*Research Hospital*

# Department of Medicine (Department of Hematology-Oncology)

*Our department has been challenging to cure a variety of fatal hematological disorders with currently available methods. The mainstay to achieve our goal is, nowadays, hematopoietic stem cells (HSC) including bone marrow, peripheral blood and umbilical cord blood stem cells, transplantation and cytokine therapy. These projects have been under way at the HSCT and Hematology wards with the excellent assistance of nurses and comedical staffs. We annually take care of more than 40 patients with allogeneic HSCT. In an attempt to expand therapeutic benefits of HSCT, we are actively participating to the nation-wide project of unrelated bone marrow transplantation as the largest HSCT center in Japan. In 1998, we furthermore have established Tokyo cord blood bank services in our hospital. Cord blood stem cell transplantation has become strong weapon for patients sufferred from hematological malignancy without any related or unrelated stem cell donors. We have alredy treated more than 25 adult patients until the end of March, 2001. Also since 1998, we had started clinical gene therapy for stage IV renal cancer patients as collaboration with other clinical departments in our hospital as well as other hospitals including Juntendo University Hospital, Tsukuba University Hospital, and National Cancer Center.*

## 1. Reports on Clinical Studies of Immunogene Therapy Using Autologous GM-CSF Transduced Tumor Vaccines(GVAX) for Stage IV Renal Cell Cancer

**K. Tani, S. Asano, et al.**

There is no effective treatment for patients with Stage IV renal cell cancer (RCC). The introduction of new therapy is required. The results of preclinical animal studies indicated that vaccination using autologous GM-CSF–transduced renal tumor cells (GVAX®,) is one of the most promising treatments to overcome this poor situation. We have produced six GVAX using MFGs retroviral vector from six Japanese patients suffering from stageIV RCC with metastasis at our hand. All of the GM-CSF gene-transduced RCC cells were safely produced. Four out of six GM-CSF cDNA transduced cells were demonstrated to produce more than 40ng/$10^6$ cells /24hr GVAX, minimally insured GM-CSF secretion to induce specific antitumor immunity in preclinical mice studies, and were permitted to be intradermally administered to four each patient. All of the four patients received more than $1.4x10^8$ GVAX, ranging from $1.4x10^8$ to $3.7x10^8$ cells, over 6-17 times. During the total of 48 vaccinations, there was no severe adverse events directly related to the vaccination. In all of the patients, DTH skin tests showed positive reaction to intradermally injected autologous RCC tumor cells as well as to normal renal cells after repetitive vaccinations. The T cell repertoire analysis demonstrated that the oligoclonal expansions of T cells in the peripheral blood and DTH sites in 3 patients after the start of vaccination. Biopsy of the metastasized sites were done in two patients and autopsy was

done in one patient. CD8 T cells became dominant after the vaccination and the oligoclonal expansions of T cells were identified in the biopsied as well as autopsied metastasized tumors. *In vitro* T cell assay demonstrated that the CTL activity against autologous tumor cells was increased in 3 patients in the course of vaccination. No visible reduction of the metastasized tumor was observed in all of the patients during the course of vaccinations. Two patients successively received low dose systemic interleukin-2. Interestingly, the decrease of tumor growth curve after 1 month of systemic low dose interleukin-2 was observed in both of the patients. The RCC/GVAX was considered to be safely administered to patients suffering from stage IV RCC with metastases. Our immunological data suggested some clinical benefits would be expected in patients receiving GVAX. The simultaneous administration of GVAX and low dose interleukin-2 may be one of choices for patients in stage IV RCC.

### 2. Unrelated Cord Blood Transpiantation in Adult Patients With Hematological Malignancy: A Single Institution Experinece

**T.Iseki, J.Ooi, A.Tomonari et al.**

Although the number of Cord Blood Transplantation (CBT) has been rapidly increasing, the indication to adults is restricted because of relatively lower cell number which had been defined as an unfavorable factor in previous reports. However, the important issues such as the eligibility of patient and cord blood unit and the proper regimen of CBT for adult patients have not been defined yet.

Between August 1998 and July 2001, 30 adult patients with hematological malignancy including AML(17), ALL(6), MDS(4),CML(1) and NHL(2) received unrelated HLA-mismatched CBT at our institute. Median age and weight of patients and infused nucleated cell number were 38 years, 52kg and $2.39x10^7$/kg. All patients received our standard conditioning regimen according to the disease status. All patients received cyclosporin A and 26 received methotrexate for GVHD prophylaxis. Three patients died shortly after CBT, because of RRT (2) and infection (1). One patient failed to achieve remission after conditioning and one patient developed autologous recovery. Among evaluable patients, median time to neutrophil and platelet recovery was 22(n=26) and 38 days (n=22), respectively which appeared to be faster than the previous reports. Eight patients developed Grade II and one patient developed Grade III acute GVHD. Four patients died of relapse at 107-307 days after CBT. Overall survival rate at 36 months was 76±9% for all patients and all of 15 patients with standard risk are alive 1-23 months (median 12 months) after CBT. Although the number of patients and the periods of observation are insufficient, the current result comparable to that of standard bone marrow transplantation was promising.

### 3. Effect of Cyclophosphamide at Conditioning of Hematopoietic Stem Cell Transplantation on Serum Cyclosporine Level

**F.Nagamura, T.Takahashi, et al.**

It is sometimes difficult to maintain therapeutic range of cyclosporine (CsA) after hematopoietic stem cell transplantation (HSCT). One reason of that is the existence of substances that show drug interactions. We analyzed the factors that may affect serum CsA concentration at early periods of HSCT, until day14. We performed 179 cases of allogeneic HSCT from April 1995 to March 2001, and 103 patients were evaluable for this analysis. No statistically significant relationships between CsA concentrations and gender, age, serum creatinine level, AST/ALT levels, and antibiotics and fluconazole administration were observed. The means of median CsA concentrations of each conditioning regimen were as follows, TBI+CY (n=14): 153.6 ng/ml, TBI+ G-CSF combined high dose Ara-C (G/Ara) (n=40): 143.3 ng/ml, TBI+ G/Ara (n=33): 200.3 ng/ml, TBI+VP-16 (n=16): 233.6 ng/ml. Differences of between the former two regimens and the latter two regimes were statistically significant: TBI+CY vs. TBI+G/Ara (p=0.0198); TBI+G/Ara+CY vs. TBI+G/Ara (p=0.0002); TBI+CY vs. TBI+VP16 (p=0.0055); and TBI+G/Ara+CY vs. TBI+VP-16 (<0.0001). The mean of median CsA concentrations of CY containing regimen (146.0 ng/ml), was statistically lower than that of non-CY regimen (211.2 ng/ml) (p<0.0001). These results strongly suggested that CY at conditioning has the effect to reduce CsA levels.

### 4. A clinical comparison of unrelated cord blood transplantation and unrelated bone marrow transplantation for adult patients with acute leukemia in complete remission

**J. Ooi, T. Iseki et al.**

Recently, several studies showed the results of unrelated cord blood transplantation (UCBT) in adults patients. However, the usefulness of unrelated cord blood as an alternative stem cell source remains unclear. We studied the clinical comparison of UCBT (n=8) and unrelated bone marrow transplantation (UBMT) (n=8) in adult patients with acute leukemia in complete remission (CR) who received the same conditioning regimen, graft-versus-host disease (GVHD) prophylaxis and supportive treatment in a single institute. The hematopoietic recovery was delayed in the UCBT group and the incidence of acute GVHD seemed to be similar, however, that of chronic GVHD was higher in the UCBT group. The

probability of disease free survival at 2 years was similar (85.7 % of UCBT versus 75.0 % of UBMT; p=.51). These preliminary results suggest that adult acute leukemia patients in CR without suitable related or unrelated bone marrow donors should be considered as candidates for UCBT.

### 5. Successful unrelated cord blood transplantation for relapse after autologous transplantation in non-Hodgkin's lymphoma

**J. Ooi, T. Iseki et al.**

We report a 34-year-old male with relapsed non-Hodgkin's lymphoma (NHL) after autologous peripheral blood stem cell transplantation successfully treated with unrelated cord blood transplantation (CBT). The conditioning regimen included 12 Gy total body irradiation and cyclophosphamide. After the conditioning, a total of $3.14 \times 10^7$ /kg cord blood nucleated cells was infused on 14 February 2000. An absolute neutrophil count greater than $5 \times 10^8$/l and a self-sustained platelet count greater than $50 \times 10^9$/l were achieved on days 21 and 43, respectively. During the follow up period, grade I acute graft-versus-host disease (GVHD) and limited chronic GVHD occurred but both were successfully treated with a dose modification of cyclosporine. At a follow-up period of 16 months, the patient is alive and free of disease. To our knowledge this is the first report of a successful unrelated CBT for an adult NHL patient who relapsed after autologous transplantation.

### 6. Successful allogeneic bone marrow transplantation for hepatosplenic gd T cell lymphoma

**J. Ooi, T.Iseki et al.**

Hepatosplenic gd T cell lymphoma is generally very refractory to regular chemotherapy, and to date, no curative treatment modality for hepatosplenic gd T cell lymphoma has been established. We report a case of hepatosplenic gd T cell lymphoma successfully treated with allogeneic stem cell transplantation (SCT). The patient was a 23-year-old male who was admitted to our hospital in June 1999 and subsequently diagnosed with hepatosplenic gd T cell lymphoma. After two courses of chemotherapy, a complete response was achieved and he subsequently received with fractionated TBI (12 Gy) and high-dose etoposide (60mg/kg) followed by allogeneic bone marrow transplantation from his HLA-matched younger sister. During the follow up period, grade II acute GVHD and extensive chronic GVHD occurred but both were successfully treated with a dose modification of cyclosporine (CyA). At a follow-up period of 23 months, the patient is alive and free of disease.

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*Research Hospital*

# Department of Infectious Diseases and Applied Immunology

*Department of Infectious Diseases and Applied Immunology (DIDAI) was founded in 1981. In 1986, clinic for patients with human immunodeficiency virus (HIV) infection was opened by former professor, K. Shimada. In 2001, approximately 150 patients with HIV infection visit the out-patient clinic on a monthly basis, and 3-5 beds for HIV-infected patients in the in-patient ward are usually occupied. Since the number of the staff members of DIDAI is too small to care both out-patients and in-patients, members of the Division of Infectious Diseases (DID) and the Division of Clinical Immunology of the Advanced Clinical Research Center join the clinic. Supported by clinicians of three department and divisions, basic scientists of immunology and virology in DID, and dedicated medical and paramedcal stuffs, IMSUT hospital provides the most up-to-date medical treatment to HIV-infected patients in Japan. DIDAI is also a treatment center for international infectious diseases such as malaria and typhoid fever.*

## 1. Treatment of and clinical research on HIV-infection and related diseases

**Tetsuya Nakamura, Takashi Takahashi[1], Hitomi Nakamura[1], Tomohiko Koibuchi[1], Toshiyuki Miura[1], Tokiomi Endoh[1], Miou Sato[1], Akihiro Hitani[1], Mieko Goto[1] and Aikichi Iwamoto[1]:[1]Department of Infectious Diseases**

### a. Treatment of HIV infection in IMSUT hospital

#### i) Statistical characteristics of HIV-infected patients in IMSUT hospital this year

Twenty-nine new patients with HIV-1 infection visited our hospital this year, and as of the end of this year, 152 patients in total are under medical management in our out-patient clinic. The numbers of admission reached peak in 1996, and then started to decline as shown in the figure below. This is due to the success of highly active anti-retroviral therapy (HAART) which was introduced to our clinic in 1997.This tendency is also observed in US and Europe, and Centers for Disease Control and Prevention (CDC) in US reported that the number of AIDS death in 1997 decreased by half compared to the previous year. Although the number of admission this year and last year increased again, the reasons for their admission in many cases were the adverse effects caused by HAART, and the number of patients with serious opportunistic infections was low.

#### ii) A Randomized, Open-Label, Phase III, International Study of Subcutaneous Recombinant IL-2 (Proleukin) in Patients With HIV-1 Infection and CD4+ Cell Counts ≥300/mm3 : Evaluation of Subcutaneous Proleukin in a Randomized International Trial (ESPRIT)

We joined last year the international clinical study called "ESPRIT" which is organized by National Cancer Institute in US. Approximately 4,000 subjects are scheduled to participate in this study from around the world. The study is scheduled to be conducted over the course of 6 years and ten patients are assigned to IMSUT hospital. We continued the clinical study this year, too and one patient was enrolled in this study.

IL-2 is a substance that is normally produced in the body, and act to increase CD4 cells. CD4 cells assist in defense against infection. The concentrations of IL-2 produced in patients with HIV infection have been determined to be lower than in normal persons. The object of this study is to determine whether bringing about an increase in the number of CD4 cells by administration of IL-2 leads to a decrease in the incidence of onset of HIV-related diseases. In clinical studies conducted thus far, increases in CD4 cells have been observed in the vast majority (but not all) patients administered IL-2. It is still unknown, however, whether these increases really improved the health of the patients. This study will be conducted in order to investigate the following matters.

* Whether or not IL-2 reduces serious infections related to HIV and prolongs the survival period in the case it is used concomitantly with other HIV drugs.
* Whether or not IL-2 can be administered safely over an extended period of time to HIV infection patients.

