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 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 autologous or 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 cell transplantation has become strong weapon for patients sufferred from hematological malignancy without any related or unrelated stem cell donors. We have already 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. Cases Reports on Clinical Studies of Immunogene Therapy Using Autologous GM-CSF Transduced Tumor Vaccines(GVAX) for Stage IV Renal Cell Cancer

Kenzaburo Tani, Shigetaka Asano, et al.

There are no effective treatment for patients with Stage IV renal cell cancer (RCC). The introduction of new therapy is required. Vaccination using autologous GM-CSF transduced renal tumor cells (GVAX) is one of promising choices to overcome this situation according to preclinical animal studies. Our preliminary data showed that we could safely produce lethally irradiated autologous GM-CSF transduced RCC at our hands in our hospital. We report four Japanese patients suffered from Stage IV RCC with metastasis. We have so far done total of 43 vaccinations of GM-CSF transduced autologous RCC cells every two weeks for 3 patients. For our 4th patients, 4 vaccinations have been done so far. The safeties of these injections were confirmed besides minimum adverse events including grade II localized skin reactions including reddness, swelling and itching at the vaccinated sites, which spontaneously were resolved within 48 hours without special treatment. Serum GM-CSF levels were not increased after vaccination, but the number of eosinophils was significantly increased at 24-48 hours after vaccinations. One patient experienced grade I systemic adverse effects of low grade fever. Immunological studies showed the oligoclonal expansions of T cells in the peripheral blood as well as DTH sites and metastatic lesions after vaccinations in three patients using PCR-SSCP analysis of TCR Vb CDR 3 length. CTL activities to autologous RCC cells were demonstrat-

ed in all of the three patients but did not persist in the 1st patient sufferred originally from right RCC with multiple lung and liver metastases. His CTL activities decreased after 5th vaccination. Our 2nd patient, a 71 year old man who had right RCC with the large sacral metastasis, received 3.7x108 cells over 17 times. His sacral metastasis was kept stable for one year after the final vaccination. Our 3 rd patient, a 57 year old female who had left RCC with multiple liver, lung and contralateral kidney metastasis. She had been injected with $3.2x10^8$ cells over 15 times. Her main liver metastasis progressed, but both of the small lung and contralateral kidney metastasis was kept stable during the course of vaccinations for 7 months.

2. Cord blood transplantation for adult patients, including nuclear accident patient

Tohru Iseki, Jun Ooi, et al.

Although the number of Cord Blood Transplants (CBT) has been rapidly increasing, most of the reported cases are for children. Several reports from us and Europe described relatively high mortality rate of CBT patients as a result of infection. Low stem cell number resulted in delayed engraftment and restricted the use of cord blood in adults. The important clinical issues such as the patient eligibility, optimal cell doses and the proper regimen of conditioning and GVHD prophylaxis for adult patients have not been defined yet. We report our experience with CBT for adult patients.

Between August 1998 and May 2000, 15 adult patients with hematological malignancy received CBT from HLA mismatched unrelated donor at IMSUT. Median age of patients was 41 years (16-51), weight was 53kg (40-68) and number of nucleated cells infused was $2.25X10^7$/kg (1.2-4.1). All patients received the standard conditioning regimen of IMSUT according to disease status. All patients received cyclosporin A and 11 received short-term methotrexate (MTX) for GVHD prophylaxis. No patients received ATG. All cases received G-CSF administration after CBT.

Three patients died shortly after CBT (27-39 days), because of RRT (2) and infection (1). The patient with ALL received full TBI and cytoxan and developed autologous recovery. Among evaluable 11 patients, median time to neutrophil and platelet engraftment was 24.5 days (9-41) and 50 days (35-164, n=9), respectively which appears to be faster engraftment than previously reported, despite concomitant use of MTX. Three patients developed Grade II and one developed Grade III acute GVHD. Three patients died of relapse 107-307 days after CBT. Overall survival rate at 22 months was 40 ± 19% for all patients and 53 ± 23% for 11 patients of first transplantation. Survival rate of poor risk group (n=11) was low (33± 17%), but all four patients with standard risk are alive 70-270 days after CBT. Although the number of patients and periods of observation are insufficient, these results are comparable to that of standard bone marrow transplantation.

