Affiliated Facilities

Laboratory Animal Research Center
実験動物研究施設

Professor	Chieko Kai, D.V.M., Ph.D.	教　授　農学博士	甲　斐　知恵子
Associate Professor	Misako Yoneda, D.V.M., Ph.D.	准教授　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
Assistant Professor	Tomoyuki Honda, M.D., Ph.D.	助　教　医学博士	本　田　知　之

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.

The nonstructural proteins of Nipah virus play a key role in pathogenicity in experimentally infected animals.

Misako Yoneda, Vanessa Guillaume[1], Hiroki Sato, Kentaro Fujita, Marie-Claude Georges-Courbot[2], Fusako Ikeda, Mio Omi, Yuri Muto-Terao, T. Fabian Wild[1], Chieko Kai: [1]Institut National de la Sante et de la Recherche médicale, U758, Lyon, France, [2]Laboratory P4 INSERM Jean Mérieux, Lyon, France.

Nipah virus (NiV) P gene encodes phosphoprotein (P) and three accessory proteins (V, C and W). It has been reported that all four P gene products have IFN antagonist activity when the proteins were transiently expressed. However, the role of those accessory proteins in natural infection with NiV remains unknown. We generated recombinant NiVs lacking V, C or W protein, rNiV(V-), rNiV(C-), and rNiV(W-), respectively, to analyze the functions of these proteins in infected cells and the implications in in vivo pathogenicity. All the recombinants grew well in cell culture, although the maximum titers of rNiV(V-) and rNiV(C-) were lower than the other recombinants. The rNiV (V-), rNiV(C-) and rNiV(W-) suppressed the IFN response as well as the parental rNiV, thereby indicating that the lack of each accessory protein does not significantly affect the inhibition of IFN signaling in infected cells. In experimentally infected golden hamsters, rNiV(V-) and rNiV(C-) but not the rNiV(W-) virus showed a significant reduction in virulence. These results suggest that V and C proteins play key roles in NiV pathogenicity, and the roles are independent of their IFN-antagonist activity. This is the first report that identifies the molecular determinants of NiV in pathogenicity in vivo.

Peroxiredoxin 1 is Required for the Efficient Transcription and Replication of Measles Virus

Akira Watanabe, Misako Yoneda, Fusako Ikeda, Hiroki Sato, and Chieko Kai

Measles is a highly contagious human disease caused by the measles virus (MeV). In this study, by proteomic analysis, we identified peroxiredoxin 1 (Prdx1) as a host factor that binds to the *C*-terminal region of the nucleoprotein (N_{TAIL}) of MeV. GST pull-down experiments showed that the Prdx1-binding site overlapped with the MeV P protein-binding site on N_{TAIL}, and that Prdx1 competed the binding to N_{TAIL} with the P protein, which is a component of RNA-dependent RNA polymerase (RdRp). Furthermore, RNA interference for Prdx1 resulted in a significant reduction in MeV growth in HEK293-SLAM cells. Minigenome assay indicated that Prdx1 suppression affected the viral RNA transcription and/or replication step. Quantitative real-time (QRT) PCR analysis showed that Prdx1 suppression not only reduced viral RNA transcription and replication but also enhanced polar attenuation in viral mRNA transcription. Furthermore, surface plasmon resonance analysis showed that the binding affinity of Prdx1 to MeV-N was 40-fold lower than that of MeV-P to MeV-N, which suggested that Prdx1 might be involved in the early stage of MeV infection, when the expression level of Prdx1 was much higher than tht of MeV-P. Prdx1 was required for efficient viral RNA synthesis regardless of vaccine or wild-type strain. These results indicate that Prdx1 is the first example of an inherent host factor implicated in MeV RNA synthesis.

CD147/EMMPRIN acts as a functional entry receptor for measles virus on epithelial cells.