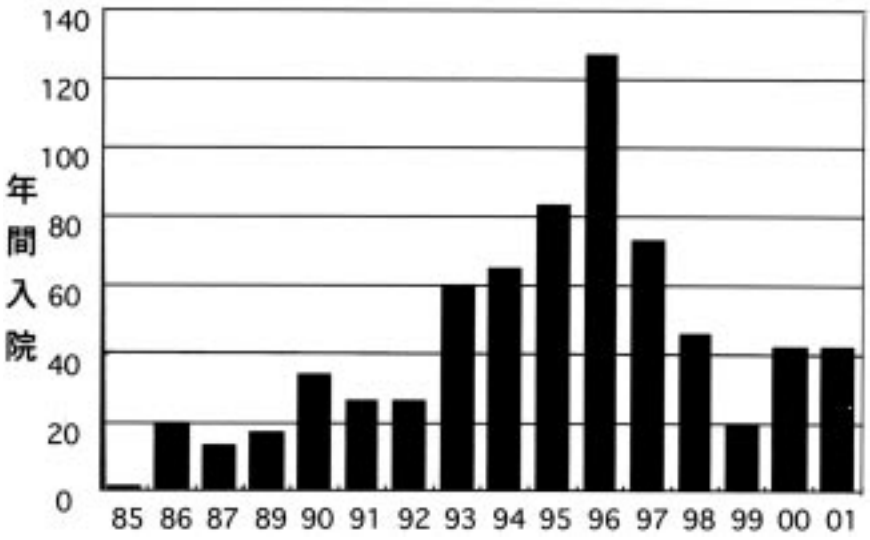
### b. Clinical research on Infectious Diseases

#### i) Case report of a patient with acute disseminated encephalomyelitis following dengue fever

We report this year a patient with acute disseminated encephalomyelitis occured after dengue fever. Acute disseminated myeloencephalitis (ADEM) is immune-mediated inflammation caused by antecedent viral infectoin and vaccination. The molecular mechanism of ADEM is thought that host immunity against the virus and vaccine crossreacts with the proteins in central nervous system. The pathological hallmark consists of widely scattered small foci of perivenular inflammation and demyelination. Postinfectious ADEM usually begins late in the course of viral infection including measles, chickenpox, rubella, mumps, influenza, parainfulenza, EB virus infection, and non-specific respiratory infection. The illness is sudden in onset with a monophasic course and may be self-limited or leave permanent neurological deficits. We recently experienced a case with acute onset of neurological disturbance after classical dengue fever. Although there have been no reports of ADEM following dengue fever, we could made the diagnosis of ADEM for this patient from the typical clinical course and magnetic resonance images.

#### ii) Surfactant Protein D and KL-6 as Serologic Indicators of *Pneumocystis carinii* Pneumonia in a Child with Acute Lymphoblastic Leukemia

We reported this year the clinical study regarding serologic indicators of *Pneumocystis carinii* pneumonia. Surfactant of lung is a complex of proteins and lipids that produce the interface between the air in the alveolar space and the aqueous film on the alveolar epithelium. It reduces the surface tension at the air/fluid interface in the alveolar air space. The surfactant-related lung-specific proteins have been classified into four surfactant proteins A (SP-A), B, C, and D. SP-D was purified from cultures of type II alveolar cells in rat and had collagen-like structure similar to SP-A. SP-D is an important factor for innate immune response to microbial challenge, and binds to glycoconjugates and lipid moieties expressed by most of microorganisms and other organic particles, *in vitro*. It also contains the capacity to modulate leukocyte function and to enhance the killing of microorganisms in some circumstance. It has been shown that PCP induces selective alterations in surfactant component expression including decreases in alveolar SP-B and SP-C levels and re-



sultant increases in alveolar surface tension in mice with PCP. In a clinical study, the measurement of SP-D in sera was reported to provide an easily identifiable and useful clinical marker to predict the severity of IPF. It has recently been described that a combination of the assays for SP-A and SP-D may be helpful for predicting the prognosis of patients with IPF. To our knowledge, this is the first demonstration that SP-D level in sera was a biomarker suggesting some inflammatory processes in PCP.

KL-6, a human MUC1 mucin, is expressed on type II alveolar cells, bronchiolar epithelial cells, and alveolar macrophages in human lungs. It was firstly detected as a soluble tumor-associated antigen in sera and effusions derived from patients with lung adenocarcinoma. Pulmonary epithelial cell injury is reflected to the serum level of KL-6, since it was highly expressed on injured, regenerating lung epithelium and absent from interstitial cells, and KL-6 in sera was related to alveolar capillary permeability changes that resulted from alveolar epithelial cell damage in berylliosis. Moreover, KL-6 in pulmonary epithelial lining fluids might produce the fibrosis in the alveolar space because it was demonstrated as one of chemotactic factors for human fibroblasts. It has recently been reported that measurements of serum KL-6 might be useful for evaluating the disease activities of PCP.

Measurements of LDH and CRP in sera are frequently used as indicators for PCP activities. However, there is a disadvantage that both LDH and CRP are not lung-specific materials. In addition, serum LDH levels reflected impaired hepatic function as well as PCP activities in this patient. CRP levels in sera were influenced by administration of methylprednisolone despite the progression of opacities in chest X-ray films. We determined both concentrations of SP-D and KL-6 in sera of two patients with PCP, of whom underlying diseases were malignant lymphoma and HIV infection (Table 1). These biomarkers were elevated according to the opacities in chest X-ray films and CT scans. Both patients were successfully treated with TMP/SMX and prednisolone for 3 weeks. The indicators were decreased together with the improvement of the opacities in chest X-ray films and CT scans. In conclusion, serum levels of SP-D and KL-6 can be novel lung-specific indicators suggesting some inflammatory processes in PCP and a combination of the assays for these biomarkers may be helpful in predicting the treatment results for PCP.

## 2. Diagnosis and Treatment of Tropical Diseases

**Tetsuya Nakamura, Akihiro Hitani[1], Mikio Kimura[2] and Aikichi Iwamoto[1]:[2]Infectious Disease Surveillance Center, National Institute of Infectious Disease**

This year, we treated 14 malaria patients. In September 2001, Ministry of Health, Labour and Welfare aproved mefloquine which is used for treatment and prophyraxis of malaria. We already prescribed mefloquine to 7 patients for malaria prophylaxis by the end of this year. This year we also treated four cases with dengue fever, three typhoid fever, one dysentery, one amebiasis, one tapeworm, and one lyme diseases.

We not ony have treated patients with tropical diseases but also have accepted consultations via telephone and E-mails from people who travel in tropical areas. We received 92 consultations this year; 35 (38%) of them were regarding malaria prophylaxis, 23 (25%) were treatment of malaria, 13 (15 %) were vaccination for rabies, and 6 (7%) were other vaccination. We will continue this consultation activity in addition to mefloquine prescription as prophylaxis of malaria in out-patient clinic.

**Table 1. Changes of serum levels of surfactant protein D and KL-6 in two patients with Pneumocystis carinii pneumonia**

| | Patient 1 (Malignant lymphoma) | | Patient 2 (HIV infection) | |
|---|---|---|---|---|
| Times after starting Treatment for PCP | SP-D (< 110 ng/ml) | KL-6 (< 500 U/ml) | SP-D (< 110 ng/ml) | KL-6 (< 500 U/ml) |
| 0 | 128.0 | 349 | 474.0 | 5500 |
| 2 weeks | 115.0 | 842 | 271.0 | 3740 |
| 4 weeks | 70.0 | 596 | 254.0 | 2120 |
| 6 weeks | 32.3 | 426 | 248.0 | 1460 |
| 8 weeks | 30.7 | 361 | 173.0 | 919 |

PCP, Pneumocystis carinii pneumonia; SP-D, surfactant protein D;
HIV, human immunodeficiency virus.
Underlying diseases in two patients are indicated in parentheses following patient 1 and 2. Normal ranges are showed in parentheses following each indicator.

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# *Research Hospital*
# Department of Pediatric Hematology-Oncology

*Our major goal is to cure children suffering from a variety of life-threatening hematological disorders. Attempting to achieve this, we continue the commitment to treatment and follow-up care of such children, and to clinical and laboratory research that ultimately will help us devise better therapeutic approaches to these deseases. Currently efforts are directed toward hematopoietic stem cell transplantation including ex vivo expansion of human hematopoietic stem cells, gene therapy, immunotherapy and analysis of pathogenesis of hematopoietic disorders.*

## 1. Hematopoietic capability of cord blood CD34$^+$ cells: A comparison with that of adult bone marrow CD34$^+$ cells

**Takahiro Ueda, Hiroshi Yoshino, Yasuhiro Ebihara, Atsushi Manabe, Kohichiro Tsuji**

Hematopoietic progenitor and stem cells (HPC/HSC) vary with the ontogenic stage in mammals. Human HPC/HSC in umbilical cord blood (CB) are increasingly being used as an alternative to those in adult bone marrow (BM) for treating patients with various hematological disorders. However, the difference in the hematopoietic activity of HPC/HSC between CB and adult BM is still not clear. We then compared CD34$^+$ cells, a hematopoietic cell population, in CB with those in adult BM, using the phenotypic subpopulations analyzed by flow cytometry, the colony-forming activity in methylcellulose clonal culture and the repopulating ability in NOD/SCID mice. Although the proportion of CD34$^+$ cells was higher in adult BM than CB mononuclear cells, the more immature subpopulations, CD34$^+$CD33$^-$ and CD34$^+$CD38$^-$ cells, were present at higher proportions in CB CD34$^+$ cells. Clonal culture assay showed that more multipotential progenitors were present in CB CD34$^+$ cells. When transplanted into NOD/SCID mice, adult BM CD34$^+$ cells could not reconstitute human hematopoiesis in recipient BM, but CB CD34$^+$ cells achieved a high level of engraftment, indicating that CB CD34$^+$ cells possess greater repopulating ability. Altogether, the present results demonstrated that human hematopoiesis changes along with the development from fetus to adult, and that CB CD34$^+$ cells contained more primitive hematopoietic cells including HSC, suggesting the usefulness of CB CD34$^+$ cells not only as a graft for therapeutic HSC transplantation, but as a target cell population of ex vivo expansion of transplantable HSC and gene transfer for gene therapy.

## 2. Exclusive expression of granulocyte colony-stimulating factor (G-CSF) receptor on myeloid progenitors in bone marrow CD34$^+$ cells

**Yasuhiro Ebihara, Ming-jiang Xu, Atsushi Manabe, Kohichiro Tsuji**

G-CSF has been reported to act on cells of neutrophilic lineage. However, the administration of G-CSF to mice and human induces an increase of circulating hematopoietic progenitor cells including not only myeloid but also erythroid, megakaryocytic and multipotential progenitors. We then analyzed the expression of receptors for G-CSF (G-CSFR) on human BM and G-CSF mobilized peripheral blood (PB) CD34$^+$ cells, and examined the proliferation and differentiation capability of sorted CD34$^+$G-CSFR$^+$ and CD34$^+$G-CSFR$^-$ cells using methylcellulose clonal culture. Flow cytometric analysis showed that G-CSFR was expressed on 18.7±9.8% of BM CD34$^+$

cells, most of which were included in $CD34^+CD33^+$ and $CD34^+CD38^+$ cell fractions, suggesting that G-CSFR is predominantly expressed on mature subpopulations in hematopoietic progenitors. In clonal culture, $CD34^+$G-$CSFR^-$ cells produced erythroid bursts, megakaryocyte and multilineage colonies, while $CD34^+$G-$CSFR^+$ cells produced only myeloid colonies including granulocyte, macrophage, eosinophil and granulocyte-macrophage colonies. When incubated with the cytokine cocktail for 5 days, CD34+G-$CSFR^-$ cells generated $CD34^+$G-$CSFR^+$ myeloid progenitors. In G-CSF mobilized PB, $CD34^+$ cells contained 10.8 ± 5.8 % of G-$CSFR^+$ cells, most of which were also myeloid progenitors, although $CD34^+$G-$CSFR^-$ cells contained a substantial number of myeloid progenitors in addition to erythroid, megakaryocytic and multipotential progenitors. These results have indicated that the expression of G-CSFR on $CD34^+$ cells is restricted to myeloid progenitors, and erythroid, megakaryocytic and multipotential progenitors do not express G-CSFR, suggesting that the specific activity of G-CSF on myelopoiesis depends on the exclusive expression of its receptor on myeloid progenitors, and that the mobilization of various hematopoietic progenitors is not a direct effect of G-CSF in human.

### 3. Impaired Granulopoiesis in the Truncated G-CSFR-Transgenic Mice

**Tetsuo Mitsui, Sumiko Watanabe[1], Kohichiro Tsuji:[1]Division of Molecular and Developmental Biology, IMSUT**

Severe congenital neutropenia (SCN), or Kostmann syndrome, is characterized by persistent absolute neutropenia and BM morphology that suggests maturational arrest of neutrophil precursors at the promyelocytic stage. In approximately 15-20% of the cases, mutations are found in the gene encoding the G-CSFR, resulting in a cytoplasmic truncation of the receptor. It is supposed that these truncated receptors act in a dominant negative manner to block granulocyte maturation and transduces a strong growth signal. Some patients with the mutations were reported to become acute myeloid leukemia after recombinant G-CSF therapy. Recently, McLemore et al. generated mice carrying a targeted one of these truncations, using homologous recombination in embryonic stem cells. Mice heterozygous or homozygous for the mutaion had normal levels of circulating neutrophils and no evidence for maturational arrest. In addition, Bernard et. al. described that these truncations were detected only in a minor percentage of transcripts from SCN and the mutations could spontaneously disappeared, and concluded that the gene abnormality has no role in etiology of SCN patients and is a bystander phenomenon. On the other hand, Hermans et al. reported the mice, being generated for the mutaion in the same way with McLemore et al, that have reduced number of neutrophils in PB.

To elucidate the role of these gene abnormalities in SCN, we generated three types of transgenic mice having two types of truncated murine G-CSFR and a wild type receptor as a control. We made two kinds of truncated G-CSFR DNA fragments (Q717-stop codon and Q730-stop codon), and wild type one as a control. These fragments were inserted to the expression vector LD2 that has murine MHC class I promoter, and transgened. The mice having the truncated recptors showed lower neutrophil counts in PB than those having the wild ones (225 ± 225, 230 ±118, and 731±301/ml in 717-truncation, 730-truncation and wild type mice). BM myelogram of the mice having the truncated receptors revealed reduced ratio of mature myelocytes. These results suggest that the truncated receptors have some role in the occurrence of neutropenia in SCN.

