the native pIX promoter. Moreover, when the expression unit was inserted in the rightward orientation, not only the pIX protein but also a fusion protein of the transgene-product/pIX were sometimes co-expressed with the transgene product through an aberrant splicing mechanism. The fused protein generated in the rightward orientation should also be immunogenic just like the native pIX protein. In *in vivo* experiments, a LacZ-expressing AdV bearing the CAG promoter caused an elevation of alanine-aminotransferase (ALT) in *i.v*., but an AdV bearing the EF1α promoter produced no detectable levels. While the FG AdV expressing human growth hormone under the CAG promoter maintained a high hormone level for less than one month, the FG AdV under the EF1α promoter maintained a high level for at least six months. These results suggest that pIX co-expression may be one of the main causes of AdV-induced immune responses of the short inflammation period for about 1-2 weeks and the long-term immune response for months. The EF 1α promoter hardly induced pIX co-expression and, probably for this reason, the immune response was mostly avoided. Therefore, the EF1α promoter is probably valuable for the long-term expression of FG AdV. Thus, the *in vivo* utility of FG AdV should be re-evaluated. These works should be very important in the study of specific cancer gene therapy, because avoiding the immunogenicity in normal tissue is crucial in such therapy. This work was published as Nakai *et al*., Hum. Gene Ther. 18:925-936, 2007.

2. Identification of stuffer sequences for improvement of helper-dependent adenovirus vector

Saki Kondo, Miho Terashima, Sou Hai, Yuki Takata, Yumi Kanegae and Izumu Saito

Although adenovirus vector induce strong immune responses applied in *in vivo* assay, it has been reported that such immune responses could be escaped by using a helper-dependent adenovirus vector (HD vector), whose genome is entirely consists of foreign DNA except for viral packaging signal and ITRs. Because the HD vector dose not have viral genes, it is necessary to be supplied all viral proteins via helper virus by trans. Moreover, for efficient packaging of the HD genome to viral particle, the genome length has to be adjusted to adenovirus packaging size (28～36 kb). For this purpose, lambda phage DNA previously has been used as a stuffer sequence. Recently, however, it has been reported that the stuffer sequences derived from lambda genome would not be desirable for HD vector generation, and would repress the transgene expression.

In this study, we tried to identify better stuffer sequences for HD vector. It is desirable that the stuffer sequence would not influence upon mammalian cell functions. Therefore, we subcloned hypoxanthine-guanine phoshoribosyltransferase (HPRT) genomic sequence, as a well-characterized mammalian gene, for a stuffer DNA candidate. Although the first intron of HPRT gene has already applied for a stuffer of HD vector, we cloned other sequences around the cording sequence of the HPRT gene. The candidate fragments of either direction were cloned into a plasmid for generating HD vector. To compare strength of transgene expression before HD vector generation, nine plasmids con-

taining each stuffer sequence and a lambda DNA was transfected to 293 cells, and the strength of GFP expression was measured by FACS analysis. The result showed that not only a difference of stuffer sequences but also a direction of inserted stuffers into a plasmid greatly effected on the GFP expression. The GFP expression of any plasmid containing a stuffer derived from HPRT gene was stronger than that of lambda DNA. We also compared the efficiency of HD vector generation using these plasmids. The result demonstrated that the stuffer sequences derived from HPRT, which were used in this study, were more efficient in vector generation than that from lambda DNA. Moreover, some of the stuffer sequences we used were much more efficient than the reported HPRT stuffer sequence. Therefore, we conclude that some of the stuffers we cloned here could be valuable for HD vectors.