3. Cord blood transplantation for nuclear accident patient

Tohru Iseki, Jun Ooi, et al.

A 39-year-old male was systemically irradiated by nuclear processing accident in October 1999. Radiation dose was estimated around 8 gy equivalent. Severe and prompt decrease of lymphocyte count was observed and extremely hypocellular bone marrow without hematopoietic progenitor cells was ascertained by repeated examination from different sites. Although the possibility of uneven radiation exposure and subsequent autologous hematopoietic recovery was considered possible at that point, it was judged that a hematopoietic stem cell transplant was necessary. An HLA identical sibling donor was not available. The risk of GVHD, which could make organ damage worse over radiation damage, was considered less likely to occur with CBT. Further, mixed chimeric phase and late graft rejection after CBT can be obtained relatively easily compared to other stem cell sources. Therefore, we selected CBT as stem cell source. However, because hematological recovery after CBT is regarded to be slow in general, we decided to cope with this risk by strict prevention of infections and introduction of intensive supportive hematopoietic factors with TPO in addition to G-CSF. ATG, methylprednisolone and cyclosporin A were administered as conditioning and GVHD prophylaxis. To avoid additional damage to organs and residual autologous hematopoietic stem cell, if there is any, we used no cytotoxic agents such as cytoxan or MTX.

Recovery of neutrophils was observed on Day 16 after CBT followed by reticulocyte and platelet recovery. No significant infection was observed. Engraftment was ascertained by chromosome analysis on Day 9 after CBT. Serial FISH analysis revealed that mixed chimerism was established shortly after CBT, following which the ratio of graft gradually decreased and was absolutely rejected three months after CBT when autologous hematopoietic recovery was achieved. The patient died of multiple organ failure caused by radiation injury seven months after the accident. No clinical and pathological evidence of transplantation related toxicity and GVHD were noted through whole his clinical course. CBT was considered to be successful in the sense to rescue bone marrow aplasia without any adverse effect.

4. Establishment of Stem Cell Transplantation Nursing Network in JAPAN

Yuko Ogami, Japan Stem Cell Transplant Nursing Network

Nowadays, about 1,800 patients annually receive stem cell transplantation in Japan. As the number of patients as well as transplantation facility has been increasing rapidly here, it has become very important for nursing staff to catch up with the development of the new treatment system. To answer various requests by nurses working at the front in this field, we have established Stem cell Transplantation Nursing Network in Japan since 1997.

Now the Network consists of more than 200 members and the annual meeting is held at the time of Annual Meeting of The Japan Society of Stem Cell Transplantation. Our activities include the planning of nursing research, lectures and symposium to educate members, biannual publication of periodical, and the organization of annual meeting. Current research projects include the research protocols concerning how to grade the severity of oral mucositis and help patients overcome this difficulties according to the grade.

Transplant Nursing, highly specialized profession, requires very matured technique and knowledge to take care of patients. Such expert nurses should also strongly support the patient's family members and donor bank. Such issues are supposed to be educated in the Network. Our Network service would provide the place where every transplant nurse, not only members of this Network, can learn and freely communicate each other. We really hope our network would be helpful to improve more transplant nursing in Japan.

5. Comparative Review of Oncology Phase I Dose Escalation Designs of New Molecular Entities

Fumitaka Nagamura, Steven Hirschfeld , et al.

We surveyed the design and conduct of Phase I studies for new molecular entities (NME) submitted to the Division of Oncology Drug Products of the Center for Drug Evaluation and Research at the Food and Drug Administration (FDA) from 1986 to 1997. We identified eighty-two cytotoxic or cytostatic NME administered systemically as monotherapies. Eleven NME submissions that used pharmacokinetically guided dose escalation (PGDE) were evaluated, and 10 NMEs used modified continual reassessment method (mCRM) were evaluated. Only 7 of the 14 PGDE studies were able to implement the design. Of these, 6 used fewer patients than predicted using alternative designs and 5 were able to predict the Phase 2 dose. Sixteen studies of the proposed 21 mCRM studies were able to implement the design. Of these, 4 used more patients than predicted using alternative designs and only 1 was able to predict the Phase 2 dose. Based on this experience we conclude that PGDE can be alternative is challenging as implemented, while mCRM has not demonstrated.