### 4. Growth of human T-cell acute lymphoblastic leukemia lymphoblasts in NOD/SCID fetal thymus organ culture

**Feng Ma, Atsushi Manabe, Wong Dang, Miyuki Ito, Kohichiro Tsuji**

T-cell acute lymphoblastic leukemia (T-ALL) is a malignant clonal desease which covers about 20 percent of all cases of ALL. Little has been understood about the in vitro proliferation of T-ALL leukemic cells because of a lack of a appropriate culture system. We have recently established an NOD/SCID mouse fetal thymus organ culture (FTOC) that is capable of supporting the development of T lymphoid cells from human $CD34^+$ hematopoietic stem/progenitor cells *in vitro*. By applying this NOD/SCID FTOC, we found that leukemic cells from fresh (1 case) and frozen (7 cases) bone marrow (BM) samples of children with T-ALL proliferated grossly over 4 weeks in the FTOC. Re-seeding of FTOC-derived T-ALL leukemic cells into second FTOC generated the same growth pattern. A detailed investigation of the FTOC-derived leukemic cells showed a similar phenotype to the original one moprphologically and immunophenotypically. Culturing of these FTOC-derived leukemic cells in suspension culture led to an expeditious death, suggesting that these FTOC-dependent cells were not cell lines. These FTOC-derived T-ALL leukemic cells were able to generate leukemia in NOD/SCID mouse and still shared clonal characteristics when probed by a PCR method using consensus primers for TCRγ chain rearrangement. Furthermore, a comparison of the original and FTOC-derived T-ALL leukemic cells revealed that the proportion of the cells that expressed IL-7R increased in all 7 samples. After 4 week FTOC, the experiment applying sorting and re-seeding of

IL-7R$^+$ and IL-7R$^-$ cells into second FTOC resulted in a predominant generation of IL-7R$^+$ cells from both fractions, while IL-7R$^-$ cells proliferated more potently in the second FTOC than IL-7R$^+$ cells did, suggesting that a conversion of IL-7R$^-$ to IL-7R$^+$ pathway existed in the proliferational process of T-ALL lymphoblasts. Our current study provides a novel assay system for the research on T-ALL lymphoblasts, especially in the exploration of the the hierarchy within human T-lymphoid leukemic cells, and may finally contribute to a novel therapeutic modality.

### 5. Gene therapy for recurrent neuroblastoma

**Atsushi Manabe, Imiko Hirose, Naohide Yamashita[2], Kohichiro Tsuji:[2]Department of Advanced Medical Science, IMSUT**

Although a notable improvement of cure rate in childhood cancer has been achieved by the development of multiagent chemotherapy, approximately one half of children with cancer do not survive with a contemporary treatment. Even a mega-dose chemotherapy combined with stem cell support can not eradicate the disease completely in this subpopulation of patients. A novel approach is needed for these patients. Recently, cancer immunotherapy employing a gene therapy technique was proposed. Actually, gene therapy using autologous tumor cells transduced with granulocyte-macrophage colony-stimulating factor (GM-CSF) has been performed for renal cell cancer patients in IMSUT. The aim of this project is to establish a feasibility of this modality in treating retractable cancer in children.

Neuroblastoma (NB) is one of the most frequent solid tumors in children and NB in children over one-year-old is known to be very difficult to cure even with stem cell transplantation. It has been observed that NB in some infants regresses spontaneously and its mechanism has not yet been elucidated. NB is derived from a neural crest which also derives malignant melanoma for which immunotherapy has already been established after identification of melanoma-associated tumor specific antigens such as gp100, MART-1/Melan-A, tyrosinase and MAGE-1. It is possible that some immunological mechanism may play a role in infants with NB.

Currently, we are preparing a gene therapy for recurrent NB. In collaboration with Dr. Malcolm Brenner at Baylor College in Houston, USA, we plan to use a tumor vaccine transduced with IL-2 and lymphotactin. It was shown that the vaccination of NB cells transduced with IL-2 into patients with relapsed NB exerted regression of tumors. It is possible that gene-transduced NB cells may become immunogenic and evoke an antitumor effect of the body. The addition of lymphotactin to IL-2 is expected to attract lymphocytes to the vaccinated NB cells. Preclinical experiments showed the feasibility of this therapy. The protocol was already approved by the institutional review board, and is now discussed by the committees of the goverment.

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*Research Hospital*

# Department of Advanced Medical Science

*Department of Advanced Medical Science was established in September 1997. We are investigating (1) Identification of genes involved in outflow tract formation during embryonic heart development, (2) Nuclear receptor in cardiovascular systems, (3) Pyk2/RAFTK signaling in hMVEC (human micro vascular endothelial cells), (4) human immunotherapy for malignancies using dendritic cells, and (5) human gene therapy for neuroblastoma. We are planning and progressing several projects described below to develop a new therapy for several diseases, including GH deficiency and carcinomas.*

## 1. Identification of genes involved in outflow tract formation during embryonic heart development

**Nakaoka T. et al.**

Malformations of the cardiovascular system account for most of the premature deaths caused by congenital abnormalities. Of these, the majority are congenital heart defects that arise from the abnormal remodeling of the single heart tube into four separate and properly aligned chambers. The importance of understanding the origin and fate of the outflow tract segment and associated cushions is that this site is most likely to be associated with birth defects of the heart in humans and, in fact, is a very common site of malformations that result from a wide variety of experimental perturbations in vertebrate models of congenital heart disease. The heart defect (hdf) mouse is a recessive lethal that arose from a transgene insertional mutation on chromosome 13. Embryos homozygous for the transgene die in utero by some 11.5 d.p.c. The future right ventricle and outflow tract fail to form and endocardial cushions are absent in this homozygote. In order to elucidate the mechanism of defective heart formation in hdf mouse, we collected total RNA from the embryonic heart of homozygote, heterozygote and normal littermate at 9.5 d.p.c. After subtractive hybridization, several candidate clones for differentially expressed gene in hdf mouse at 9.5 d.p.c. were obtained. We are now narrowing down the genes, truly up regulated or down regulated among them by using real-time PCR and virtual northern analysis. And we also are trying to examine the expression pattern of these candidate genes in mouse embryo in order to elucidate their roles during mouse development.

## 2. Nuclear receptor in cardiovascular systems

**Nakaoka T. et al.**

The importance of nuclear receptor is more and more being recognized in variety of systems. This prompted us to address the involvement of nuclear receptors in cardiovascular systems. Firstly, we investigated the expression and the role of NGFI-B, which is one of the orphan nuclear receptor, in vascular smooth muscle cells (VSMCs). Pyrrolidinedithiocarbamate (PDTC), a modulator of an oxidative state, induces apoptosis only when the density of VSMCs is low. At low VSMC density, expression of NGFI-B mRNA was induced 1 hour after the addition of PDTC, peaking at 6 hours, and persisted for up to 12 hours. The protein level of NGFI-B was increased 4 hours after PDTC addition and persisted for up to 12 hours. Moreover, PDTC-induced expression of NGFI-B mRNA was correlated with the magnitude of apoptosis. We next investigated whether the NGFI-B gene may act as a transcription factor under treatment

with PDTC by measuring the promoter activity of luciferase reporter plasmids that contained typical NGFI-B-responsive elements. The PDTC-induced transcriptional activity of NGFI-B was 2-fold higher at low cell density than at high cell density. These data demonstrate that NGFI-B can be induced in VSMCs and suggest that NGFI-B may play a role in PDTC-induced VSMC apoptosis

Secondly, two types of estrogen receptors are known so far, however, much is not known about their functional difference. Therefore in order to address how estrogen receptor alfa and beta are individually involved in cardiovascular systems, we are investigating the cells over expressing either estrogen receptor alfa or beta by a transfection of adenovirus.

### 3. Pyk2/RAFTK signaling in hMVEC (human micro vascular endothelial cells)

**Kuwabara K. et al.**

Pyk2/RAFTK is a cytoplasmic tyrosine kinase and a member of focal adhesion kinase gene family. Pyk2 is known to play a role downstream of a signaling through a chemokine receptor such as CXCR4. In order to elucidate the role of pyk2 in endothelial function, we analyzed HMVEC infected with adenovirus expressing two type of dominant negative pyk2, AxY402F and AxK457A. HMVEC infected with AxY402F showed higher migration activity than HMVEC infected with control adenovirus, whereas infection of AxY402F inhibited growth of HMVEC. ERK1/2 activation was also detected in HMVEC infected with AxY402F. Increased migratory activity of HMVEC infected with AxY402F was considered to be at least partly through ERK1/2 activation, since U0126, ERK1/2 inhibitor, inhibited migration of HMVEC infected with AxK457A. These data suggest anti-migratory signaling mechanisms in HMVEC.

### 4. Immunotherapy for malignancies

**Yamashita N. & Morishita M. et al.**

Malignant melanoma is an intractable disease and its prognosis is poor when the disease progresses to stage IV. We are finishing phase I study of immunotherapy using dendritic cells (DCs) to stage IV melanoma patients. The procedure to make mature DCs is as follows: periferal mononuclear cells are corrected using apheresis. Adherent cells to culture dishes are collected and GM-CSF and IL-4 are added to culture medium to make immature DCs. Tumor lysate is applied to immature DCs and further cultured in the presence of TNF-$\alpha$. Thus obtained mature DCs are intracutaneously injected to the patients once a week. In conjunction with application of DCs rIL-2 is subcutaneoulsy injected. During 10 weeks the administration of DCs is continued. After therapy clinical evaluations were done. From 1999 to 2000 ten patients entered this study protocol. Tumor progression was stopped in one patient. In two patients obvious regressions of metastatic tumors were observed. Safety of this therapy was proved and the activation of tumor immunity has been suggested.

Thyroglobulin (Tg) plays a central role in thyroid pathophysiology. Most differentiated thyroid carcinomas and some anaplastic thyroid carcinomas express thyroglobulin, and the tissue-specific origin of Tg has led to its use as a marker for thyroid cancer especially in patients without residual normal thyroid tissue. In general, the majority of patients with differentiated thyroid carcinoma have a good prognosis, but some high risk cases with large metastases cause death despite surgical resection, radioiodine, or external beam irradiation. Therefore we intended to treat metastatic thyroid cancer by immunotherapy using Tg pulsed dendritic cells (DCs). Some experimental autoimmune thyroiditis caused by Tg have been reported in animals. Now we are trying to detect human T cell clones stimulated by Tg pulsed DCs in vitro and to project clinical study.

### 5. Human gene therapy for neuroblastoma

**Yamashita N. et al.**

Neuroblastoma is the most common extracranial solid tumor of childhood. When the tumor occurs in infants (< 1 year age), it is frequently localized and responds well to therapy. However in older children (> 1year age) the prognosis is far worse. Although patients with localized disease may still be cured by conventional therapy, 80% or more of those with disseminated tumor can be expected to relapse within 3 years, and virtually none of this subgroup will become long-term survivors. Over the past decade, attempts to improve the outcome of advanced neuroblastoma have focused on greater intensification of the induction and consolidation phases of chemo-radiotherapy, with or without stem cell rescue. Although remission rates have been increased, there is no evidence at all of significant improvement in long-term survival. This failure has lead to a resurgence of interest in alternative methods of disease eradication, immune modulation in particular. We have finished the preparation of clinical gene therapy protocol for neuroblastoma in collaborations with Professor Brenner in Baylor University of Texas. The aims of this study are as follows; (1) to determine the safety up to four subcutaneous (SC) injections of autologous neuroblastoma cells, which have been genetically modified by adenoviral vectors to secrete lymphotactin and interleukin-2, (2) to determine the

safety of up to eight (total) injections in patients who have received the first four injections without unacceptable toxicity and have evidence of stable disease or better after receiving these injections, (3) to determine whether MHC restricted or unrestricted antitumor immune responses are induced by SC injection of modified autologous neuroblastomas and the cell doses required to produce these effects, (4) to obtain preliminary data on the antitumor effects of this treatment regimen. After permission by ethical committees this clinical study is going to start.

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*Research Hospital*

# Department of Surgery

*We have been engaged in the surgical treatment of solid tumors and the renal transplantation. We have also been offering services, including upper and lower endoscopic examination, ultrasonic examination, and angiography, in the Department of Clinical Examination. The principal goal of our department is to develop and conduct clinical trials (Phase I and II) for patients at Reseach Hospital. We have initiated phase I clinical trials of melanoma vaccine using gp100 derived peptides.*

## 1. Summary of surgical treatment and other procedures performed in 2001

**Hideaki Tahara, Masazumi Eriguchi, Shinji Tomikawa, Takuya Tsunoda, Yasutaka Takeda, Iwao Yoshizaki, Yoshifumi Beck, Hironobu Yanagie, Takuya Takayama, Yuichi Ando, Yasumasa Nonaka, Syogo Nakano, Hiroyuki Mushiake, Kenichi Ohyama, Hiroaki Tanaka**

Surgical operations have been performed in 112 cases under general anesthesia and spinal anesthesia. As shown in Table 1, major operations were performed in 69 patients with malignant diseases, and in 47 patients with benign diseases. Renal transplantation was performed in 2 patients.

Procedures other than surgical operations performed in 2001 are as follows: angiography including trans-arterial embolization and trans-arterial chemotherapy (35 cases), gastroduodenal endoscopy (490 cases), and colorectal endoscopy (212 cases).