3. Characterization of FLP recombination activity in mammalian cells introduced using adenovirus vectors

Saki Kondo, Yuki Takata, Masakazu Nakano, Yuzuka Takahashi, Izumu Saito and Yumi Kanegae

Site-specific recombinases are widely utilized to regulate gene expression. Although Cre recombinase is the most commonly used recombinase, especially in mammals, its cytotoxicity has recently been reported. Another well-characterized recombinase, FLP, has a lower activity than that of Cre, but its cytotoxicity has not been reported. To apply FLP in mammals efficiently, a thermo-stable mutant of wild-type (wt) FLP, named FLPe, has been reported. Despite this improvement, however, the recombination efficiency of FLPe in mammalian cells remains quite low; at most, a chromosome DNA recombination rate of only 6% was achieved in ES cells, which was 4- to 10-fold better than of wtFLP, when FLPe was expressed using plasmid DNA electroporation. Therefore, further improvement to obtain efficient recombination activity in mammalian cells is desired. Moreover, a detailed examination of the recombination activity of FLPe in mammalian cells has not yet been performed.

To improve FLP recombination activity in mammals, we utilized a recombinant adenovirus (rAd), and precisely compared the recombination efficiency of wtFLP and FLPe in mammalian cells. Unexpectedly, although FLPe showed thermo-stability, its recombination activity per enzyme was lower than that of wtFLP in mammalian cells. We constructed another mutant FLP, "humanized" FLPe (hFLPe), containing a codon usage suitable for mammals. (In the annual report two years ago the hFLPe we wrote found mutated at the fourth amino acid and the hFLPe written here was correct.) hFLPe showed a high activity in transfection experiments, as expected; however, an rAd expressing hFLPe could not be generated. Moreover, the large quantity of wtFLP protein expressed using rAd caused repression of the recombination in 293 cells. These results suggested that FLP may cause a deleterious effect on cells when an extremely high amount of FLP enzyme is expressed using rAd in 293 cells, in which the rAd genome is highly amplified. So, we conclude that FLPe-expressing rAd may be the most effective tool because, unlike wtFLP, it did not cause any problem when an rAd was used. However, FLPe is less efficient than wtFLP, hFLPe would be applicable when using plasmid transfection, tissue-specific promoter in rAd and studies using cell lines and mice.

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2. Nakai, M., Komiya, K., Murata, M., Kimura, T., Kanaoka, M., Kanegae, Y. and Saito, I. Expression of pIX Gene Induced by Transgene Promoter: Possible Cause of Host Immune Response in First-Generation Adenoviral Vectors. Hum Gene Ther. *18*: 925-936, 2007.
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Affiliated Facilities

Medical Proteomics Laboratory
疾患プロテオミクスラボラトリー

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Assistant Professor	Hiroshi Sagara, Ph.D.		助　教	医学博士	相　良　　洋
Project Assistant Professor	Masaaki Oyama, Ph.D.		特任助教	医学博士	尾　山　大　明

Protein analyses with high levels of techniques are now essential for understanding onset of many diseases especially in post-genomic research areas. Therefore, a proteomics research laboratory, which can serve various data leading to understanding systematic view of protein behaviors or networks, should be timely set up. Medical proteomics laboratory has been established in October 2006 in our institute due to such a need. This laboratory has four groups; first and second groups perform identification of proteins or protein complexes using two distinct types of mass spectrometry, third group demonstrates visualization of protein behaviors using electron microscopic techniques and fourth group prepares and serves highly purified water and sterile experimental apparatus. In addition to supporting other laboratories, these groups except the last one have their own research projects, which are described in this annual report.

〈Group I〉

1. Analysis of post-translational modification of proteins during cell death

Cell death involves various biochemical reactions. Among them, post-translational modification of proteins is intensively investigated in this laboratory. First, we describe transglutamination of a death receptor Fas. Intracellular proteolytic enzymes are also activated prior to and during apoptosis. Caspases are now established as pivotal apoptosis-executing enzymes that cleave various substrates. Endogenous or viral proteins and synthetic substances inhibitory for caspases suppress the apoptotic cascade and rescue cells from cell death. On the other hand, proteasomes drive the cell cycle by degrading cyclins etc., and also play important parts in apoptosis, since proteasome inhibitors induce apoptotic cell death in growing cells but suppress apoptosis of some cells that is in quiescent state. Furthermore, in some specific cells such as polymorphonuclear leukocytes, other proteases might be involved in cell death.