6. Unrelated Cord Blood Transplantation for adult patients with MDS-related secondary acute myeloid leukemia

Jun Ooi, Tohru Iseki, et al.

Between August 1998 and June 2000, 6 adult patients with MDS-related secondary acute myeloid leukemia (sAML) were treated with total body irradiation, cytosine arabinoside (AraC) and cyclophosphamide followed by unrelated cord blood transplantation at the Institute of Medical Science, the University of Tokyo. Granulocyte colony-stimulating factor was infused continuously at a dose of 5 μg/kg/day, starting 12 hr before AraC therapy until the end of AraC therapy to increase anti-leukemia effect of AraC. The median recipient age was 41 years (range, 27 to 50 years), weight was 50 kg (range, 43 to 63 kg) and number of infused nucleated cells was 2.56 x 10^7/kg (range, 2.09 to 4.1 x 10^7/kg). Cyclosporine plus short-course methotrexate were used for GVHD prophylaxis for all patients. All patients had myeloid reconstitution and the median time to >0.5 x 10^9/L ANC was 24 days (range, 19 to 35 days). A self-sustained platelet count greater than 50 x 10^9/L was achieved in 5 patients at a median time of 49 days (range, 35 to 164 days). Acute GVHD appeared in 4 of 6 patients (grade I; n=2, grade II; n=1, grade III; n=1) and chronic GVHD in 3 of 4 evaluable patients. Four patients remain alive in CR. Two patients relapsed. Among 4 patients who remain alive in CR, 3 patients had not received remission induction therapy before transplantation. These preliminary results suggest that adult sAML patients without suitable related or unrelated donor should be considered for cord blood transplantation.

7. Establishment of a IL-6-independent myeloma cell line, IMS-MY1, from pleural effusion of a patient with multiple myeloma

Yasushi Soda, Arinobu Tojo, Kenzaburo Tani , et al.

A human myeloma cell line, IMS-MY1, was newly established from pleural effusion of a patient with advanced IgG-λ multiple myeloma. Mononuclear cells in pleural effusion (PEMC) of the patient were isolated by Ficoll-Hypaque centrifugation. These PEMC consisting of more than 99% of myeloma cells were cultured in RPMI1640 medium supplemented with 10% FBS and 10 ng/mL rhIL-6 and a half of media was replaced biweekly. Myeloma cells began growing without any support of adherent cells at day 80 after the start of culture and acquired IL-6-

independence at day 160. IMS-MY1 cells were positive for cytoplasmic IgG-λ immunoglobulin and secreted the same type of immunoglobulin into the culture media. IMS-MY1 cells and parental PEMC similarly showed immature phenotypic cell surface markers of CD38 (+), CD49e (-) and MPC-1 (-), but not CD45. All of CD10, CD56 and CD138 (syndecan-1) were also expressed in both cells. Chromosome analysis of IMS-MY1 showed tetraploidy with numerous abberations like parental cells. Double t(11;14)(q13;32) were found in all cells and *bcl-1-IgH* fusion gene was recognized by fluorescence in-situ hybridization, and the rearrangement of c-myc was also detected by Southern blot analysis. IFN-α induced cell death dose-dependently, but many cytokines, including IL-6, did not affect the cell growth. Secretion of immunoglobulin from IMS-MY1 cells, however, was increased by IL-6. PCR analysis for HHV-8/KSHV genome was positive, but not EB virus genome in IMS-MY1 cells. Both genomes were not detected in parental cells. These results suggested that aberrant fusion gene, not IL-6 stimulation, is rather important for the survival and the growth of IMS-MY1 cells. And the integrated HHV-8 genome might contribute to the progression of malignancy magnitude in this cell line. Further molecular analysis is required to prove the role of the integrated HHV-8 genome for the establishment of IMS-MY1 cells.