Akira Watanabe, Misako Yoneda, Fusako Ikeda, Yuri Terao-Muto, Hiroki Sato and Chieko Kai

The wide tissue tropism of MeV suggests that it involves ubiquitously expressed molecules besides signaling lymphocytic activation molecule (SLAM) known as a cellular receptor for morbilliviruses, We identified cyclophilin B (CypB) as host factors binding to MeV-N by proteomic analysis. Western blot analysis of purified viral particles showed that CypB was incorporated in viral virions through the binding to N protein. As CypB is known as a ligand for CD147/EMMPRIN (extracellular matrix metalloproteinase inducer), a cell surface glycoprotein, we examined whether CD147 acts as a receptor for MeV. Anti-CD147 antibody or recombinant CypB inhibited MeV-infection on HEK293 cells which are SLAM-negative epithelial cells, while overexpression of human CD147 on CHO cells enhanced MeV-infection. Furthermore, prevention of CypB-incorporation in virions significantly reduced their infectivity to SLAM-negative HEK293 cells, while it had no effect on their infectivity to SLAM-positive HEK293-SLAM cells. These results indicated that CD147 is used as an entry receptor for MeV through incorporated CypB in the virions on SLAM-negative cells.

Comparative and mutational analyses of promoter regions of rinderpest virus.

Chieko Imai, Kentaro Fujita, Fusako Shimizu, Akihiro Sugai, Misako Yoneda, Chieko Kai

Comparative and mutational analysis of promoter regions of rinderpest virus was conducted. Minigenomic RNAs harboring the genomic and antigenomic promoter of the lapinized virulent strain (Lv) or an attenuated vaccine strain (RBOK) were constructed, and the expression of the reporter gene was examined. The activities of the antigenomic promoters of these strains were similar, whereas the activity of the genomic promoter (GP) of the RBOK strain was significantly higher than that of the Lv strain, regardless of cell type and the source of the N, P and L proteins. Increased replication (and/or encapsidation) activities were observed in the minigenomes that contained RBOK GP. Mutational analysis revealed that the nucleotides specific to the RBOK strain are responsible for the strong GP activity of the strain. It was also demonstrated that other virulent strains of RPV (Kabete O, Saudi/81 and Kuwait 82/1) have weaker GPs than that of the RBOK strain.

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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, Takeshi Kuraishi, Motonori Ohno[1], Naoko Oda-Ueda[2], Takahito Chijiwa[1], Aichi Yoshida[3], Yoshihiro Hayashi[4], Michihisa Toriba[5] and Tomohisa Ogawa[6],: [1]Department of Applied Life Science, Faculty of Bioscience, Sojo University, [2]Department of Biochemistry, Faculty of Pharmaceutical Science, Sojo University, [3]School of Health Science, Faculty of Medicine, Kagoshima University, [4]Department of Veterinary Anatomy, Faculty of Agriculture, University of Tokyo, [5]The Japan Snake Institute, [6]Faculty of Agriculture, Tohoku university.

Snake bites by the venomous snake Habu, *Protobothrops flavoviridis*, have been reported annually about 60 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. Unique structural characteristics and evolution of a cluster of venom phospholipase A_2 isozyme genes of *Protobothrops flavoviridis* snake

Naoki Ikeda[1], Takahito Chijiwa[1], Kazumi Matsubara[7], Naoko Oda-Ueda[1], Shosaku Hattori, Yoichi Matusda[7], Motonori Ohno[1]: [7]Division of Biological Scinece, Graduate School of Science, Hokkaido University.

Protobothrops flavoviridis (Crotalinae) venom gland phospholipase A_2 (PLA_2) isozyme genes have evolved in an accelerated manner to acquire diverse physiological activities in their products. For elucidation of the multiplication mechanism of PLA_2 genes, a 25,026 bp genome segment harboring five PLA_2 isozyme genes was obtained from Amami-Oshima *P. flavoviridis* liver and sequenced. The gene *PfPLA2* encoded [Lys^{49}]PLA_2 called BPII, the gene *PfPLA4* neurotoxic [Asp^{49}]PLA_2 called PLA-N, the gene *PfPLA5* basic [Asp^{49}]PLA_2 called PLA-B, and *PfPLA1*(ψ) and *PfPLA3*(ψ) were the inactivated genes. The 5′ truncated reverse transcriptase (RT) elements, whose intact forms constitute long interspersed nuclear elements (LINEs), were found in close proximity to the 3′ end of PLA_2 genes and named PLA_2 gene-coupled RT fragments (PcRTFs). The facts that PcRTFs have the stem-loop and repetitive sequence in the 3′ untranslated region (UTR) which is characteristic of CR1 LINEs suggest that PcRTFs are the debris of *P. flavoviridis* ancestral CR1 LINEs, denoted as *Pf*CR1s. Since the associated pairs of PLA_2 genes and PcRTFs are arranged in tandem in the 25,026 bp segment, it is thought that an ancestral PLA_2 gene-*Pf*CR1 unit (*PfPLA-Pf*CR1) which was produced by retrotransposition of *Pf*CR1 by itself to the 3′ end of PLA2 gene duplicated several times to form a multimer of *PfPLA-Pf*CR1, a cluster of PLA_2 genes, in the period after Crotalinae and Viperinae snakes branched off. Recombinational hot spot of a 37 bp segment, named Scomb, was found in the region 548 bp upstream from the TATA box of PLA_2 genes. Thus, it could be assumed that multiplication of *PfPLA-Pf*CR1 occurred by unequal crossing over of the segment, -Scomb-*PfPLA-Pf*CR1-Scomb-. The *Pf*CR1 moieties were afterward disrupted in the 5′ portion to PcRTFs. The detection of two types of PcRTFs different in length which were produced by elimination of two definitive sequences in *Pf*CR1 moiety possibly by gene conversion clearly supports such process but not multiplication of the PLA_2 gene-PcRTF unit. (Abstract, Gene, 461: 15-25, 2010)