a. Down-regulation of Fas-mediated apoptosis by plasma transglutaminase that polymerizes the Fas molecule

Hidehiko Kikuchi, Fotoshi Kuribayashi and Shinobu Imajoh-Ohmi

A cell death-inducing monoclonal antibody CH11 mimics Fas ligand and triggers apoptotic signal mediated by Fas molecule. Plasma transglutaminases are found to involved in down-regulation of apoptosis induced by a cytotoxic anti-Fas monoclonal antibody in Jurkat cells. When cells were treated with the antibody in fetal calf serum-containing media, Fas was polymerized to higher-molecular-weight polypeptides as judged by immunoblotting. Un-

der conditions where the transglutaminase activity was eliminated or supprressed, the polymerization of Fas was not observed, and concurrently cell death was hastened. Furthermore, an antibody against blood coagulation factor XIII strongly accelerated the Fas-mediated apoptosis, indicating that plasma transglutaminases catalyze polymerization of Fas and down-regulate apoptotic cell death.

b. Limited proteolysis of actin in polymorphonuclear leukocytes

Junko Ohmoto and Shinobu Imajoh-Ohmi

Polymorphonuclear neutrophils (PMNs) undergo spontaneous apoptosis during cultivation in vitro. Various proteases are also activated and many target proteins have been reported in apoptotic PMNs. Actin is proteolyzed to a 40-kDa fragment that lacks amino-terminal region involved in polymerization. To investigate the role of actin proteolysis we made a cleavage-site-directed antibody for the 40-kDa form of actin using synthetic peptide as a hapten. The antibody stained the 40-kDa polypeptide but did not recognize native actin abundant in cell lysates. First, we found that the 40-kDa fragment is generated during isolation of PMNs from peripheral bood. By using diisopropyl fluorophosphate, an inhibitor for serine proteases, PMNs with native actin could be prepared. Furthermore, elastase was identifited as the enzyme responsible for the limited proteolysis of actin. In fact, when isolated PMNs were incubated with elastase, the 40-kDa fragment was observed, providing us with a question how extracellular elastase attacks actin.

2. Establishment of novel antibodies as tools available for in situ analyses of post-translational modification of proteins

After biosynthesis proteins undergo various post-translational modifications, and their functions are modulated. In order to understand such biochemical reactions in a single cell, we have been making modification-specific antibodies as probes for such in situ analyses; cleavage-site-directed antibodies for proteolysis , phosphorylation-site-specfic antibodies, myristoilated peptide-specific antibodies , ubiquitination-specific antibodies , inhibitor-bound enzyme-specific antibodies etc. These antibodies should be useful tools for research in cellular biochemistry.

a. Evaluation of polyclonal cleavage-site directed antibodies and their fractionation into more easy-to use probes

Tsuyoshi Katagiri, Chidzuko Takamura, Nozomi Ichikawa and Shinobu Imajoh-Ohmi

Cleavage-site directed antibodies are convenient tool for in situ analysis of proteolysis, since they do not bind unproteolyzed native proteins that retain the same sequence internally. To obtain such antibodies, peptides corresponding to the terminal regions around the cleavage are synthesized chemically and used for haptens, where molecular design of the peptides is critical for quality of the antibodies. Too short peptide results in generation of useless antibodies recognizing the short peptide but not the terminus of cleaved proteins. On the other hand, when a longer sequence is selected for immunogenic peptide, antibodies raised bind unproteolyzed proeins as well as the cleaved ones. Thus, an evaluation system is necessary for cleavage-site directed antibodies. Phage display liblaries were used for evaluation of antigenic specificity of cleavage-site directed antibodies. Randomized sequences of synthetic oligonucleotide were introduced into phage DNA in order that a fusion protein with randomized sequences of amino- or carboxyl-terminal region. A library was applied to immobilized antibodies, and phages bound were subjected to sequence analysis for terminal regions. When antigenic specificity of a cleavage-site derected antibody was examined by this method, the antibody was found to be a mixture of three types of antibodies that bind to terminal and two internal regions of the peptide used for immunogen. Quality of the antibody was successfully improved by affinity chromatography immobilized three peptides according to the evaluation method.