## 2. Phase I clinical trial of melanoma vaccine using gp100 derived peptides restricted to HLA-A*0201 or -A*2402

**Table1. Major Operations Performed in 2001**

| Malignant Diseases | | Benign Diseases | |
|---|---|---|---|
| Cancer of the stomach | 10 | Cholelithiasis | 17 |
| Cancer of the colo-rectum | 22 | Inguinal hernia | 9 |
| Cancer of the liver | 5 | Miscellaneous | 21 |
| Cancer of the bile duct | 1 | Total | 47 |
| Cancer of the pancreas | 3 | | |
| Cancer of the kidney | 3 | Renal transplantation | |
| Cancer of the breast | 10 | Cadaveric | 2 |
| GIST | 2 | | |
| Cancer of the thyroid | 5 | | |
| Miscellaneous | 8 | | |
| Total | 69 | | |

**Takuya Tsunoda, Yoshifumi Beck, Hiroyuki Mushiake, Toshiyuki Baba, Shogo Nakano, Kenichi Ohyama, Hiroaki Tanaka Hideaki Tahara**

Epitope peptides derived from gp100, a melanoma associated antigen, are used for the cancer vaccine to treat the patients with advanced malignant melanoma. Patients with HLA-A*0201 were treated with a gp100 derived peptide (ITDQVPFSV) and another peptide with a mutation (IMDQVPFSV). Patients with HLA-A*2402 were treated with a gp100 derived peptide. All of the peptides were used with IFA in order to augment anti-tumor immunity. To analyze the immune response of the vaccinated patients, HLA-Tetramer was prepared and used for staining of the peripheral blood lymphocytes taken from the patients enrolled in this protocol. Our goals in this clinical trial are to examine its safety and immune responses associated with the peptide vaccination. This clinical trail is considered to be novel in; 1) synchronously injecting wild type and mutant gp100 peptides, and 2) using a newly mapped gp100 peptide restricted to HLA-A*2402. We have enrolled 4 patients for HLA-A*2402 peptide and 3 patients for HLA-A*0201 peptides during year 2001.

### 3. Development for detection method of minimal residual disease for gastrointestinal cancer

**Kenichi Ohyama, Takuya Tsunoda, Hiroaki Tanaka, Shogo Nakano, Yuichi Ando, Takuya Takayama, Yoshifumi Beck, Hideaki Tahara**

Hematogenous metastasis is one of the most frequent cause of treatment failure following gastrointestinal cancer surgery. In these cancer patients, dormant minimal residual disease (MRD) is often asymptomatic and clinically undetectable until relapse. Thus, detection of MRD is needed to improve the treatment of gastrointestinal cancer, and may provide useful information for selecting candidates for adjuvant chemotherapy. Recent molecular biological evidence has demonstrated that microscopic cancer cells are detectable by immunohistological staining and RT-PCR. In this study, immunohistological staining is assayed using anti cytokeratin antibody and anti CEA antibody in bone marrow aspirates and primary tumors. Furthermore, real-time RT-PCR assay is performed for measuring CEA, CK-19 and CK-20 mRNA in bone marrow aspirates and peripheral blood and primary tumors. Bone marrow aspirations are obtained from sternum after operation. Peripheral blood samples are obtained from femoral artery before and after operation. A total of 60 gastrointestinal cancer patients who undergo curative operation will be enrolled in this study. The present study investigates the feasibility and clinical utility of this procedure for sensitive detection of hematogenous metastasis in gastrointestinal cancer patients.

### 4. The results of cadaveric renal transplantation in our institute

**Shinji Tomikawa, Yoshifumi Beck, Yuichi Ando, Naoya Ichikawa, Hideaki Tahara**

Between March 1986 and December 2001, we performed a total of 46 cadaveric renal transplantation under cyclosporine-based (n=33) or tacrolimus-based (n=13) immunosuppression at our institute. The transplanted kidney from non heart-beating donor developed a high incidence of delayed graft function (DGF) that was defined as the need for dialysis during the first week after transplant. We studied deleterious effects of delayed graft function in cadaveric renal transplant recipients in our institute. Thirty recipients were males and 16 were females. Of the 46 transplanted kidneys, two (4.3%) were primary nonfunction owing mainly to irreversible kidney damage from long ischemia, 34(73.9%) developed DGF which lasted mean of 15±17 days and 10 (21.7%) showed immediate function. When compared with the kidneys with DGF, the kidneys without DGF showed a shorter duration of the average of warm ischemic time (1.50±3.20 vs. 9.85±10.87 minutes, $p<0.01$), total ischemic time (361±272 vs. 679±313 minutes, $p<0.01$) and a lower level of the average of the nadir of serum creatinine by the time of discharge (1.03±0.21 vs. 1.67±0.72, $p<0.01$) and lower incidence of acute rejection during three months after transplant (10.0% vs. 32.4%, statistically not significant). Graft survival rates of the kidneys with DGF at 1, 3, 5, and 10 years were 100%, 84.8%, 81.2% and 71.4%, respectively. On the other hand, graft survival rates of the kidneys without DGF at 1, 3, 5, and 10 years were 100%, 100%, 100% and 85.7%, respectively. DGF was identified as one of the principal correlates of poor graft survival in cadaveric renal transplantation.

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# *Research Hospital*
# Department of Radiology

*Our department consists of three major divisions: diagnostic radiology, nuclear medicine and radiation oncology. Diagnostic radiology plays a critical role in evaluating various neoplastic and infectious diseases. Clinical studies are conducted mainly using magnetic resonance imaging, supported by other departments and other institutions. In nuclear medicine, we develop analytic methods to estimate in vivo physiology, as well as studying the kinetics of radiotracers and physical characteristics of detectors. In radiation oncology, total body irradiation prior to bone marrow transplantation provides valuable advantage.*

## 1. Validation study of the simplified technique for cerebral tissue segmentation

**Kohki Yoshikawa, Yusuke Inoue, Masaaki Akahane, Morio Shimada[1], Tsuyoshi Matsuda[2], Seizoh Takahashi[3], and Takashi Ogino[4]:[1]Department of First Radiology, Toho University;[2]GE Yokogawa Medical System Ltd.;[3]Department of Chemical & Biological Sciences, Japan Women's University; and [4]National Institute of Neuroscience**

The aim of this study is validation of our developed simplified technique for cerebral tissue segmentation. The phantom with mixed various concentrations of agar and six normal volunteers were used for comparing the accuracy of the tissue segmentation. The images used in our simplified method were oblique T2W- and PDW- images with slice thickness/slice gap of 6mm/2mm and those in the usual method were axial T2W- and PDW-images with 2mm/0mm. In our simplified method, the linear interpolation was used for reconstructing the serial image section with 1 to 1 correspondence of each pixel. Although the accuracy of our method deteriorated slightly as compared with the usual method using gap-less images, it was possible to perform fairly good tissue segmentation using thick oblique images with slice gaps. The technique for tissue segmentation, which can be performed easily in a short time, is quite important, because such technique will play an important role for quantification of the volume of the targeted tissues or organs and for quantitative analysis of metabolic substrates in the brain or other organs. Although we do not perform at present, we intend to apply our simplified-mould technique to proton magnetic resonance spectroscopic imaging (MRSI) of the brain, and more accurate and detailed analysis of the metabolic substrates in the targeted tissues or lesions.

## 2. Fatty-Meal MR Cholangiography to Evaluate Biliary Motor Function

**Yusuke Inoue, Yutaka Komatsu[5], Kohki Yoshikawa, Masaaki Akahane, Kuni Ohtomo[6], and Masao Omata[5]:Departments of [5]Gastroenterology and [6]Radiology, Graduate School of Medicine, University of Tokyo**

Magnetic resonannce cholangiography (MRC) is being accepted as a safe alternative to a diagnostic endoscopic cholangiography. Its ability to reveal biliary morphology has been well described, however, that to evaluate biliary motility has yet to be elucidated. The aim of this studyis to invesitigate the possibility of the evaluation of biliary function with MRC. Twenty patients with gallstones and 30 normal control subjects were studied using fatty-meal MRC, MRC before and after the ingestion of fatty meal. After the completion of baseline MRC, they encouraged to drink 250 ml of milk and underwent

postprandial MRC every 10 min for 60 min. Postprandial changes in the gallbladder volume and diameter of the common duct were assessed as indicators of gallbladder contractility and biliary obstruction, respectively. Percent gallbladder volume was defined as gallbladder volume at each time point expressed in percent of the baseline volume, and gallbladder ejection fraction was calculated as percent volume reduction at maximal contraction. A change of more than 1 mm in duct diameter was considered significant, and postprandial dilatation at 60 min after milk ingestion was defined as a finding indicative of persistent biliary obstruction. MRC images before and after fatty meal ingestion were successfully obtained in every subject with no serious complications. In the controls, mean percent gallbladder volume decreased soon after the ingestion of fatty meal and remaind almost constant from 30 min to 60 min. In the gallstone patients, percent gallbladder volume was significantly larger at 20-60 min than in the controls. The gallbladder ejection fraction ranged widely and was significantly reduced when compared with the controls ($p < 0.01$). In two gallstone patients with co-existing common bile duct stones, postprandial dilatation associated with transient stone impaction at the ampulla was observed. The impaction was relieved spontaneously in both patients, and the duct diameter decreased. Persistent biliary obstruction was indicated in no subjects. The results of this study suggest the feasibility of fatty-meal MRC and its potential usefulness in evaluating biliary motor function in addition to biliary morphology.

### 3. Measurement of Left Ventricular Volume and Function by MR Imaging

**Yusuke Inoue, Shuhei Komatsu[6], Masaaki Akahane, Kohki Yoshikawa, Ikuo Yokoyama[7] and Kuni Ohtomo[6]:[7]Department of Cardiovascular Medicine, Graduate School of Medicine, University of Tokyo**

Cardiac MR imaging is an evolving field through the development of fast imaging technique. It has been recognized as a gold standard method to measure left ventricular volume and function, however, various problems disturbs its wide use in clinical settings. We aim at developing convenient, accurate, and reproducible method to evaluate left ventricular volume and function by MR imaging. Fifteen normal subjects underwent cardiac MR imaging using different imaging sequences, different slice thicknesses and different planes. These techniques were compared in view of image quality and estimates of volume and function. Although contiguous short-axis slices are usually utilized to estimate left ventricular volume based on the Simpson's rule, we demonstrated that multiple thin-slice images with wide intersection gaps provide comparable estimates with clearer demarcation of the cavity. The effect of slice thickness on image quality was more prominent for long-axis images than for the short-axis images. Signal of the left ventricular cavity was higher on thin-slice images than on thick-slice images. In addition to decrease in partial volume effect, increased inflow effect appears to add advantages to thin-slice imaging. At present, thin-slice imaging fails to offer better accuracy in quantitative indices, probably due to reduction in signal-to-noise ratio. We plan to utilize novel fast imaging sequence which will permit imaging with high signal-to-noise ratios.

### 4. Influence of Collimators on Iodine-123 Imaging

**Yusuke Inoue, Ichiro Shirouzu[8], Akira Suzuki[9], and Toru Machida[8]:Departments of [8]Radiology and [9]Cariovascular Medicine, Kanto Medical Center NTT EC**

Chemistry of iodination is well understood and versatile, and various tracers labelled with iodine-123 are used to evaluate flow, metabolism, neutotransmission, and antibodies. Iodine-123 emits high-energy photons other than 159 KeV photons which is used to create gamma camera images, and scatter from high-energy photons induce difficulties. Choice of collimators plays a crucial role in determining the quality of gamma camera imaging, and a new collimator specifically designed for imaging 159-KeV photopeak (special low-energy collimator, SLE) is recommended for the use of iodine-123 imaging. We compare this collimator with a low-energy high-resolution collimator (LEHR) and a medium-energy collimator (ME). There were no substantial differences in sensitivity and system resolution for technetium-99m between SLE and LEHR. ME exhibited higher sensitivity and lower resolution. In imaging iodine-123, the count of the high energy window was the largest for LEHR and the smallest for ME. So was the count of the photopeak window in the periphery of the collimator surface. SLE may provide advantage over LEHR in improving the detection of small abnormalities, however, ME would be still recommended for quantitative measurement. We are now testing this hypothesis, focuing on nuclear cardiology. In addition, the utility of scatter correction is being examined for each collimators.

### 5. Curved Surface Reformation in Multi-slice CT: A Novel Technique for Evaluating Diffuse Lung Disease

**Masaaki Akahane, Manabu Minami[6], Yusuke Inoue, Shigeru Kiryu[6], Masashi Miyazawa[6], Naoki Yoshioka[6], Kohki Yoshikawa, and Kuni Ohtomo[6]**

High resolution computed tomography (HRCT) plays an important role in the initial diagnosis and follow-up study of diffuse lung diseases, and contiguous HRCT of the whole lung is realized by multi-slice CT. We developed curved surface reformation (CSR), a novel three-dimensional visualization technique for evaluating diffuse lung diseases in order to handle large data sets of multislice CT efficiently. CSR were obtained by depicting the average CT value of the first three voxels below the segmented lung surface facing the viewer with an integral algorithm. All post-processing and image saving were automatically performed within three minutes. Alterations of normal structures are observed on CSR images in pathological conditions, and the location of pathologic changes within secondary lobules can be recognized easily because they display these lobules in an ideal manner. Such images may eliminate the need for three-dimensional reconstruction of contiguous axial slices in the mind. Furthermore, numerous lobules can be observed at a glance on CSR images. This technique seems to permit the fast, accurate assessment of the distribution and extent of pathologic changes. CSR images are obtained with minimal labor in an automatic manner, which contributes to convenience of use and reproducibility of image processing. The technique described here seems suitable for follow-up studies as well. In conclusion, CSR images appear to be useful for accurately determining the localization of pathologic changes within secondary lobules and for surveying the distribution and characteristics of diffuse lung diseases as a complement to axial images. Further evaluation of the clinical usefulness of CSR images is being undertaken.

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# *Research Hospital*
# Department of Laboratory Medicine

*Our department consists of five divisions of clinical physiology, hematology, biochemistry, bacteriology and pathology, and engage in laboratory analysis and diagnosis of clinical material submitted from the Research Hospital. Since our department's primary mission is to examine materials in routine bases and not for research purpose, our scientific output is currently quite limited. However, along with the ongoing practice of advanced exploratory medicine in the research hospital, we manage to develop research-oriented analysis and are now evolving to function as an integrated diagnosis & monitoring laboratory.*

## 1. Pathological evaluation of cancer immuno-therapy conducted in the research hospital

**Haruo Onoda, Mamiko Sato and Naoki Oyaizu**

We have initiated to intensively examine the surgical materials obtained from patients under cancer immuno-therapy conducted in the research hospital. By applying sophisticated immunohistochemical technique, we have completed analysis of cases of GM-CSF-based gene theraphy for renal cell carcinoma and dendritic cell-based anti-melanoma immuno therapy. Our goal is to evaluate the effectiveness and to elucidate the mechanisms of anti-tumor immune response elicited by the therapy

## 2. Immunopatholgical analysis of hematological disorder

**Members of Department of Medicine and Naoki Oyaizu**

By applying intensive immunohistochemical analysis, we are now elucidating the new category of disease designated as an autoimmune MDS-like syndrome (AMLS). Among the patients with hematological disorder such as myelodysplastic syndrome(MDS), —refractory anemia, aplastic anemia, or pure red cell aplasia, we noticed common patho-immunological mechanism is operative under these disease condition. That is the destruction of developing hematopoietic cells by immune-based mechanisms in the bone marrow, but not in the periphery. Criteria for the diagnosis of AMLS includes: no major chromosomal abnormality in the hematopoietic stem cell; presence of lymphoid aggregates in the bone marrow; and improvement of hematological symptoms by the administration of immunosuppressant(s). Our goal is to solidly establish the disease concept by elucidating the immuno-pathological mechanism(s) and to develop new therapeutic maneuver.