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 3～7 newborns are given annually by 24 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.

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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
Assistant Professor	Saki Kondo, D.M.Sc.	助　教　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 and laws. 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 adenovirus vector (AdV) expressing various genes efficiently. And recently we developed the new cosmid cassette for AdV construction 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; Kondo et al., J. Molec. Biol., 2009). These methods continuously promote studies of various fields of molecular biology and medicine. We recently identified adenovirus pIX gene as a main cause of inflammation observed in AdV infection (Nakai *et al*., Hum. Gene Ther. 18: 925-936, 2007). The research activities in 2010 were shown below.

1. The positions and orientations of the expression unit are important to obtain high expression in adenovirus vector

Naoki Goda, Mariko Suzuki, Yumi Kanegae and Izumu Saito

Adenovirus vectors (AdV) are valuable because of high efficiency of transduction and expression to broad range of cell types. In the first-generation AdV a foreign expression unit is substituted for viral E1A and E1B genes (called E1 substitution). The right orientation of the expression unit is used worldwide simply because

original E1 genes are located in this orientation in the adenovirus genome. However, we previously showed that viral titers are sometimes low when an expression unit containing a potent CAG promoter was located in this orientation and therefore we always recommend the left orientation for E1 substitution (Miyake *et al*., PNAS 93: 1320-1324, 1996). Besides E1 substitution, E3 substitution and E4 insertion (Saito et al, J. Virol., 58: 544-560, 1986) have been used as the position of the expression unit. Difference of expression level among the three substitution/insertion sites, if any, are very important if a weak, specific promoter is used. Such information is probably very important, for example, especially for construction of a cancer-specific replication-competent AdV.

To examine influences on position and orientation of transgene expression, we constructed six different AdVs containing the "Switch unit" of Cre gene under the control of α-fetoprotein (AFP) promoter. A small amount of expressed Cre turned on a potent EF1α promoter in the "Target unit" and a high-level of dsRed was expressed (double infection method). The result showed that the expression level was quite different depending on the site and the orientation. The order of expression level was: E1/left＝E1/right＞E4/left＝E3/left＞E4/right＞E3/right. Because the specificity of AFP promoter was low when using at E1 site in the right orientation, we concluded that E1/left is the best site/orientation for use of a specific promoter in AdV. The results possibly give valuable information for users of AdV bearing cancer-specific promoter.

2. Cancer-targeting gene therapy using a novel adenovirus vector: a mouse model of disseminated hepatocarcinoma

Yumi Kanegae, Naoki Goda, Miho Terashima and Izumu Saito

Development of curing method against disseminated and recurrent microtumors is important for the next generation of anti-cancer treatment. Tissue/cancer specific promoter is regarded as a variable tool. We have developed the method of an "excisional expression" adenovirus vector (AdV). This vector, which possesses Cre driven by tissue/cancer specific promoter as "Switch unit" and the expression unit of a purpose gene by EF1α promoter under control of Cre as "Excisional unit", can express a high level of purpose-gene while maintaining very strict specificity; using α-fetoprotein (AFP) promoter, it can express 40-90 fold more the purpose-gene in hepatocellular-carcinoma cells (HuH-7 and HepG2) than the AdV bearing the authentic AFP promoter.