b. A novel method for hunting substrates of caspases in apoptotic cells

Maiko Okada, Chidzuko Takamura, Hiroyuki Fukuda, Masahiko Kato and Shinobu Imajoh-Ohmi

Caspases catalyze limited proteolysis of many proteins in apoptotic cells. Hundreds of substrates have been identified as targets of caspases so far. Previously, nonmuscle myosin heavy chain-A and a component of DNA-dependent proein kinase, Ku80, are found to be cleaved during apoptosis in human Jurkat T cells. We used first a cleavage-site derected antibody against the amino-terminal fragment of

caspase 3/7-catalyzed calpastatin. Carboxyl-terminal region of caspase-proteolyzed fragments resemble each other, and such antibodies are expected to misrecognize the target molecules. We further investigated the apoptotic Jurkat cells for the anitobody-stained polypeptides. Cells were selectively extracted with salt- and denaturant-containing buffers, and extracts were subjected to two-dimensional gel electrophoresis/immunoblotting. Candidate polypeptides stained with antibodies were digested with trypsin and analyzed by mass spetrometer. Isoforms of ribonucleoprotein were thus identified.

c. Identification of cysteine proteases in *Caenorhabditis elegans*

Yohei Kato and Shinobu Imajoh-Ohmi

E64c, [L-3-trans-carbonyloxirane-2-carbonyl]-L-leucine(3-methylbutyl)amide, is a synthetic inhibitor for cysteine proteases such as cathepsins B, H, L and calpain. To inhibit intracellular cysteine proteases E 64 d , [L-3-trans-ethoxycarbonyloxirane-2-carbonyl]-L-leucine (3-methylbutyl)amide, a membrane-permeable derivative of E64c is used instead of E64c. E64d penetrates into the cell membranes where cellular esterases convert it to E64c that covalently binds to the SH group of active center in enzymes. Thus, anti-E64c antibody is a useful probe for *in vivo* analysis of cysteine proteases.

We have succeeded in making an antibody to E64c. First, we tried to establish an antibody against E64c-bound calpain. A peptide corresponding to the active center of calpain was synthesized by using the multiple-antigen peptide system. E64c was chemically introduced into the SH group of active center cysteine under reducing conditions. Rabbits were immunized with the E64c-conjugated calpain-derived peptide without further conjugation with a carrier protein. Unexpectedly, an antibody thus prepared reacted not only with E64c-inactivated calpain but also with E64c-bound other cysteine proteases such as papain and cathepsins. Low antigenicity of peptide region in the immunogen may result in such broad specificity of the antibody. Our antibody is expected to be used for identification of E64c-targeted novel proteases. When cells were treated with E64d, cell growth was suppressed and several proteins were labeled by E64c that is visualized with this antibody on immunoblotting. Structural analysis of these proteins may lead identification of novel cysteine proteases.