### Publications

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# *Research Hospital*
# Department of Applied Genomics

*Our department was established in April, 2001 to support the translational researches of our hospital. Ongoing projects which we are participating in are immune therapy for melanoma and thyroid cancer utilizing dendritic cells immunized with tumor antigens. We are also conducting researches to find responsible SNPs in genes related to drug sensitivity, disease progression, and prognosis in various hematological disorders.*

## 1. Studies to find SNPs responsible for the immune response to dendritic cell therapy in genes related to cytokine signalings

**Satoru Yoshida, Nozomi Yusa, Mayumi Karikomi and Noriharu Sato**

Patients with intractable melanoma or thyroid cancer were enrolled for the immune therapy and were treated with dendritic cells immunized with their tumor antigens in association with interleukin-2 injections. Some patients showed favorable, albeit a little response to the therapy. In order to analyze genetic background of the diversity of tumor response, we are examining SNPs in several genes related to cytokine signalins using JSNP database.

## 2. Genetic study on CML

**Nozomi Yusa, Satoru Yoshida, Mayumi Karikomi and Noriharu Sato**

Before the advent of STI571, interferon has been the first choice drug for patients with CML who had no HLA-identical sibling donors. Since the long-term effect of STI is still unclear, interferon may have some position in the treatment of CML. We are going to determine SNPs in genes related to the sensitivity of interferon in patients with CML.

## 3. Genetic study on MDS

**Satoru Yoshida, Nozomi Yusa, Mayumi Karikomi and Noriharu Sato**

Myelodysplastic syndorme (MDS) is heterogeneous diseases with different prognosis and different drug sensitivities. Some MDS respond to steroid therapy and some do so to cytokine therapies. We are studying on the SNPs in genes related to cytokine signaling or apoptosis whether there is some association between these SNPs and drug sensitivities or disease prognosis.

## 4. Genetic study on GVHD

**Satoru Yoshida, Nozomi Yusa, Mayumi Karikomi and Noriharu Sato**

In hematopoietic stem cell transplantation, we sometimes observe severe GVHD (graft-versus-host disease) in HLA-matched transplant. There are reports suggesting an important role of some cytokines in GVHDs. In fact, promoter polymorphism of TNF gene is reported to be involved in severe GVHD. In order to find other genes affecting the severity of GVHD in addition to TNF, we are studying SNPs in genes related to immunologic responses.

## 5. Functional characterization of alkaline phosphatase in granulocytes

**Satoru Yoshida, Nozomi Yusa, Mayumi Karikomi and Noriharu Sato**

Alkaline phosphatase is expressed only in cells belonging to neutrophilic lineage in the hematopoietic tissue and called neutrophil alkaline phosphatase (NAP). Although NAP is regulated by G-CSF *in vivo*, its function in neutrophils is largely unknown. In order to explore the function of NAP, we transfected alkaline phosphatase cDNA into U937 cells and established stable NAP-expressing cell line. The resulting transformants showed increased capacity to phagocytose zymosan, suggesting the role of alkaline phosphatase in active phagocytosis. In addition they showed increased ability to adhere to type I collagen, suggesting its role in the case of neutrophil migration in the extravascular tissue.

## 6. Assay for phagocytic activity of neutrophils and other hematopoietic cells

**Mayumi Karikomi, Satoru Yoshida, Nozomi Yusa and Noriharu Sato**

We developed E.coli expressing green fluorescent protein (GFP). Using these cells as the targets of phagocytic cells, we can measure the percentage of cells that phagocytized these GFP positive-cells. In addition this system may pave the way for multi-color analysis with various kinds of antibodies to define the characteristics of individual phagocytozing cells.

## Publications

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佐藤典治　ゲノム医療 日本臨症 59:2445-2450, 2001

# *Research Hospital*
# Division of Clinical Trial Safety Management

*Division of Clinical Trial Safety Management (DCTSM) was established on April 2001 in the Research Hospital to watch the safety and ethics of clinical trials. DCTSM also deals with the risk management of diagnosis and treatment for general medicine. The staffs of DCTSM (doctors and nurses) are doing their work in collaboration with Translational Research Coordinators (TRC), which are organized by co-medical staffs, including pharmacist, dietician, psychologist and clinical laboratory technologist. The aim of DCTSM is to carry out the safe and ethically-protected clinical trials in the Research Hospital in addition to the data management. In order to accomplish it we are doing the following activities.*

## 1. Contribution of QOL on NDA approval process of Oncologic Drugs at U.S. Food and Drug Administration

**Fumitaka Nagamura**

At the U.S. Food and Drug Administration (FDA), the major concern on New Drug Application (NDA) approval process for oncologic drugs has been the prolongation of survival and improvement of quality of life (QOL). In 1986, this concept was recommended by Oncologic Drugs Advisory Committee. It took ten years from this recommendation that the first oncologic drug which QOL was the main basis for approval. The drug was gemcitabine for advanced pancreatic cancer. The primary endpoint of its pivotal study was clinical benefit response. This criterion was mainly based on the evaluation of pain and its control. This QOL score improvement and modest but significant prolongation of survival were the basis for approval of this drug. Mitoxantrone for prostate cancer with severe pain and pamidoronate for breast cancer with osteolytic bone metastases are the representative drugs, which QOL was the major basis for approval. Other drugs that QOL was the main basis for approval were toxicity-reducing drugs. The ratio of NDA that QOL was the major concern for approval is still limited. QOL analyses had some weaknesses in methodology, problems with dropouts and missing data, and subscale profiles that overlapped with toxicity and symptoms, which should be dissolved. Further methodological and analytical improvements on QOL evaluation according to the kind of malignancy and disease status are necessary.

## 2. Review of clinical study protocols from Divisions in IMS/UT Research Hospital

**Fumitaka Nagamura**

After the reorganization of IMS/UT and establishment of our division, protocols of translational researches and intra-research hospital clinical studies should be reviewed by our division before the submission to the Institutional Review Boards (CHIKEN SINSA IINKAI). The purposes of this review are: to assist the planning of appropriate clinical studies; to help the writing of protocols; to keep the scientific aspects of studies; to assure that studies are ethically planned and conducted.

From July to December of 2001, we received two protocols and numerous questions. The first protocol was "Phase I study of Dendritic Cell Therapy for advanced/metastatic thyroid cancer" from Department of Advanced Medical Science, and the second one was "Phase I study of Dendritic Cell Therapy combined with local irradiation to sub/in-

tra-cutaneous lesions for advanced/metastatic solid tumors" from Department of Surgery (both English names were translated by our Division). Pre-review of both study protocols were handed within two weeks from the receipt. The format of pre-review is based on the style of U.S. Food and Drug Administration. Our opinion is summarized into three sections: safety issue (most concern), major problem, and minor problems and suggestions. These opinions are not obligations to have enforcement, but the options to improve clinical studies. Final decision should be made at the Institutional Review Boards.

To assist the planning of clinical studies and writing protocols, we wrote "Guideline". This "Guideline" will be disclosed within the first quarter of 2002. New Good Clinical Practice and Guidelines of International Conference on Harmonization has been settled, and many guidelines for gene therapy and gene analysis have been announced. Our division prepared these guidelines, and tries that intra-institutional clinical studies respect such guidelines.

## 3. Roles of Ethics and Psychology on Translational Research

**Momoyo Ohki, Hajime Kotaki, and Naohide Yamashita**

The concept of Translational Research (TR) is a new one, and the role of psychologists on TR has not been settled clearly. To seek the role of psychologist, we focused on three points in patients enrolled gene therapy for renal cell cancer: 1) tends of emotions and autonomy of participants; 2) acts and effects of psychologist; 3) role of psychologist on TR and problems.

Trends of emotions were generally summarized as calm, acceptance of disease, fear. Most of patients accepted their disease status calmly, however, some patients expected overwhelmingly. As for autonomy of participants for treatments, five of six patients were score 100/100 for "requirement of information". Participants to TR tend to want information and to have preparedness. On the other hand, mean score of "self decision" varied from 7/100 to 66/100.

Psychologist had understood the emotions of patients mentioned above, and then tried to make patients to be able to control emotions through counseling as a direct intervention. The result was that patients recognized their fear and understood their circumstances. While, as an indirect intervention, psychologist pointed out the personality of patients, mental status, appropriated attitudes of medical staff, and psychological problems to be emerged at TRC meeting, and gave suggestions. Especially, we succeeded to made the relationship between patients/their family and medical staff trustworthy by revealing the differences of recognition between medical staff and patients and by the direction to act from the patients' point of view.

TRC, as a check system, is necessary for preventing the right of patients. When considering the ethics of patients who are participating to TR with fear after informed their status, it is not enough to obey the law and/or to pay attention to the safety. For mental care of patients who are restricted to hospitalization, psychologist should play an important role as a member of TRC as a specialist. The future theme is to establish the intervention, that psychologists can apply their specialties considering the characteristics of TR. One role of psychologist is to sophisticate the content and the degree of interventional methods according to the progress of protocols based on the personal events.

## 4. Education program for Translational Research Coordinator

**Fumitaka Nagamura, Hajime Kotaki and Naohide Yamashita**

The major purposes of settling translational Research Coordinator are to keep patients' right, to conduct translational research more ethically, and to perform translational research scientifically. The role of TRC is not the same as that of Clinical Research Coordinator CRC) in terms of the aggressive intervention to keep translational research ethically conducted. The problem of education for research coordinators including CRC is the new but the critical in Japan. To educate TRCs, we take place seminars after the weekly TRC meeting.

However, there was no good example in Japan. So, we prepared textbooks by ourselves. One textbook is the one used at the CRC educational program held at the U.S. Dr. N. Yamashita joined the Society and attended the meeting. We translated it into Japanese and modified to meet the Japanese environment. Almost all of TRCs attended the seminar based on this, and mastered the content. Another textbook is the one that we have been writing originally. The content is the following: accidents happened during the development of clinical researches; lows and regulations related to clinical studies; what is the clinical research; the reason why clinical studies are necessary; classification of studies, and so on. The latter one has not been completed yet. Seminars based on these textbooks will be planned regularly.

To collect information and to keep up with the development of medicine and environment of clinical studies, we attended the educational programs and lectures. These results were reflected into the education for TRCs. We try to settle the education system for TRC. After the completion of this plan, IMS/UT will be the base for education as well as the one for conducting translational research.

## 5. Risk management of Research Hospital

**Fumitaka Nagamura, Yuko Ogami, Naohide Yamashita**

To perform clinical trials it is indispensable to assure the safety of daily medical diagnosis and treatment. Staffs of DCTSM also work as risk mangers in the Research Hospital. Medical accidents are reported to DCTSM by report forms. When the urgent response to the patient is required, the meeting is immediately held to discuss the first lines of action to protect the involved patient. This meeting also determines the preventive measures. Medical accidents and the responses of DCTSM are reported in the Council of Risk Management in the Research Hospital, which is held every three months. Medical incidents are also reported to DCTSM by report forms and the ways for prevention are considered. When necessary, the urgent meeting is held. To educate the risk management to the medical staffs several seminars are held by DCTSM.

## Publications

探索型臨床研究におけるトランスレーショナル・リサーチ・コーディネーター（薬剤師、看護婦、臨床心理士、栄養士）体制の確立．小瀧　一、福田直子、今野嘉子、小野寺公枝、大木桃代、稲沢健志、山崎知子、中岡隆志、濱尾房子、山下直秀、浅野茂隆：病院薬学，27(2): 113-122, 2001.

臨床研究における医師とトランスレーショナル・リサーチ・コーディネーター（薬剤師、看護婦、臨床心理士、管理栄養士)の連携．福田直子、今野嘉子、小野寺公枝、大木桃代、稲沢健史、山崎知子、濱尾房子、小瀧　一、山下直秀：臨床薬理，32(1): 147S-148S, 2001.