We improved the "excisional expression" vector, because the AdV genome has position effect for the purpose-gene expression level and the previous insertion site of "Switch unit" was worse than E1-substitution region. We newly constructed "E1L-type excisional expression" vector and were able to increase the expression level of herpes thymidine-kinase (TK). These high-level expression and strict specificity of this vector have been shown in cultured cells but appropriate animal models for *disseminated* hepatocellular-carcinoma hardly reported.

As a breakthrough, we have established such a mouse model in collaboration with CLEA Japan Inc. We injected HuH-7 cells highly-expressing the luciferase from the portal vein into nude mice and succeeded in generating disseminated liver tumors in a high efficiency. The mice can be used for *in vivo* imaging and can supply from CLEA Japan. The model will be valuable for study of disseminated hepatocarcinoma and could also be useful for assay of chemical anti-cancer substances.

3. Keratinocyte growth factor gene transduction ameliorates pulmonary fibrosis induced by bleomycin in mice

Seiko Sakamoto[1], Takuya Yazawa[2], Yasuko Baba[1], Hanako Sato[2], Yumi Kanegae, Toyohiro Hirai[3], Izumu Saito, Takahisa Goto[1], Kiyoyasu Kurahashi[1]: [1]Department of Anesthesiology and Critical Care Medicine, [2]Department of Pathology, Yokohama City University Graduate School of Medicine, and [3]Department of Respiratory Medicine, Graduate School of Medicine, Kyoto University.

Pulmonary fibrosis has high rates of mortality and morbidity, but there is no established therapy at present. We investigated whether bleomycin-induced pulmonary fibrosis in mice would be improved by intratracheal administration of keratinocyte growth factor (KGF)-expressing adenovirus vector (Ad-KGF). Progressive pulmonary fibrosis was created by continuous administration of 120 mg/kg of bleomycin subcutaneously using an osmotic pump twice from Day 1 to 7 and Day 29 to 35. Those mice initially experienced subpleural fibrosis then advanced fibrosis in the parenchyma of the lungs. These histopathological changes were accompanied by reduced lung compliance (0.041 ± 0.011 vs. 0.097 ± 0.004; $p < 0.001$), reduced surfactant protein D messenger expression (0.409 ± 0.119 vs. 1.006 ± 0.081; $p < 0.009$), and reduced KGF messenger expression in the lungs at 4

weeks compared with naïve group. Intratracheal instillation of Ad-KGF at 1 week after the administration of bleomycin increased KGF mRNA expression in the lungs compared with the fibrosis induced mice that were instilled saline alone. This phenotype was associated with alveolar epithelial cell proliferation, increased pulmonary compliance (0.062 ± 0.005 vs. 0.041 ± 0.011; $p = 0.023$), and decreased mortality (survival rate on Day 56: 68.8% vs. 0%; $p = 0.002$), compared with those received only the saline vehicle. These observations suggest the therapeutic utility of a KGF-expressing adenoviral vector for pulmonary fibrosis.

Publications

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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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Professor	Kouhei Tsumoto, Ph.D.	教　授	工学博士	津　本　浩　平
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Associate Professor	Masaaki Oyama, Ph.D.	准教授	医学博士	尾　山　大　明
Assistant Professor	Hiroshi Sagara, Ph.D.	助　教	医学博士	相　良　　洋

The mission of our laboratory is to develop technologies for protein research that enable us to analyze complex cellular systems leading to a variety of diseases such as cancer and infection. We mainly focus on the researches based on advanced technologies regarding mass spectrometry and electron microscopy for precise measurement of dynamic behaviors of functional protein networks.
We are also engaged in collaborative researches regarding electron microscopy, mass spectrometry, peptide synthesis and purification of proteins and their functional analyses and have made a substantial contribution to many scientific achievements.

‹Group I›

1. Biology of calcium-dependent proteases in *Caenorhabditis elegans*

Caenorhabditis elegans, a free-living nematode in the soil, consists of no more than 1,000 cells, but retains various functions similar to those of higher-order animals. We have been interested in biology of vertebrate calpain, an intracellular proteolytic enzyme activated with calcium ions. Genes related to mammalian calpain are also present in C. elegans, and they are expected to be translated into proteinous forms, but their function have not been characterized biochemically.

a. Identification of cysteine proteases in *Caenorhabditis elegans*

Yohei Kato, Nozomi Ichikawa 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 E64d, [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 endogenous esterases convert it to enzyme-inhibitable E64c that covalently binds to the SH group of active center in cysteine proteases. 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 means of the multiple-antigen peptide system using Fmoc chemistry. 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 fur-

ther 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.