Homogenates of *C. elegans* were treated with E64c in the presence or absence of calcium ion, and subjected to electrophoresis/immunoblotting using an anti-E64c antibody. A 55-kDa polypeptide (p55) was labelled with E64c in a calcium ion-dependent manner. In *C. elegans* several calpain-related gene products were identified at the mRNA level, but their physiological function remains to be elucidated

3. Myosin IIC regulates the division of intercellular bridge of cytokinesis.

Akira Nakanishi[1], Yoshio Miki[1,2] and Shinobu Imajho-Ohmi: [1]Department of Genetic Diagnosis, The Cancer Institute, Japanese Foundation for Cancer Research, [2]Depertment of Molecular Genetics, Medical Research Institute, Tokyo Medical and Dental University

Cytokinesis is the final step of cell division and leads to the physical separation of the daughter cells. Nonmuscle myosin IIs play an essential role during cytokinesis, but its exact role in cell division remains poorly understood. Here, we report an essential role for human nonmuscle myosin heavy chain (NMHC) IIC in both the stability of the bridge and its final abscission in the A549 human lung tumor cell. NMHC IIC was recruited between both of the plus-ends of microtubules of the intercellular bridge during the late telophase-to-cytokinesis. To investigate the significance of these observations, we used RNA interference to deplete NMHC IIC in A549 cells. Using time-lapse video imaging, A549 cells tended to divide with the two places of the microtubules plus-ends of the intercellular bridge. However, using specific siRNAs to decrease NMHC IIC in A549 cells resulted in failure to complete a functional cleavage of the intercellular bridge. The daughter cells still connected each other by intercellular bridges that contain microtubules and midbody components. These findings demonstrate that NMHC IIC may play an essential role in the terminal phase of cytokinesis.

4. Plectin, a novel protein which interacts with BRCA2

Akira Nakanishi[1], Yoshio Miki[1,2] and Shinobu Imajho-Ohmi: [1]Department of Genetic Diagnosis, The Cancer Institute, Japanese Foundation for Cancer Research, [2]Depertment of Molecular Genetics, Medical Research Institute, Tokyo Medical and Dental University

BRCA2 is known as tumor-suppressor gene, and the mutation of BRCA2 gene causes breast and ovarial cancers. It is known that the BRCA2 protein localizes to nucleus mainly, and it works accompany with recombinant repair for the

double-stranded DNA damage. Recently, we reported that BRCA2 had a nuclear export signal (NES) and located around a centrosome. To search novel protein which interacts with BRCA 2, HeLa cells extracts were separated by glycerol density gradient centrifugation. The endogenous BRCA2 consisted of a 700-800-kDa complex, and plectin was identified in the complex by analysis of tandem mass spectrometry. We confirmed that endogenous BRCA2 was immunoprecipitated with endogenous plectin. Furthermore, we have used the PLEC repeats (2738-3021 aa.) of plectin Ni(2+)-affinity pull-down assay and showed that PLEC repeats interacts with BRCA 2. It was already reported that plectin interacts with intermediate filament, or with nuclear membrane protein. Therefore, we thought that plectin might have a possibility of having played a role of the anchor protein, that is, BRCA2-plectin complex connects centrosomes to a nucleus. We planned future research to ascertain this.

〈Group II〉

1. Comprehensive analysis of phosphotyrosine-dependent signaling network dynamics by quantitative proteomics

Masaaki Oyama, Hiroko Kozuka-Hata, Shinya Tasaki, Ayumu Saito[1], Masao Nagasaki[1], Seiya Imoto[1], Ryo Yoshida[2], Kentaro Semba[3], Sumio Sugano[4], Jun-ichiro Inoue, Satoru Miyano[1] and Tadashi Yamamoto[5]: [1]Laboratory of DNA Information Analysis, Human Genome Center, IMSUT, [2]Department of Statistical Modeling, Institute of Statistical Mathematics, [3]Department of Life Science and Medical Bio-Science, Waseda University, [4]Laboratory of Functional Genomics, Department of Medical Genome Sciences, Graduate School of Frontier Sciences, The University of Tokyo, [5]Division of Oncology, Department of Cancer Biology, IMSUT