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# *Research Hospital*
# Department of Transfusion Medicine

*Improving the clinical outcome of hematopoietic stem cell transplantation, we have been engaged in researches to clarify the cytokine network acting on normal hematopoiesis, and to establish the ex vivo expansion system of hematopoietic stem cells. For the successful engraftment of cord blood cells, we are engaging in the basic researches to establish novel culture systems for progenitor B cells, and to clarify the mechanisms how transplanted stem cells are homing to the bone marrow environment. For the future development of regenerative medicine, we also study the differentiation processes of mesenchymal stem cells to various types of cells and/or tissues.*
*We are also engaging in the development for the new therapeutic strategies using antisense oligodeoxynucleotides. As the clinically-based department, we supply purified hematopoietic stem cells to clinical trials for allogeneic bone marrow and peripheral blood transplantation. The depletion of T lymphocytes from the donor cells is undertaken to reduce the rate of graft-versus-host disease, and the purification of CD34$^+$ cells is performed in order to purge grafts of tumor cells. These clinical-oriented researches are focused not only on cytokine therapy and immunotherapy, but also on gene therapy, antisense therapy, and cell therapy that are being generated in the Institute.*

## 1. Purification of human hematopoietic progenitor and stem cells for bone marrow, peripheral blood transplantations in the clinical setting

**Taira Maekawa, Kazuo Ogami, Yuka Wada, Shinobu Hosoda, Tetsu Yoshimasu, Hitomi Nagayama[1], Tsuneo A. Takahashi[1], Tohru Iseki[2], Jun Ooi[2], Ryuhei Tanaka[3], Atsushi Manabe[3], Kouichiroh Tsuji[3], and Shigetaka Asano[2]:Departments of [1]Cell Processing, [2]Hematology-Oncology, and [3]Pediatrics, The Institute of Medical Science, The University of Tokyo**

Cell surface antigen CD34$^+$ cells contain the majority of human hematopoietic progenitors and stem cells, that can produce a variety of hemopoietic colonies and reconstitute the hematopoiesis after myeloablative chemotherapy. Several methods to purify a large number of CD34$^+$ cells from the bone marrow (BM) and peripheral blood (PB) samples have been developed such as the panning and the culumn filtration methods with immunobeads. The purity and recovery efficiencies after separation using immunobeads in our department are more than 98% and 50-60%, respectively. The administration of more than 5 x $10^5$/kg CD34$^+$ cells purified from bone marrow and 2 x $10^6$/kg CD34$^+$ cells purified from PB after mobilization by granulocyte colony-stimulating factor (G-CSF) are capable of inducing a rapid and permanent recovery of the hematopoiesis after transplantation. Allogeneic peripheral blood stem cells have now been used as an alternative method for clinical transplantation (allo-PBSCT). In order to obtain less graft versus host disease (GVHD), bone

marrow and peripheral blood transplantations using allograft of purified $CD34^+$ cells are now used in the clinical settings.

## 2. Establishment of enzyme linked immunosorbent assay to detect soluble HLA class I antigens in serum from patients received allogeneic stem cell transplantations

**Taira Maekawa, Shinobu Hosoda, Yuka Wada Y, Kazuo Ogami, Tetsu Yoshimasu, Tohru Iseki, Hitomi Nagayama, Jun Ooi, Arinobu Tojo, Kenzaburo Tani, Ryuhei Tanaka, Atsushi Manabe, Kohichiro Tsuji, and Shigetaka Asano[2]**

Besides being expressed on the membrane of most nucleated cells, HLA class I antigens are present in serum. We established the emzyme linked immunosorbent assay to detect soluble HLA class I antigens in serum. An increase in the serum HLA class I antigen level has been seen in acute rejection episodes following heart, liver, and kidney transplants. We found that soluble HLA class I level significantly increases in patients suffering from acute graft versus host disease (GVHD) episodes following allogeneic bone marrow transplantation (allo-BMT) whereas it does not change in patients without GVHD. We are now investigating whether the increase of this soluble HLA Class I antigen levels in serum from patients received allo-BMT, -PBSCT, and cord blood transplantation (CBT) can modulate the immunoregulatory systems leading to less onsets of GVHD, comparing with other cytokine levels including interleukins, interferons, and tumor necrosis factor.

## 3. Development of antisense therapeutics for hematological malignancies

**Taira Maekawa, and Shigetaka Asano[2]**

Cloning and sequencing of pathogenic genes have provided useful informations for preventive medicine and conventional therapies through molecular diagnosis of various diseases including hereditary disease, cancer, and AIDS. They have also made possible new therapeutic approaches through gene manipulation. Oligodeoxynucleotides (ODNs) show great promise as therapeutic agents because of their potential to inhibit gene expression by sequence-specific mechanisms. The elegant specificity of Watson-Crick base pairing between the antisense (AS) ODNs and the target mRNA or gene could form the basis for a highly specific and effective drug. Clinical trials are now in progress to the United States, Italy and the United Kingdom. However, because chemically modified AS ODNs, especially PS ODNs, have been reported to cause a number of nonspecific effects as described above, we are now examining the efficacy of new ODN analogues with mixed backbone structure and N3'→P5' phosphroamidates targetting oncogenes such as BCR-ABL, c-myc, and Bcl-2, and investigating the feasibility to establish antisense therapeutics for leukemias and lymphomas. We have recently establish the novel drug delivery system named transmembrane carrier system (TCS) to increase the efficacy of cellular uptake of AS ODNs.

## 4. Megakaryopoiesis of cord blood cells are effectively enhanced by stromal cells derived from bone marrow mesenchymal stem cells

**Taira Maekawa, Kazuo Ogami, and Shigetaka Asano**

Mesenchymal stem cells (MSCs) give rise to marrow stromal cells that produce the spongy stromal matrix comprising the bone marrow microenvironment. These marrow stromal cells contribute directly to blood cell formation by producing the extracellular matrix where blood cell development takes place and by providing cytokines and other molecules that direct or stimulate the production of mature blood cells. MSCs are rarely contained in cord blood, that may cause the delayed engraftment of megakaryopoiesis in a clinical setting of cord blood transplantation. Preclinical studies in animals have demonstrated that culture-expanded mesenchymal stem cells can be used to repair bone defects, full-thickness articular cartilage defects, bone marrow stroma, and tendon. We are now seeking to develop human therapeutic products based on the role of hMSCs in megakaryocytopoiesis. We found that stromal cells derived from MSCs effectively suuport the megakaryocytopiesis of cord blood cells *in vitro*.

## 5. Establishment of Room for Clinical Cellular Technology (RCCT)

**Taira Maekawa, Tsuneo A. Takahashi[1], Kenzoburo Tani[2], Naohide Yamashita[8], Tatsutoshi Nakahata[5], and Shigetaka Asano[2]:[8]Department of Advanced Medical Science, The Institute of Medical Science, The University of Tokyo**

Cell therapy including stem cell transplantation and gene therapy being ultimate therapeutic approaches for incurable diseases, their establishments are urgently needed. It is also mandatory to separate and manipulate cells under quality-controlled sterilized circumstances that can meet with GMP approvals, and provide powerfully enegineered cells to clinical settings. For this purpose, the center having clean rooms (Room for Clinical Cellular Technology:RCCT) with clinical P2 and P3 facilities is now operating in the Institute. The banking of cord blood cells for cord blood transplantation, the inser-

tion of GM-CSF gene to renal tumor cells using retrovirus vector for gene therapy, and the generation of antigen-pulsed denderitic cells against malignant melanoma cells are on going in RCCT.

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# *Research Hospital*
# Surgical Center

*To improve quality of surgical patients and those suffering from severe diseases or pain is our main purpose. Our research is directed to 1) investigate the interaction of various mechanisms of pain and develop new analgesics, and 2) improvement of the quality of the blood products, especially MAP red blood cell concentrates. In addition, we support some investigation and clinical works in other instututions.*

## 1. Analgesic interaction between intrathecal clonidine and glutamate receptor antagonists on thermal and formalin induced pain in rats

**Tomoki Nishiyama, Laszlo Gyermek, Chingmuh Lee, Sachiko Kawasaki-Yatsugi, Tokio Yamaguchi, Kazuo Hanaoka**

Clonidine, an $\alpha_2$ adrenergic receptor agonist inhibits glutamate release from the spinal cord. In the present study, the interaction of intrathecally administered clonidine and glutamate receptor antagonists on acute thermal or formalin induced nociception was studied. Sprague-Dawley rats with lumbar intrathecal catheters were tested for their tail withdrawal response by the tail flick test and paw flinches produced by formalin injection after intrathecal administration of saline, clonidine, AP-5 (a N-methyl-D-aspartate (NMDA) receptor antagonist), or YM872 (anα-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) receptor antagonist). The combinations of clonidine and the other two agents were also tested by isobolographic analyses. Motor disturbance and behavioral changes were observed as side effects. The $ED_{50}$ values of clonidine decreased from 0.26μg (tail flick), 0.12μg (phase 1) and 0.13μg (phase 2) to 0.036μg, 0.006μg, and 0.013μg with AP-5, and 0.039μg, 0.057μg, and 0.133μg with YM 872, respectively. Side effects were attenuated in both combinations. In conclusion, spinally administered clonidine and AP-5 or YM 872 exhibited potent synergistic analgesia on acute thermal and formalin induced nociception with decreased side effects in rats. These results may lead to a new clinical approach for the management of acute and inflammatory pain.

## 2. Synergistic analgesic effects of intrathecal midazolam and NMDA or AMPA receptor antagonists in rats

**Tomoki Nishiyama, Laszlo Gyermek, Chingmuh Lee, Sachiko Kawasaki-Yatsugi, Tokio Yamaguchi**

The aim of the study was to investigate the interaction of midazolam and N-methyl-D-aspartate (NMDA) receptor or α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) receptor antagonist in the effects on persistent inflammatory nociceptive activation. Male Sprague-Dawley rats were implanted with lumbar intrathecal catheters and were tested for their responses to subcutaneous formalin injection into the hindpaw. Saline, midazolam (1 to 100μg), AP-5 (1 to 30μg), a NMDA receptor antagonist, or YM872 (0.3 to 30μg), an AMPA receptor antagonist was injected intrathecally 10 minutes before formalin injection. The combinations of midazolam and AP-5 or YM 872 in a constant dose ratio based on the 50% effective dose ($ED_{50}$) were also tested and were analyzed with an isobologram. Dose-dependent effects were observed with midazolam ($ED_{50}$ was 1.34μg and 1.21μg in phase 1 and 2 of the formalin test, respectively.), AP-5 (7.64μg and 1.4μg) and YM872 (0.24μg and 0.21μg). Significant synergistic effects in both phases were obtained when combining midazolam with AP-5 or YM872. The $ED_{50}$ of midazolam decreased to 0.012μg (phase 1) and 0.27μg (phase 2) with AP-5 and to 0.09μg (phase 1) and 0.35μg (phase 2) with YM 872 ($P<0.01$). These results suggest a functional coupling

of benzodiazepine-γ-aminobutyric acid $(GABA)_A$ receptor with NMDA and AMPA receptors in acute and persistent inflammatory nociceptive mechanisms in the spinal cord.

### 3. Synergistic interaction between midazolam and clonidine in spinally mediated analgesia in two different pain models of rats

**Tomoki Nishiyama, Kazuo Hanaoka**

Both midazolam, a benzodiazepine-γ-aminobutyric acid $(GABA)_A$ receptor complex agonist, and clonidine, an $\alpha_2$ adrenergic receptor agonist, are known to have spinally mediated analgesic effects. The aim of the present study was to investigate the analgesic interaction of spinally administered midazolam and clonidine in their effects on acute and inflammatory nociceptive stimulation. Rats implanted with lumbar intrathecal catheters were injected intrathecally with saline (control), midazolam (1 to 100μg), or clonidine (0.1 to 3μg) to test for their responses to thermal stimulation to the tail (tail flick test) and subcutaneous formalin injection into the hind paw (formalin test). The effects of the combination of midazolam and clonidine on both stimuli were tested by isobolographic analysis using the 50% effective doses ($ED_{50}$s). The general behavior, motor function, pinna reflex, and corneal reflex were also examined as side effects. When combined, the ED50s of midazolam (clonidine) decreased from 1.57μg (0.26μg) to 0.29μg (0.05μg) in the tail flick test, and from 1.34μg (0.12μg) and 1.21μg (0.13μg) to 0.05μg (0.005μg) and 0.13μg (0.015μg) in the phase 1 and 2 of the formalin test, respectively. Side effects did not increase by the combination. These results suggest a favorable combination of intrathecal midazolam and clonidine in the management of acute and inflammatory pain.

### 4. Hemolysis in stored red blood cell concentrates: Modulation by haptoglobin or ulinastatin, a protease inhibitor

**Tomoki Nishiyama, Kazuo Hanaoka**

Polymorphonuclear leukocyte elastase (PMNE) may injure various tissues. The release of PMNE induced by various stimuli was reported to be inhibited by a protease inhibitor, ulinastatin. In stored blood preparations, PMNE increases depending on the storage days as hemolysis increases. We hypothesized that PMNE might be one of the factors inducing hemolysis in stored blood. Haptoglobin binds to free hemoglobin to reduce hemolysis. The purpose of the study was to investigate the effects of ulinastatin on hemolysis in blood preparations in comparison with haptoglobin. Nine, two-day-old packs of red blood cell concentrates (CRC) in mannitol, adenine, glucose, phosphate and citrate (MAP) (MAP-CRC) of 400 mL were obtained from the Japan Red Cross Society. Each MAP-CRC was divided into three different packs of equal amount and treated either with 10 mL of saline (control group), 200 units of haptoglobin, or 50,000 units of ulinastatin. They were stored at 4°C. Supernatant concentrations of total and free hemoglobin, total haptoglobin, PMNE and potassium were measured for 25 days. Free haptoglobin concentration was calculated. Total and free hemoglobin concentrations increased significantly dependent on the storage days in the control group while haptoglobin and ulinastatin groups showed no increase. Total and free haptoglobin concentrations were significantly higher in the haptoglobin group than in the other two groups. Free haptoglobin concentrations were 0 after five days of storage in the control and ulinastatin groups. PMNE concentrations increased with the increase in storage days without any differences among the three groups. Potassium concentration increased according to the storage and showed the highest value in the control group. Adding haptoglobin or ulinastatin to MAP-CRC was useful to suppress hemolysis during storage of the preparation. The PMNE might not be involved in the mechanisms of hemolysis in MAP-CRC stored for 25 days.