2. Development 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 chemically synthesized 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 proteins as well as the cleaved ones. Thus, an evaluation system is necessary for cleavage-site directed antibodies. Phage display libraries 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 directed antibody was examined by this method, the antibody was found to be a mixture of three or more types of antibodies that bind to terminal and 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.

3. Post-translational modification of proteins during apoptotic cell death

Apoptotic cell death involves various biochemical reactions. Among them, post-translational modification of proteins is intensively investigated in this laboratory. First, intracellular proteolytic enzymes are 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. Limited proteolysis of actin in apoptotic neutrophils

Junko Ohmoto and Shinobu Imajoh-Ohmi

Neutrophil actin is proteolyzed to a 40-kDa fragment during preparation/isolation from peripheral blood. The truncated actin lacks amino-terminal region of native protein and presumably cannot copolymerize to F-actin. The 40-kDa actin-derived fragment is apparently related to spontaneous apoptosis of neutrophils. To investigate the role of actin proteolysis, especially cause-and-effect relationship to neutrophil apoptosis, we have made a cleavage-site-directed antibody (#1090pAb) for the 40-kDa form of actin using synthetic peptides as haptens. The antibody reacted with the 40-kDa polypeptide but not with unproteolyzed native actin which remain abundant in the cell. Using this antibody, we have found that (1) the truncated actin is generated during isolation of neutrophils from peripheral blood, (2) neutrophils without the truncated actin can be prepared in the presence of diisopropyl fluorophosphate, and (3) leukocyte elastase is possibly responsible for this limited proteolysis.

Herein we analyzed cellular localization of the truncated actin using #1090pAb. Confocal laser microscopic observation indicated that the plasma membrane of neutrophils were strongly stained with #1090pAb, but that intracellular regions near the membrane were sometimes stained weakly. We examined here whether or not the amino-teriminal region of the 40-kDa actin is on the cell surface of neutrophils using at the same time established antibodies for components of superoxide-generating system composed of transmembranous cytochrome and cytosolic activator proteins. Furthermore, flow-cytometric analysis revealed that #1090pAb stained the cell-surface antigen under the conditions that antibodies for cytosolic proteins did not. Our findings suggest that the truncated actin is, at least in its amino-terminal part, on the surface of neutrophils. However, another antibody against the amino-terminal region of native actin did not stain neutrophils from outside suggesting that the cleavage site is inaccessible to exogenous proteinases.

b. Fas, a death receptor, is polymerized to high-molecular weight forms during Fas-mediated apoptosis in Jurkat T cells

Hidehiko Kikuchi, Fotoshi Kuribayashi and Shinobu Imajoh-Ohmi

An apoptotic receptor Fas mediates death signal from Fas ligand. 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. Under 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.

4. BRCA2 binds to motor domain of nonmuscle myosin heavy chain IIC

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Cytokinesis is the final step of the M phase of the cell cycle and completed by scission of the midbody, a narrow intercellular bridge between daughter cells. We reported that human nonmuscle myosin heavy chain (NMHC) IIC and phosphorylated BRCA2 by Plk1 was recruited between both of the plus-ends of microtubules of the midbody. Furthermore, when a cell lysate was fractioned by a glycerol density gradient

centrifugation, endogenous BRCA2 and NMHC IIC were observed to sediment at approximately 700-800 kDa.

To examine the interactions BRCA2 of NMHC IIC, we performed coimmunoprecipitation analysis and determined whether BRCA2-FLAG can associate with HA-NMHC IIC in COS7 cells. Indeed, we found that the HA-NMHC IIC can be coimmunoprecipitated with BRCA2-FLAG. In reciprocal experiment, BRCA2-FLAG also coprecipitates with HA-NMHC IIC. Next, we investigated which subunit of NMHC IIC associated with BRCA2, we divided the NMHC IIC into two fragments (IIC-N: motor domain (1-1000 a. a.) and IIC-C: coild-coil domain (887-2003 a.a.)). The lysates of COS-7 cells transfected with deletion mutants of NMHC IIC were incubated with BRCA2-FLAG or FLAG. As a result, BRCA2 interacted with IIC-N in the N-terminal region. To further narrow down the binding region, we divided the IIC-N region into three fragments (IIC-N/1: 1-341 a.a., N/2: 331-670 a.a., N/3: 661-1000 a.a.). As a result, the interaction with BRCA2 was observed only in the IIC-N/1 region near the N-terminal end of motor domain. These results indicate that BRCA2 associated with the N-termini of NMHC IIC.