Signal transduction system is known to widely regulate complex biological events such as cell proliferation and differentiation. As phosphotyrosine-dependent signaling networks play a key role in transmitting signals, a comprehensive and fine description of their dynamics would contribute substantially toward understanding the regulatory mechanisms that result in each biological effect. Recent proteomics approaches using a highly sensitive nano-liquid chromatography tandem mass spectrometry (nanoLC-MS/MS) system have enabled us to obtain a comprehensive view of the focused proteome such as phosphoproteome. Based on the SILAC (Stable Isotope Labeling by Amino acids in Cell culture) technology, we developed a highly sensitive and simple method for making a temporal quantitative analysis of phosphotyrosine-related proteins. In order to automatically extract quantitative information from large volumes of nanoLC-MS/MS raw data, we also developed software named AYUMS, which enabled us to obtain fine activation profiles of the signaling molecules with the high time-resolution necessary for a systems biology approach.

Highly time-resolved analysis of the EGF-dependent signaling pathways in human A431 cells revealed a global view of their multi-phase network activation, comprising the spike signal transmission within one minute followed by the prolonged activation of multiple Src-related molecules. Experimental perturbation of Src-family kinases with PP2 in the second activation phase led to the drastic downregulation of the molecules related to cell adhesion, cytoskeletal rearrangement and receptor degradation, whereas the canonical MAPK and PI3K cascades as well as EGF receptor maintained their activities.

We also investigated signaling networks in NIH3T3 cells transfected with either of wild type or mutated EGFR to analyze the effect of the mutation on their signaling network dynamics. In order to perform *in silico* simulation based on our proteome data, we have constructed a mathematical model of the EGFR pathway using Cell Illustrator (Tasaki et al., Genome Inform., 17: 226-238, 2006), indicating some possible regulations caused by the mutation.

2. Description of the human short ORFeome through large-scale identification of small proteins by 2DnanoLC-MS/MS system

Masaaki Oyama, Hiroko Kozuka-Hata, Kentaro Semba[3], Jun-ichiro Inoue, Sumio Sugano[4] and Tadashi Yamamoto[5]

In parallel with the human genome projects, human full-length cDNA data has also been intensively accumulated. Large-scale analysis of their 5′-UTRs revealed that about half of these had a short ORF upstream of the coding region. Experimental verification as to whether such upstream ORFs are translated is essential to reconsider the generality of the classical scanning mechanism for initiation of translation and define the real outline of the human proteome. Our previous proteomics analysis of small proteins expressed in human K562 cells provided the first direct evidence of translation of upstream ORFs in human full-length cDNAs (Oyama et al., Genome Res., 14: 2048-2052,

2004). In order to grasp an expanded landscape of the human short ORFeome, we have performed an in-depth proteomics analysis of human K562 and HEK293 cells using a two-dimensional nanoLC-MS/MS system. The results led to the identification of eight protein-coding regions besides 197 small proteins with a theoretical mass less than 20 kDa that were already annotated coding sequences in the curated mRNA database. In addition to the upstream ORFs in the presumed 5′-untranslated regions of mRNAs, bioinformatics analysis based on accumulated 5′-end cDNA sequence data provided evidence of novel short coding regions that were likely to be translated from the upstream non-AUG start site or from the new short transcript variants generated by utilization of downstream alternative promoters. Protein expression analysis of the *GRINL1A* gene revealed that translation from the most upstream start site occurred on the minor alternative splicing transcript, whereas this initiation site was not utilized on the major mRNA, resulting in translation of the downstream ORF from the second initiation codon. These findings reveal a novel post-transcriptional system that can augment the human proteome via the alternative use of diverse translation start sites coupled with transcriptional regulation through alternative promoters or splicing, leading to increased complexity of short protein-coding regions defined by the human transcriptome.

〈Group III〉

The main activity of this group is to offer supports for the research projects of other laboratories using electron microscopic techniques. The electron microscopic techniques available in this group are the conventional thin section transmission electron microscopy, immuno-electron microscopy, negative staining techniques and scanning electron microscopy. By using these individual techniques or combination of some of these, we can offfer direct visual evidence that can not be acquired by other methods.