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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 creat and conduct clinical trials (Phase I and II) for patients at Research Hospital. We are preparing clinical phase I trial for cancer vaccine using gp100 derived peptides.

1. Summary of surgical treatments and examinations performed in 2000

Hideaki Tahara, Masazumi Eriguchi, Shinji Tomikawa, Takuya Tsunoda, Yasutaka Takeda, Iwao Yoshizaki, Yoshifumi Beck, Hironobu Yanagie, Takuya Takayama, Yasumasa Nonaka, Syogo Nakano, Hiroyuki Mushiake

Surgical operations have been performed in 128 cases under general anesthesia and spinal anesthesia. As shown in Table 1, major operations were performed in 70 patients with malignant diseases, and in 55 patients with benign diseases. Renal transplantation was performed in 3 patients.

Examinations performed in 2000 are as follows: angiography for 85 cases, including trans-arterial embolization and trans-arterial chemotherapy, gastroduodenal endoscopy for 506 cases, and colorectal endoscopy for 166 cases.

2. Clinical phase I trial for Cancer Vaccine using gp100 derived peptides restricted HLA-A*0201 and -A*2402 against the advanced malignant melanoma

Takuya Tsunoda, Hideaki Tahara, Yoshifumi Beck, Yushiyuki Mushiake, Toshiyuki Baba, Shogo Nakano

Table 1. Major Operations Performed in 2000

Malignant Diseases		Benign Diseases	
Cancer of the stomach	10	Cholelithiasis	14
Cancer of the colo-rectum	27	Inguinal hernia	6
Cancer of the liver	4	Miscellaneous	35
Cancer of the bile duct	3	Total	55
Cancer of the pancreas	5	Renal transplantation	
Cancer of the kidney	2	Living	1
Cancer of the breast	14	Cadaveric	2
Miscellaneous	5	Total	3
Total	70		

Melanoma associated antigen, gp100, derived epitope peptides are used for the cancer vaccine against malignant melanoma. In the restriction for HLA-A*0201, the wild type gp100 derived peptide (ITDQVPFSV) and mutated gp100 peptide (IMDQVPFSV) are used, whereas in the restriction for HLA-A*2402, the newly mapped gp100 derived peptide (VYFFLPDHL) is used in this clinical trial. As the adjuvant, GMP grade IFA is also utilized in order to augment for anti-tumor immunity. To analyze the immunoresponse from the vaccinated patients, HLA-Tetramer is prepared for this purpose. Our goal in this clinical trial is to examine its safety, adverse effect and the immunoresponse. The unique points of this clinical trail are that 1) wild type gp100 peptide and mutate gp100 peptide are synchronously injected and 2) the newly mapped gp100 peptide restricted HLA-A*2402 is injected. We also investigate the clinical effects by this cancer vaccine.

3. Determination of chemosensitivity for colorectal cancer using macroarray

Takuya Tsunoda[1], Kazuto Nishio[2], Hiroshi Tanimura[3]:[1]Department of Surgery and Bioengineering (IMSUT), [2]Pharmacology division, National Cancer Center Research Institute and [3]Second Department of Surgery, Wakayama Medical School

It is essential for promote the clinical efficacy to predict the chemosensitivity for the solid tumor especially the colorectal cancer. MTT assay has been performed for this purpose from the freshly isolated tumor. We have successfully performed the MTT assay even the freshly isolated colorectal cancer, which has potentially the contaminations. Furthermore, MTT assay from the freshly isolated colorectal cancer was utilized to choose the anticancer agents. Especially, CPT-11 (Topoisomerase I inhibitor) was newly developed the anticancer agent that has wild spectrum including colorectal cancer. However, it is difficult to predict the combination with other anticancer agents. It is demonstrated that CTP-11 has synergistic effects with CDDP and MMC. It is significant to analyze the chemosensitivity and gene expression in order to promote the clinical efficacy and protect the adverse effects. Our focus is the determination for the gene related chemosensitivity from the freshly isolated colorectal cancer using macroarrey. Based on that, cDNA from the freshly isolated colorectal cancer needs to be determined. We successfully have demonstrated that the clinical samples are able to be analyzed, and have showed the unregulated expression of angiogenesis genes and down regulation of cell cycle genes determined by cDNA macroarray.