Conversely, we tried to narrow down the NMHC IIC-binding region of BRCA2. A series of overlapping FLAG-fused deletion mutants of BRCA2 (R1: 1-157 a.a., R2: 113-685 a.a., R3: 639-1508 a.a., R4: 1475-1620 a.a., R5: 1596-2280 a.a., R6: 2241-2940 a.a., R7: 2611-3318 a.a., R8: 3119-3418 a.a.) were incubated with COS-7 cell lysates transfected with HA-NMHC IIC. HA-NMHC IIC specifically associated with R3, R5, R 6 and R7. We demonstrated that there was strong (R5 and R6), intermediate (R3) and weak (R7) binding to NMHC IIC. The results suggest that the residues in the 1596-2940 a.a. regions of BRCA2 appear to be required for its interaction with NMHC IIC. EMSY is capable of silencing the activation potential of BRCA2. The EMSY-binding resion (23-44 a.a.) of BRCA2 did not bind to NMHC IIC, suggesting that the NMHC IIC binding region did not overlap with the EMSY interaction surfaces.

‹Group II›

1. Integrative analysis of phosphoproteome and transcriptome dynamics defines drug-resistance properties of breast cancer

Masaaki Oyama, Takeshi Nagashima[1], Hiroko Kozuka-Hata, Noriko Yumoto[1], Yuichi Shiraishi[1], Kazuhiro Ikeda[2], Yoko Kuroki[1], Noriko Gotoh[3], Satoshi Inoue[2], Hiroaki Kitano[4] and Mariko Okada-Hatakeyama[1]: [1]RIKEN, [2]Research Center for Genomic Medicine, Saitama Medical University, [3]Division of Systems Biomedical Technology, IMSUT, [4]Sony Computer Science Laboratories, Inc.

Signal transduction system, in orchestration with subsequent transcriptional regulation, widely regulates complex biological events such as cell proliferation and differentiation. Therefore, a comprehensive and fine description of their dynamic behavior provides a fundamental platform for systematically analyzing the regulatory mechanisms that result in each biological effect. Here we developed an integrated framework for time-resolved description of phosphoproteome and transcriptome dynamics based on the SILAC-nanoLC-MS and GeneChip system. In this study, we analyzed cellular information networks mediated by estrogen receptor/ErbB2 pathways, which have long been implicated in drug response of breast cancer. Through shotgun identification and quantification of phosphorylated molecules in breast cancer MCF-7 cells, we obtained a global view of the dynamics regarding breast cancer-related signaling networks upon estrogen (E2) or heregulin (HRG) stimulation. Comparative analysis of wild-type and tamoxifen-resistant MCF-7 cells revealed altered behaviors of signaling hub dynamics, indicating distinct signaling network properties between these two cell types. Pathway and motif activity analyses using the transcriptome data suggested that deregulated activation of GSK3β and MAPK1/3 signaling might be associated with altered activation of CREB and AP-1 transcription factors in tamoxifen-resistant MCF-7 cells. Thus, our integrative analysis of phosphoproteome and transcriptome in human breast cancer cells revealed distinct signal-transcription programs in tamoxifen-sensitive and insensitive tumor cells, which potentially defines drug-resistance properties against tamoxifen.

2. System-level analysis of CagA-dependent signaling network dynamics by Helicobacter pylori infection

Hiroko Kozuka-Hata, Masato Suzuki[5], Kotaro Kiga[5], Shinya Tasaki, Jun-ichiro Inoue, Tadashi Yamamoto[6], Chihiro Sasakawa[5] and Masaaki Oyama: [5]Division of Bacterial Infection, Department of Microbiology and Immunology, IMSUT, [6]Division of Oncology, Department of Cancer Biology, IMSUT.