1. Thin section electron microscopy and immuno-electron microscopy

Thin section electron microscopy is the most widely used technique to observe the fine structure of the cells and tissues. In this method, samples are fixed and embedded in epoxy resin, thin sections with about 70nm thickness are cut and observed in the electron microscope. In case of immuno-electron microscopy, thin sections are obtained by similar procedure, and the antigen epitopes exposed on the surface of the sections are marked by sequentially reacted with appropriate primary antibodies and colloidal gold labeled secondary antibody. This year, thin section electron microscopy combined with immuno-electron microscopy were used in many collaborative works.

a. Mapping of the VP40-binding regions of the nucleoprotein of Ebola virus.

Noda, T[1]., Watanabe, S[1]., Sagara, H. and Kawaoka, Y[1].: [1]Division of Virology, Department of Microbiology and Immunology

To understand the mechanisms involved in Ebola virus assembly, we have been analyzing the function(s) of viral proteins ultrastructurally and have already revealed that binding of Ebola virus nucleoprotein (NP) with the matrix protein VP40 is important for nucleocapsid incorporation into virions. This year, functions of the region(s) on the NP molecule were examined by analyzing the interaction of a series of NP deletion mutants with VP40 in mammalian cells. We found that both termini of NP (amino acids 2 to 150 and 601 to 739) are essential for its interaction with VP40 and for its incorporation into virus-like particles (VLPs). We also found that the C terminus of NP is important for nucleocapsid incorporation into virions. These results were published in the Journal of Virology (ref: Noda, Watanabe, et al).

b. Ultrastructural analysis of the role of Bysl during mouse embryo development.

Adachi, K[2]., Soeta-Saneyoshi[2], C., Sagara, H. and Iwakura, Y[2]. [2]Division of Cell Biology, Center of Experimentl Medicine.

In this study, mouse embryos injected with small interfering RNA (siRNA) directed against Bysl (bystin-like) were analyzed ultrastructurally, and revealed to have deficiences in ribosome formation.

Some other collaborative research works using thin section electron microscopy and/or immuno-electron microscopy were done with Dr. Chida[2] *et al* (ref. Chida *et al*), Dr. Yana *et al*, in Division of Cancer Cell Research, Department of Cancer Cell Research (ref. Yana et al), and Dr. Honda *et al*, in Division of Stem Cell Engineering (ref. Honda *et al*). Also, other works are now in progress with Dr. Kawaguchi *et al* in Department of Infectious Disease Control, International Research Center for Infectious Diseases, and with Dr. Nochi[3], Dr. Igarashi[3], Dr. Sato[3] in [3]Division of Mucosal Immunology, Department of Microbiology and Immunology.

2. Negative staining techniques

Negative staining techniques are simple and quick method to observe the morphology of the macro molecules. This year, the negative staining method are used in two collaborative works. One is with Dr. Park *et al*. at Division of Biochemistry, Department of Cancer Biology. In this study, negative staining methods were used to analyze the function of heat shock protein 90 (HSP90) in N-WASP and Arp2/3 induced actin bundle formation in vitro (ref. Park *et al*). The other is with Dr. Hagiwara *et al*. at Laboratory Animal Center. In this study, negative staining techniques were used to analyze the roles of canine distemper virus proteins in nucleocapsid formation.

3. Scanning electron microscopy

Scanning electron microscopy is a technique used to examine the surface structure of the cells, tissues or other non-biological materials. The collaborative works using scanning electron microscopy were done with Dr. Iizumi *et al*, Division of Bacterial Infection, to observe the changes in the surface structure of the bacteria infected cells (ref Iizumi *et al*). Other work are in progress with Dr. Kinoshita *et al*, Department of Gerontological Nursing, Division of Health Science and Nursing, Graduate School of Medicine, The University of Tokyo, to analyze the effects of diabetes or bacterial infection during wound repair.

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