4. Differential tissular expression and localization of type IV collagen alpha1(IV), alpha2(IV), alpha5(IV), and alpha6(IV) chains and their mRNA in normal breast and in benign and malignant breast tumors

Nakano S, Iyama K[4], Ogawa M[4], Yoshioka H[4], Sado Y[4], Oohashi T[4], Ninomiya Y[4]:[4]Department of Surgical Pathology, Kumamoto University School of Medicine, Japan

Type IV collagen, the major component of basement membrane (BM), is composed of six genetically distinct alpha chains. We investigated the cellular regulation and origin of these alpha(IV) chains in normal and neoplastic breast tissues by immunohistochemistry by using alpha(IV) chain-specific antibodies and by *in situ* hybridization. In normal breast, alpha1(IV) and alpha2(IV) chains were stained in all BM, whereas alpha5(IV) and alpha6(IV) chains were restrictively localized in a linear pattern in the BM of the mammary gland. Similar immunostaining profiles were observed in benign breast tumors and in the intraductal components of invasive ductal carcinoma. However, in invasive ductal carcinoma, alpha1(IV) and alpha2(1V) chains were discontinuously or negatively stained in the cancer cell nests, and the assembly of alpha5(IV) and alpha6(IV) chains into the BM was completely inhibited. Coexpression of alpha5(IV) and alpha6(IV) chains was related to the localization of alpha-smooth muscle actin (alpha-SMA)-positive myoepithelial cells. By in situ hybridization, in fibroadenoma and invasive ductal carcinoma, the signals for alpha1(IV) and alpha2(IV) mRNA were abundant in stromal cells. However, the signals for alpha5(IV) and alpha6(IV) mRNA were not seen in any of these cells. In contrast, in intraductal papilloma, coexpression of alpha1 (IV)/alpha2(IV) mRNA and alpha5(IV)/alpha6(IV) mRNA was identified in epithelial cells. The results indicate that the mammary gland forms a second network of BM composed of alpha5(IV)/alpha6(IV) chains, in addition to the classic network of alpha1(IV)/alpha2(IV) chains. The expression of type IV collagen alpha chains seems to be differentially regulated by the epithelial-myoepithelial interaction and to be associated with the invasive potential of breast cancer.

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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 (MRI), 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. Metabolism of Tc-99m ECD in Infarcted Brain Tissue of Rats

Yusuke Inoue, Osamu Abe[1], Takeshi Kawakami[3], Takayuki Ozaki[3], Minoru Inoue[3], Ikuo Yokoyama[2], Kohki Yoshikawa and Kuni Ohtomo[1]:Departments of [1]Radiology and [2]Cardiovascular Medicine, University of Tokyo, [3]Daiichi Radioisotope Laboratories, Ltd.