The signal transduction system within a cell regulates complex biological events in response to bacterial infection. The previous analyses of cell signaling in Helicobacter pylori-infected gastric epithelial cells have revealed that CagA, a

major virulence factor of Helicobacter pylori, is delivered into cells via the type IV secretion system and perturbs signaling networks through the interaction with the key signaling molecules such as SHP-2, Grb2, Crk/Crk-L, Csk, Met, and ZO-1. Although the biological activity of tyrosine-phosphorylated CagA has intensively been studied, system-wide effects of the virulence factor on cellular signaling have yet to be analyzed. Here we performed time-resolved analyses of phosphoproteome and CagA-interactome in human gastric AGS cells by CagA-positive/negative Helicobacter pylori infection. Our highly sensitive nanoLC-MS/MS analyses in combination with the Stable Isotope Labeling by Amino acids in Cell culture (SILAC) technology defined CagA-dependent perturbation of signaling dynamics along with a subset of CagA-associated possible modulators on a network-wide scale. Our result indicated that the activation level of the phosphotyrosine-related signaling molecules in AGS cells was suppressed overall in the presence of CagA during Helicobacter pylori infection. As Helicobacter pylori infection plays pivotal roles in the progression of gastric diseases including carcinogenesis, a comprehensive and fine description of the signaling dynamics would serve as a fundamental platform to theoretically explore for the potential drug targets through analyzing the regulatory mechanisms at the system-level.

3. Phosphoproteomics-based modeling defines the regulatory mechanism underlying aberrant EGFR signaling

Shinya Tasaki, Masao Nagasaki[7], Hiroko Kozuka-Hata, Kentaro Semba[8], Noriko Gotoh[3], Seisuke Hattori[9], Jun-ichiro Inoue, Tadashi Yamamoto[6], Satoru Miyano[7], Sumio Sugano[10] and Masaaki Oyama: [7]Laboratory of DNA Information Analysis, Human Genome Center, IMSUT, [8]Department of Life Science and Medical Bio-Science, Waseda University, [9]Department of Biochemistry, School of Pharmaceutical Sciences, Kitasato University, [10]Laboratory of Functional Genomics, Department of Medical Genome Sciences, Graduate School of Frontier Sciences, The University of Tokyo.

Mutation of the epidermal growth factor receptor (EGFR) results in a discordant cell signaling, leading to the development of various diseases. However, the mechanism underlying the alteration of downstream signaling due to such mutation has not yet been completely understood at the system level. Here, we report a phosphoproteomics-based methodology for characterizing the regulatory mechanism underlying aberrant EGFR signaling using computational network modeling. Our phosphoproteomic analysis of the mutation at tyrosine 992 (Y992), one of the multifunctional docking sites of EGFR, revealed network-wide effects of the mutation on EGF signaling in a time-resolved manner. Computational modeling based on the temporal activation profiles enabled us to not only rediscover already-known protein interactions with Y992 and internalization property of mutated EGFR but also further gain model-driven insights into the effect of cellular content and the regulation of EGFR degradation. Our kinetic model also suggested critical reactions facilitating the reconstruction of the diverse effects of the mutation on phosphoproteome dynamics. This is the first phosphoproteomics-driven mathematical description of the regulatory mechanism of EGFR signaling networks, which could provide a systematic strategy toward controlling disease-related cell signaling.

4. Photo-cross-linking-based proteomics elucidates direct protein-protein interactions involving a defined binding domain

Nobumasa Hino[1], Masaaki Oyama, Aya Sato[1], Takahito Mukai[1], Hiroko Kozuka-Hata, Tadashi Yamamoto[6], Kensaku Sakamoto[1], and Shigeyuki Yokoyama[1]

Signal transduction pathways are essentially organized through the distribution of various binding domains in signaling proteins, with each domain binding to its target molecules. To identify the targets of these domains, we developed a novel proteomic approach, based on photo-cross-linking and mass spectrometry. Through the use of an expanded genetic code, a photoreactive amino acid, *p*trifluoromethyldiazirinyl-L-phenylalanine, was site-specifically incorporated into the SH2 domain of the adaptor protein GRB2 in human embryonic kidney cells. By exposing the cells to 365-nm light after an EGF stimulus, the SH2 of GRB2 was cross-linked with the endogenous proteins directly interacting with it. These targets were identified by a comparative mass-spectrometric strategy. Thus, we discovered that GRB2-SH2 directly binds to the GIT1 scaffold protein and the AF6 protein, a putative effector of the RAS protein. Furthermore, heterogeneous nuclear ribonucleoproteins F, H1, and H2 were found to be direct targets of GRB2-SH2.

5. Mass spectrometry-based annotation of the human short ORFeome

Masaaki Oyama, Hiroko Kozuka-Hata, Sumio

Sugano[10], Tadashi Yamamoto[6] and Jun-ichiro Inoue

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 (Oyama et al., Mol Cell Proteomics, 6: 1000-1006, 2007).