### 5. Do the effects of a protease inhibitor, ulinastatin on elastase release by blood transfusion depend on IL-6?

**Tomoki Nishiyama, Kazuo Hanaoka**

Blood transfusion induces polymorphonuclear leukocyte elastase (PMNE) and interleulin-6 (IL-6). IL-6 would activate neutrophils to release PMNE. Ulinastatin, a protease inhibitor inhibits PMNE release by blood transfusion. The purpose of this study was to investigate whether the effects of ulinastatin on PMNE release by blood transfusion come through inhibition of IL-6. Patients age 35 to 70 undergoing gastrectomy were enrolled in this study until the following four groups had 12 patients each. Half of the enrolled patients received ulinastatin at random. After surgery patients were divided into four groups; group A received neither blood transfusion nor ulinastatin, group B received only blood transfusion, group C received only ulinastatin, and group D received both blood transfusion and ulinastatin. The infusion of ulinastatin 300,000 units was started at manipulation of the stomach in the group C and at the start of blood transfusion in the group D. Segmented neutrophil count, plasma concentrations of PMNE and IL-6 were measured. In addition, PMNE and IL-6 concentrations in every unit of concentrated red blood cell transfused and these concentrations in the plasma of the recipient after every unit of transfusion were measured. Blood transfusion increased

plasma concentrations of PMNE and IL-6, and the PMNE release from segmented neutrophil. The increase of plasma PMNE but not IL-6 concentration after each unit of blood transfusion was inhibited by ulinastatin. However, ulinastatin did not inhibit the increase of plasma concentrations of PMNE and IL-6 by surgical stimuli of gastrectomy. Ulinastatin 300,000 units might be useful to inhibit blood transfusion-induced increase of PMNE but not Il-6. The inhibition of PMNE increase by ulinastatin was independent of IL-6.

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# Laboratory Animal Research Center

*Morbilliviruses in the family Paramyxoviridae including canine distemper virus, rinderpest virus and measles virus are highly infectious among their natural hosts. We have succeeded in establishing a system of reverse genetics for these three morbilliviruses, using originally isolated strains. Studies on the functions of viral proteins in replication, pathogenicity, and species-specificities have been performed. We have also examined the effect of full-length Hepatitis C virus genome expression on cell growth regulation. In addition, more than 30,000 mice, mainly transgenic and gene-targeted ones, are always kept for the research of IMSUT and the technical staff members contribute to their maintenance and breeding.*

## 1. Development of reverse genetics of morbilliviruses

**Chieko Kai, Ryuichi Miura, Kentaro Fujita, Fusako Shimizu, Hiroki Sato, Masahi Uema, and Misako Yoneda**

The genus morbillivirus, in the *Paramyxoviridae* family, includes 7 viruses; measles virus (MV), rinderpest virus (RPV), canine distemper virus (CDV), peste des petits ruminants virus, and three aquatic mammalian viruses. Morbilliviruses are highly contagious and are considered one of the most important pathogens in each host animal. For a decade beginning in the late 1980s, serious epidemics with high mortality rates occurred among both seal and lion populations. These outbreaks were attributed to infection with CDV, although large felids had not previously been considered susceptible to CDV. Thus, morbilliviruses are good models for analyses of the mechanisms of pathogenicity and cross-species infection for mononegavirales. Development of rescue systems of nonsegmented single and negative-strand viruses (*mononegavirales*) since 1994 have opened vast new fields of analysis for a wide range of previously inaccessible areas of these viruses.

Recently the incidence of canine distemper in both unvaccinated and vaccinated dogs has increased in Japan. We isolated prevalent viruses from affected dogs and determined that they were distinct from vaccine strains and closely related to recently prevailing viruses in dogs and wildlife according to phylogenetic analysis. Using one of the viruses, the Yanaka strain of CDV, we have successfully developed a reverse genetics system. The cDNA clone of the full genome of CDV contains restriction enzyme sites between viral protein genes. This system offers a powerful tool not only for the analysis of fundamental aspects of viral replication but also for the development of new attenuated, proliferation-defective and polyvalent vaccines.

We established an excellent animal model for RPV infections using rabbits, which exhibit natural symptoms to experimental peripheral infection. To investigate the pathogenicity of RPV, we isolated ten virus clones from homogenates of infected rabbit lymph nodes by plaque cloning in B95a cells. Two virus clones were highly virulent in experimentally inoculated rabbits and one was avirulent. The entire nucleotide sequence of the most virulent clone virus (RPV-Lv) was determined and an infectious cDNA clone was constructed. We successfully rescued the virus using the reverse genetics system from the infectious cDNA clone, and confirmed that it exhibited the same pathogenicity as the parental virus in rabbits and appeared to grow in identical tissue cultures to the original virus. This system represents an excellent tool for the investigation of RPV pathogenicity.

In addition, we established a monkey model for MV. This was the first model to demonstrate measles rash with other natural symptoms such as immunosuppression following experimental infection with MV isolated from affected humans. A reverse genetics system has been successfully developed using a field isolate, the HL strain. This could offer another powerful tool for the investigation of the mechanisms of immunosuppression and for the development of polyvalent vaccines for significant human diseases, after the reduction of virulence by genetic engineering. The recombinant MV will also offer a strong potential for the development of novel anti-tumor therapies in human.

## 2. Host range of CDV

**Kentaro Fujita, Ryuichi Miura, Misako Yoneda, Masashi Uema, Toshiya Nishi, Ken-ichi Togashi, Yasuyuki Endo, Kyoko Tsukiyama-Kohara, and Chieko Kai**

We have applied an innovative reverse genetics system to recover recombinant CDV expressing marker genes. Using this system, we constructed recombinant CDVs expressing enhanced green fluorescent protein (EGFP) (CDV-EGFP) and firefly luciferase (CDV-Luc). Recombinant CDVs were successfully recovered and expression of exogenous genes was verified by confocal microscopy and measurement of luciferase activity in infected cells. Growth kinetics of recombinant CDVs were slightly slower than in the parental virus. Using these viruses, we examined the susceptibility of various cell lines to CDV infection. The results demonstrated that these recombinant viruses can infect a broad range of cells.

CDV reportedly utilizes SLAM as a receptor in the same manner as MV. However, CDV should have receptor(s) other than SLAM, as CDV displays a wide host range, whereas SLAM is expressed only in lymphoid tissues. One possible candidate for implying the process of virus entry might be glycosaminoglycan, such as heparan sulfate. We treated 293 cells with heparin, chondroitin A or chondroitin B prior to inoculation. CDV infection was inhibited by heparin and weakly inhibited by chondroitin B, but was not inhibited at all by chondroitin A. Direct binding of CDV-EGFP to heparin was demonstrated by heparin agarose chromatography using purified CDV-EGFP. Thus, CDV possesses an intrinsic capacity to use more than one receptor and heparin-like glycosaminoglycan(s) may be involved in virus entry.

## 3. Mechanism of the persistent infection of CDV

**Toshiya Nishi, Ryuichi Miura, Motohiro Shiotani, Chiaki Wakasa, Kyoko Tsukiyama-Kohara and Chieko Kai**

The Yanaka strain, a field isolate of CDV, causes extensive cytopathic effect (CPE) followed by cell death. However, the Yanaka-BP, derived from the Yanaka strain of CDV can grow persistently in B95a cells but scarcely show syncytium formation. To analyze the mechanisms of persistence, we first investigate the functional deficiency of fusion activity of the persistent strain. After inoculation of the Yanaka and Yanaka-BP strain at an equal titer, the transcription of viral proteins of the Yanaka-BP strain was revealed to be slower than that of the Yanaka strain. The function of the membrane proteins, the fusion (F) and hemagglutinin (H) protein of the Yanaka-BP strain did not lose its fusion activity. However, the fusion formation was negatively regulated by co-expression of the matrix (M) protein with these glycoproteins. Thus, the functional alternation of the M protein is considered to implicate the functional defectiveness of fusogenicity of the Yanaka-BP persistent strain.

## 4. Host specificity and pathogenicity of Rinderpest virus

**Misako Yoneda, Ryuichi Miura, Kentaro Fujita, Motohiro Shiotani, Fusako Shimizu, Athipoo Nuntaprasert, Masahi Uema Michae D. Baron[1], Thomas Barrett[1] and Chieko Kai:[1]National Institute for Animal Health, UK**

RPV-L strain is highly pathogenic in rabbits, causing marked lymphoid lesions and severe immunosuppression similar to the results of RPV infection in cattle. Rabbits experimentally infected with RPV-L strain are thus considered a useful model for studying the pathogenicity of RPV. Recently, a reverse genetics system for recovering infectious RPV-RBOK (vaccine strain for cattle) has been established. Rabbits inoculated with the RBOK strain do not demonstrate any pathogenic signs. To examine the role of H protein, a viral surface glycoprotein, in host specificity and pathogenicity of RPV, we attempted to rescue recombinant RPV-RBOK. The H protein of the recovered virus was replaced with that of the RPV-L strain, and the virus was designated rRPV-lapH. The three strains of viruses, RPV-RBOK, RPV-lapH and RPV-L, were intravenously inoculated into rabbits. RPV-L caused clinical signs including pyrexia, leucopenia and decrease in body weight gain, whereas rabbits inoculated with RPV-RBOK or rRPV-lapH remained clinically normal. High titers of virus and histopathological lesions in the lymphoid tissues were observed only in rabbits inoculated with the RPV-L strain. Rabbits inoculated with rRPV-lapH were shown to have produced measurable titers of anti-RPV antibodies. These results indicate that the H protein plays a key role in allowing infection of

rabbits by rRPV-lapH, but does not determine pathogenicity in this species.

## 5. Development of polyvalent CDV vaccines

**Ryuichi Miura, Miho Ejima, Akiko Takenaka, Yasuyuki Endo, Yoshitsugu Matsumoto[1] and Chieko Kai:[1]Laboratory of Veterinary Applied Immunology, The University of Tokyo**

Using a reverse genetics system for CDV, we attempted to develop a polyvalent CDV vaccine against CDV and *Leishmania* infection. Leishmaniasis is one of the most serious parasitic zoonoses transmitted to humans from domestic dogs or rodents via infected sand flies. Effective vaccination of dogs against Leishmaniasis is expected to disrupt the infection cycle and thus prevent the human manifestation of the disease. To construct a recombinant CDV expressing a *Leishmania* antigen, LACK (*Leishmania* homologue of the receptor for activated C kinase), a full-length LACK cDNA was inserted between the *N* and *P* genes in the infectious cDNA clone of the Yanaka strain of CDV. We successfully rescued the virus, CDV-LACK, which was confirmed to express LACK mRNA and protein in B95a cells. Four 1-month-old beagle dogs were inoculated with CDV-LACK subcutaneously. All dogs inoculated with CDV-LACK were demonstrated to have produced high titers of antibodies against CDV at 14 days post inoculation (dpi), whereas no detectable titers of anti-LACK antibodies were observed for 8 weeks. CDV-LACK-immunized and control dogs were challenged with lethal doses of virulent CDV strain at 21 dpi. The CDV-LACK-immunized dogs demonstrated no observable clinical signs of infection, whereas unimmunized dogs developed severe symptoms, including pyrexia, leukopenia and weight loss. Different CDV-LACK-immunized and control dogs were challenged with infective-stage promastigotes of *L. major* intradermally in the ears, noses and interdigits at 56 dpi. Nodules in the ears and noses of control dogs appeared at 14 days after the challenge and continued to enlarge. CDV-LACK-immunized dogs demonstrated smaller nodules than unimmunized dogs up to 42 days after the challenge. These results indicate that vaccination with CDV-LACK induced significant protective immunity against CDV infection and effectively suppressed the proliferation of *Leishmania* at the early stage of infection.

## 6. Efficacy of recombinant cytokines expressed by a Sendai virus vector

**Yasuyuki Endo, Kentaro Fujita, Atsushi Katoh[1], Ryuichi Miura, Misako Yoneda, Chiaki Wakasa, Masashi Uema, Motohiro Shiotani, Toshiya Nishi, and Chieko Kai:[1]National Institute of Infectious Diseases**

As Sendai virus (SeV) can replicate well in a broad range of cells and produce large quantities of viral proteins with natural glycosylation, genetic engineering has allowed SeV to become a useful vector for recombinant protein synthesis. We constructed recombinant SeV (rSeV) containing canine cytokine-genes, interferon (IFN)-γ, granulocyte-macrophage colony stimulating factor (GM-CSF) and interleukin (IL)-6, and characterized the efficacy.

***IFN-γ*** Recombinant canine (rc) IFN-γ was successfully produced in CV-1 cells and embryonated chicken eggs infected with the rSeV. The rcIFN-γ was expressed as approximately 17, 20 and 25 kDa glycoproteins in eggs, in the same manner as human IFN-γ, whereas 17, 19, 20, 22, 25 and 27 kDa glycoproteins were expressed in CV-1 cells. Glycosidase treatment of rcIFN-γ produced in CV-1 cells revealed that there are two non-glycosylated variants. The rcIFN-γ derived from both CV-1 and chicken eggs enhanced the expression of cell surface MHC class II antigens on MDCK cells. In addition, both rcIFN-γ displayed anti-viral activities and rcIFN-γ produced in CV-1 cells demonstrated much higher inhibitory activity than that from chicken eggs. These results indicate that the rcIFN-γ possesses functional activity and will be an effective immunotherapeutic agent for malignancies and infectious diseases.

***GM-CSF*** Approximately 10 μg/mL rcGM-CSF was produced in allantoic fluid inoculated with rSeV-GM-CSF. The purified rcGM-CSF induced the proliferation of TF-1 cells (in which growth is dependent on GM-CSF) and bone marrow cells in a dose-dependent manner. The administration of rcGM-CSF to healthy dogs subcutaneously at the dosages of 10, 20 or 40 μg/kg/day for 7 days induced increases of total white blood cells, neutrophils and monocytes in the dogs. These results suggest that rcGM-CSF possesses adequate biological activity both *in vitro* and *in vivo* and is applicable for clinical use.

***IL-6*** Expression of rcIL-6 was observed in embryonated chicken eggs infected with rSeV. The rcIL-6 was recognized as an approximately 28 kDa protein, and stimulated the proliferation of 7TD1 murine hybridoma cells (in which growth is dependent on IL-6) in a dose-dependent manner. Therefore, the rcIL-6 is biologically functional, and will prove useful in the development of new vaccine strategies.