‹Group III›

1. Analysis of neural circuits in the retina using electron microscopic tomography and x-ray microscopy

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Neural circuits in the central nervous system are the basis of various high-order brain functions. It is also true in case of retin. In the retina, six main classes of cells connect each other systematically to make up complex neural circuits. Characteristics of the retinal functions have been examined precisely by the electrophysiological methods and models of cell connectivity have been proposed. But morphological studies of the actual neural connection, which constitute the physiological properties of higher order neurons, still not enough. In this study, we are trying to reveal the actual neural circuit morphologically by using electron microscopic computed tomography (CT) and X-ray microscopy. This year, we improved the specimen preparation procedure of the conventional electron microscopy to gain higher membrane contrast and examined by electron microscopic tomography and x-ray microscopy. Either of the techniques revealed nerve fibers and organelles including mitochondria and synapses in the 200nm thick sections of neural tissue. In the future, we will utilize that information to begin deciphering the wiring diagram of the retina.

2. Collaborative and supportive researches as electron microscope core-laboratory

This group is also engaged in collaborative researches using electron microscope. We offer supports for the research projects those need electron microscopic analysis. The services 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 technique or combination of some of these, we can offer direct visual evidence that cannot be acquired by other methods. This year, 19 projects in 13 laboratories were performed as core-laboratory works.

a. Thin section electron microscopy

Thin section electron microscopy is the most widely used technique to observe the inner structure of 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 antibodies. This year, thin section electron microscopy combined with immuno-electron microscopy were used in many collaborative works.

a-1. Ultrastructural analysis of entry and assembly of Herpes Simplex Virus

We have been performing several studies with research groups in Dr. Kawaguchi[2]'s laboratory: [2]Department of Infectious Disease Control, International Research Center for Infectious Diseases, regarding the infection/replication processes of herpes simplex virus (HSV). This year, serial thin section electron microscopy was applied to a single target cell cultured on the surface of a cover slip to figure out the virus forming compartment observed by confocal lazer fluorescent microscope. In early stages of infection, the compartments were revealed to be the intracellular structure. But in later stages, the compartments observed by confocal laser microscpe were revealed to be the accumulation of viruses in the extracellular space. This indicates that the viruses are first formed in the intracellular spaces and then exocytosed to the extracellular space.

a-2. Morphological and immuno-electron microscopic analysis of the mucosal M-cells

We have been performing several studies also with research groups in Dr. Kiyono[3]'s laboratory: [3]Division of Mucosal Immunology, Department of Microbiology and Immunology. In these studies, we analyzed the ultrastructure of the M-cells in the intestinal epithelium by thin section transmission electron microscopy and scanning electron microscopy. Molecular characteristics of the M-cells were analyzed using Immuno-electron microscopy. In another study, several species of proteins were induced to express in rice and examined the localization within the cell with immuno-electron microscopy. We revealed that the expressed proteins were accumulated in the different compartments depending on the kind of the expressed protein.

Some other collaborative research works using thin section electron microscopy and/or immuno-electron microscopy were performed with Dr. Noda[4] *et al* in [4]Division of Virology, Department of Microbiology, regarding the structure of the influenza viruses and ebola virus, Dr. Masaike[5] *et al*, in [5]Integrated Research Institute, Tokyo Institute of Technology (ref. Masaike *et al*), Dr. Hoshina[6] in [6]Division of Oncology, regarding the structure of the synapses, Dr. Ma[7] in [7]Department of Pediatric Hematology/Oncology, regarding the morphology of the eosinophilic leucocytes induced from ES/iPS cells and so on.

b. Negative staining techniques

Negative staining techniques are simple and quick method to observe the morphology of the macro molecules. This year, the negative staining techniques were used in collaborative work with Dr. Noda[4] *et al*. In this study, negative staining techniques were used to analyze the morphology of the ebola virus nucleoprotein-RNA complex (ref. Noda[4] *et al*).

c. 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. Nishizumi-Tokai[6] *et al*, regarding the surface structure of the egg cells. Other works are in progress with Dr. Sanada[8] *et al*, [8]Department of Gerontological Nursing, Division of Health Science and Nursing, Graduate School of Medicine, to analyze the effects of diabetes or bacterial infection during wound repair. Scanning electron microscopy was also used to analyze the non-biological materials as a collaborative work with Dr. Cheng[9] in [9]Olympus Co.

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