## 7 A CDV epidemic in wildlife in Japan

**Kenjiro Ohashi, Kyoko Hirama Masashi Uema, Yoko Goto and Chieko Kai**

All members of the Canidae and Mustelidas families and certain members of the Procyonidea family are susceptible to CDV infection. Moreover, CDV has

recently been isolated from wild animals including large cats and aquatic mammals, which had not been known to be susceptible to CDV previously. To understand the relation of canine distemper in domestic dogs and that in wildlife in Japan, the distribution of CDV infection in raccoon dogs was surveyed seroepidemiologically. Antibodies against CDV were detected by ELISA and virus neutralization tests in 75 blood samples collected from raccoon dogs from 1982 to 1998 in Japan. The genetic and serological analyses of a newly isolated CDV from a raccoon dog revealed that there are only four mutated portions in the amino acid sequence of the H protein and one mutated antigenic epitope, indicating that the virus spreading among Japanese raccoon dogs belongs to the same group as the CDV strains causing recent outbreaks of canine distemper in dogs.

### 9. Modification of cell growth regulation by Hepatitis C virus

**Kyoko Tsukiyama-Kohara, Kohsuke Izumi, Ying Huang, Takahiro Seki, Michinori Kohara[1] and Chieko Kai:[1]The Tokyo Metropolitan Institute of Medical Science**

Hepatitis C virus (HCV) is classified as family Flaviviridae and has a single-stranded RNA genome of positive polarity (9.4 kb). HCV causes persistent infection in hepatocytes, and this infection in turn is strongly associated with the development of hepatocellular carcinoma. To clarify the mechanisms underlying these effects, we established a *Cre/loxP* conditional expression system of the precisely self-trimmed HCV genome in human liver cells. Passage of hepatocytes expressing replicable full-length HCV RNA caused upregulation of anchorage independent growth and tumorigenicity after 44 days. In contrast, neither HCV structure nor non-structure protein expressing hepatocytes were upregulated in tumorigenicity. After the characterization of the modified regulatory pathway, activation of the cdk-Rb-E2F pathway was only observed in the full length HCV expressing cells and not activated in the structure nor non-structure HCV protein expressing cells. The detailed mechanism is now under investigation.

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# Laboratory of Molecular Genetics

*This laboratory has two main activities: developing efficient expression vectors for gene therapy and supporting the researchers by advising on recombinant DNA technology under the safety guideline.*

**Saki Kondo, Aya Okuda[1], Hiromi Sato[2], Miho Terashima, Yumi Kanegae and Izumu Saito: [1]School of Sciences and [2]School of Pharmaceutical Sciences, Kitasato University**

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 under the safety guideline. 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 gene therapy. We are maintaining more than 50 collaborations within and outside of this institute. In these collaborations, we offer and supply our efficient method (COS-TPC method: Miyake *et al.*, Proc. Natl. Acad. Sci. USA, 93:1320-1324, 1996) to construct recombinant adenoviruses expressing various genes efficiently. Nine years ago, we constructed 44 recombinant adenovirus for 14 months using this method; this number was more than double constructed in the world per year at that time. More recently we have developed a method for ON/OFF switching of gene expression in mammalian cells using a combination of Cre/*loxP* system and adenovirus vector (Kanegae *et al.* Nucleic Acids Res. 23:3816-3821, 1995; Kanegae *et al.* Gene 181:207-212, 1996). The method will promote many fields of molecular biology and medicine and may open a new field of "intracellular gene manipulation". The research activities in 2001 were shown below.

## 1. Simultaneous regulation of gene expression in transgenes located on mammalian cell chromosome using adenovirus expressing Cre recombinase

Regulation of gene expression of a transgene located on a chromosome of mammalian cells can be achieved using site-specific recombination mediated by Cre recombinase. We have reported that a transgene on a cell chromosome can be switched on or switched off by infecting Cre-expressing recombinant adenovirus (rAd), which serves as a "molecular switch" (Kanegae *et al.* Gene 181:207-212, 1996). Although this method works efficiently and has been used worldwide, it allows switching only a single gene; two or more genes cannot be regulated using this method because only a single target sequence of Cre recombinase called *loxP* is available to date. We recently identified *loxP* mutants, *loxP* V and *loxP* S, each of which recombines efficiently with identical mutant *loxP* but not with wild-type *loxP* (denoted as L) using *in vitro* recombination assay. Utilizing mutant *loxP* V (denoted as V), we here examined whether simultaneous regulation of gene expression in transgenes introduced on a cell chromosome. We constructed a transgene containing EF1α promoter - V - GFP cDNA - V - polyadenylation signal; the GFP expression will be turned off through Cre-mediated V-V recombination and excision of GFP cDNA. The transgene (called V unit) was connected with previously-reported CALNLZ transgene (called L unit), which expresses neo-resistance gene and LacZ gene before and after Cre-mediated recombination, respectively. The resultant plasmid was transfected to

CV1 cells and four independent cell lines containing intact transgenes were identified. In all four cell lines, GFP gene expression was turned off and LacZ expression was switched on after infection of a Cre-expressing adenovirus AxCANCre. Southern blot analysis showed that both V-V and L-L recombinations occurred completely after AxCANCre infection at multiplicity of infection (MOI) of 3. Although efficiency of V-V recombination is slightly lower than that of L-L recombination in our *in vitro* assay, we cannot detect any partial recombination product having a deletion only in L unit but retaining V unit intact, even after the infection at very low MOI. The result suggests that off-regulation of V unit and on-regulation of L unit simultaneously occured in there cells when using AxCANCre. The undesired V-L recombination was not detected, or less than in one cell per 1,000, by PCR analysis. Therefore, these results showed that successful simultaneous regulation of gene expression can be done using mutant *loxP* V and AxCANCre. The strategy is probably useful for identifying a cascade of signal transduction, gene function in developmental process and conditional transgenic/knockout analyses in animals.

## 2. Identification of viral gene responsible for immunogenicity of adenovirus vector

**Michio Nakai[3], Yumi Kanegae and Izumu Saito: [3]Sumitomo Pharmaceuticals Research Center, Osaka**

The adenovirus vector of E1-substituted type, so called first generation adenovirus vector, is widely used in various basic research and gene therapy. One important problem of this vector is immunogenicity when administrated in animals but the viral gene(s) responsible for the immunogenicity is not fully understood. We first found that major immunogenicity, which causes liver inflammation two weeks after administration to mice, was observed only when a foreign promoter sequence was inserted into the vector at its E1 region and even when no foreign gene downstream of the promoter was present. Based on highly-sensitive northern analysis throughout the viral genome, we found that the gene specifically activated by the inserted foreign promoter is viral polypeptide nine (pIX) gene, which is located immediately downstream of the E1 region. The expression level of pIX RNA was specifically elevated when expression unit of foreign gene under the control of promoters of CAG or SRα was inserted, and these viruses caused elevation of ALT level showing liver inflammation. In contrast, the pIX RNA level was not elevated when EF1α promoter was used. Moreover, adenovirus vectors expressing LacZ gene under the control of EF1α promoter do not cause clear elevation of ALT level after administration. These results suggest that pIX gene activated by inserted foreign promoter may be responsible for strong immunogenicity and that use of EF1α promoter for expressing a foreign gene may skip out the immunogenicity.

## 3. Development of a new method for constructing helper-dependent adenovirus vectors using double-reciprocal recombination mediated by Cre recombinase

**Yumi Kanegae, Saki Kondo, Chitose Wakai[2], and Izumu Saito**

Although the first-generation adenovirus vector (E1-substitution type) has been extensively used in various fields of basic research and for gene therapy, the next-generation adenovirus vector has also been reported. The new adenovirus vector, called "gutted vector", is devoid of all the viral genes but retains only about 0.5-kb of the left terminal and 0.2-kb of the right terminal of the viral genome. These terminal regions of the virus genome contain viral replication origins and the signal for packaging into the viral capsid. The gutted vector does not express any viral gene products and is consequently expected to cause only minimum immune reaction against its host and to achieve prolonged gene expression. However, because the gutted vector require helper virus, which supply all the viral gene products *in trans*, the method for construction and production of gutted vector is still complex and can be managed by only a limited number of groups in the world.

We have developed a new method for constructing first-generation adenovirus vector using double-reciprocal recombination mediated by Cre/*loxP* system. We have reported a mutant *loxP* V, which contains two certain transversion mutations in its 34-nucleotide sequences. The mutant *loxP* recombines efficiently with the identical mutant *loxP* but not with wild-type *loxP* (Lee and Saito, Gene, 216:55-65, 1998). A pair of DNA sequences flanked with wild-type *loxP* and mutant *loxP* V can efficiently be exchanged through double-reciprocal recombination mediated by Cre recombinase. We constructed a parent virus, called a recipient virus, which contains an inserted gene at the E1 region flanked with wild-type *loxP* and mutant *loxP* V. In addition, the packaging signal of the virus is flanked with a pair of wild-type *loxP*. We also constructed a donor plasmid containing viral packaging signal and a gene to be transferred onto the adenovirus genome, both of which were flanked as a unit with wild-type *loxP* and mutant *loxP* V. A 293 cell line constitutively expressing Cre recombinase was transfected with a donor plasmid containing a marker gene and then infected with the recipient virus. Some of the recipient viruses lacking their packaging signal by Cre-mediated excision restored the signal together with the marker gene from the donor plasmid through the double-reciprocal recombination. The resulting marker

gene-containing recombinant adenovirus became the major population of the virus stock after five cycles of serial passages through a Cre-expressing 293 cell line. Therefore, this method works for construction of the first-generation adenovirus vector. Using the same strategy, we are currently trying to construct gutted vector. We constructed a recipient virus containing mutant *loxP* V at 0.2 kb downstream from the right end of the genome. We also constructed a donor cosmid where 28 kb DNA containing expression units is flanked with wild-type *loxP* and mutant *loxP* V. Transfection of the new recipient virus and the donor cosmid into Cre-expressing 293 cells and subsequent serial passages generated virus containing a marker gene derived from donor cosmid. Further investigation is under way.

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# Amami Laboratory of Injurious Animals

*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 Habu control

**Shosaku Hattori, Yoshihisa Noboru, Hiroshi Kihara[1], Motonori Ohno, Daisuke Tsuru[2], Shigenari Terada[3], Hiro Yonezawa[4], Yoshihiro Hayashi[5], Michihisa Toriba[6], Hideki Endo[7], and Tomohisa Ogawa[8]:[1]Bioscience Research Institute, Takara Shuzo Co., Ltd., [2]Departmen of Applied Microbiology, Kumamoto Institute of Technology, [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]Department of Zoology, National Science Museum, [8]Faculty of Agriculture, Tohoku University**

Snake bites by the venomous snake Habu, *Trimeresurus flavoviridis*, have been reported annually about 100 cases in the population of 100,000 in the Amami Islands. Moreover, there is no indication that the population of 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 Habu survived the injection of Habu venom since early times. Because, some proteins in the serum of Habu blood combine to the elements of Habu venom. The research of these binding proteins has been initiated with an objective of clinical trials. Phospholipase A2 and its isozymes isolated from Habu venom have myonecrotic activity and hemorrhagic activity, and T2 protease has hemorahagic activity. The binding proteins isolated from serum of Habu inhibit myonecrotic activity of phospholipase A2 and its isozymes. We found that protein-HSF and peptide-AHP isolated from the Habu serum effectively control the hemorrhage caused by Habu, *Ovophis okinavensis*, *Agkistrodon blomhoffi brevicaudus*, *Calloselasma rhodostoma*, *Bitis arietans*, *Bothrops asper*, and,*Trimeresurus stejnegeri* venoms.

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 Habu and the result of the questionnaire surveys for the inhabitant in the Amami-oshima Is-

land, the total population of the Habu which lives in this island was estimated at about 100,000. 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 Habu is expected to decrease in the near future.

These studies are supported by grants from the National Bureau of Land Development and the Kagoshima Prefectural Government.

## 2. Reproduction of squirrel monkeys

**Shosaku Hattori, Yoshihisa Noboru, Yoshitugu Matsumoto[9], Katakai Yuko[9], Mamoru Ito[10], and Takahisa, Furuta[11]:[9]Department of Molecular Immunology, Faculty of Agriculture, University of Tokyo, [10]Laboratory of Immunology, Central Institute of Experimental Animals, [11]Department of Parasitology**

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 5 newborns are given annually by 30 adult females.

Recently, primates came to be often used to experiments of parasites. Especially the primates in the South America are the important infectious models. We use the squirrel monkeys to basic experiments on the infection and vaccination models for malaria, toxoplasma, and schistosomiasis. It was proven that the squirrel monkey was excellent as experimental model animal of the malaria.

## 3. Research of wild mammals

**Shosaku Hattori, Yoshihisa Noboru, Hideki Endo[12], Kimiyuki Tsuchiya[13], Nobuo Ishii[14] and Fumio Yamada[15]:[12]Department of Zoology, National Science Museum, [13]Experimental Animal Center, Miyazaki Medical College, [14]Japan Wildlife Research Center, [15]Wildlife Ecology Laboratory, Forestry & Forest Products Research Institute**

Amami-oshima Island is a habitat of animals and plants indigenous to the Nansei Islands. These animals occur originally in the Oriental region of Asia and include the Amami rabbit, *Pentalagus furnessi*, the Ryukyu spiny rat, *Tokudaia osimensis*, the Okinawa long-haired rat, *Diplothrix legata*, the Watase's shrew, *Crocidura watasei*, and the Musk shrew, *Suncus murinus*. These mammals are used for researches on comparative anatomy, taxonomy, and development of experimental animals. Besides, these mammals are valuable species biologically as survivors from the Miocene about 10,000,000 years ago. We have initiated the investigation for these species to protect from extinction. We have documented the feasibility of recovering large numbers of oocytes from the Watase's shrew, and some of oocytes can be induced to mature *in vitro*.

Recently, the Java mongoose, *Herpetologica javanicus* grew in the wild as invasive carnivore in the Amami-oshima Island. The population of the mongoose increases every year and the habitat range is extending to south area in the Island. It is necessary to remove the invader to defend nature. Then we are investigating the influence which the mongoose gives to wildlife in the Island. Since hairs such as Amami rabbit, Ryukyu spiny rat, Akahige were confirmed from the excrement of the mongoose, the necessity of the urgent ridding countermeasure of the mongoose was indicated. From 2000, the capture project of the mongoose was started by Environment Agency in order to protect Amami-oshima's endemic species.

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