Brain SPECT with Tc-99m ethyl cysteinate dimer (ECD) visualizes subacute cerebral infarct as a hypoactive area even in the presence of postischemic hyperperfusion. Brain retention of Tc-99m ECD depends on hydrophilic conversion mediated by enzymes, and impaired enzymatic trapping is hypothesized to depress retention efficiency in the infarcted region. The aim of this study was to determine whether the metabolic rate of Tc-99m ECD is actually reduced in infarcted brain tissue. In 50 mM phosphate buffer (pH 7.4), Tc-99m ECD was incubated for 30 min with homogenates of rat brain tissue with and without triphenyltetrazolium chloride (TTC) staining. The ratio of polar products was determined by thin layer chromatography as a function of incubation time, and metabolic rates were obtained. Permanent focal ischemia was induced by occlusion of the right middle cerebral artery (MCA) in rats. The brain was removed 24 hours after MCA occlusion, and the infarcted area was defined by TTC staining. The metabolic rate of Tc-99m ECD was determined in homogenates of infarcted tissue, contralateral noninfarcted tissue and tissue sampled from sham-operated rats. Infarct volume was measured by direct and indirect methods to assess volume expansion due to edema, and the metabolic rate in infarcted tissue was corrected for the effect of edema. TTC staining had no effect on the metabolic rate of Tc-99m ECD. The metabolic rates in the infarcted tissue were 0.222±0.054%/min and 0.285±0.064%/min before and after correction for edema, respectively, and were significantly lower than those in the contralateral noninfarcted tissue (0.426±0.028%/min) and the tissue sampled from the sham-operated rats (0.439±0.031%/min). No substantial difference in rates was observed between the contralateral tissue and tissue from the sham-operated rats. The results of this study demonstrated that infarction decreases the activity of enzymes that mediate the hydrophilic conversion of Tc-99m ECD in the brain and suggest that reduced metabolic activity is related to decreased accumulation of Tc-99m ECD in hyperperfused infarcts. We are now preparing to examine the retention and washout of the tracer under physiological and ischemic conditions using living brain slices.

2. Estimation of Differential Renal Function with Tc-99m MAG3

Yusuke Inoue, Kohki Yoshikawa, Ikuo Yokoyama[2] and Kuni Ohtomo[1]

Measurement of differential renal function is essential in determining therapeutic strategies for nephrourological patients. We have studied the evaluation of differential renal function from renal scintigraphy and developed accurate techniques applicable to both children and adults. In this study, we compared single-sample methods, proposed by Russell et al. and Bubeck et al., and camera-based methods in calculating ^{99m}Tc-MAG3 clearance, and determined camera-based methods that provide estimates comparable to those measured by the Russell method. Twenty-one patients underwent ^{99m}Tc-MAG3 renal scintigraphy, and clearance was measured by the Russell method and Bubeck method. Various renogram parameters were determined based on the slope of the renogram and area under the renogram, and correlated with the clearance measured by the Russell method. Camera-based clearance was calculated with the obtained regression equations and with equations determined previously using the Bubeck method as a standard. The Bubeck method provided lower measures than the Russell method in high renal function. Clearance measured by the Russell method was well correlated with renogram parameters, and clearance calculated with the obtained regression equation was comparable to that measured by the Russell method. When camera-based clearance was predicted with the previous equation, it was lower than the result obtained by the Russell method in high function. In conclusion, there are systematic differences in ^{99m}Tc-MAG3 clearance calculated by different methods. The camera-based methods obtained in this study appear to facilitate comparison of results obtained by the Russell method and camera-based method. We are now integrating our results into a semiautomated utility program and planning to conduct a multicenter validation study.

3. Virtual Bronchoscopy for Pulmonary Nodular Lesions Using Synchronized Reference Images

Naoki Yoshioka, Yusuke Inoue, Kohki Yoshikawa, Manabu Minami[1], Masaaki Akahane[1] and Kuni Ohtomo[1]

Conventional virtual bronchoscopy uses three orthogonal reformatted images along body axis as reference views. Synchronized reference image (SRI) mean a new technique that provides three orthogonal reformatted images along the vector of view. In this study, we examined the usefulness of this technique in virtual bronchoscopy for the evaluation of pulmonary nodular lesions and compared it with corresponding fiberoptic examinations. Twenty-one patients (sixteen men and three women) with pulmonary nodular lesions were examined with both virtual bronchoscopy and fiberoptic bronchoscopy. Virtual bronchoscopy was calculated and reconstructed from the cross-sectional images on a separate workstation. Stenoses and tumor infiltration were classified from the fiberoptic examination. These results were compared with the virtual bronchoscopy findings. Synchonized reference images offered the advantage of being able to visualize relationship of the lesion and the bronchus. Virtual bronchoscopy of diagnostic quality was achieved in 16 of 21 patients. However, on virtual bronchoscopy discrete infiltration was not visible in five patients. Virtual bronchoscopy using synchonized reference images is accurate and useful techniques in the preoperative assessment of pulmonary nodular lesions.

4. Evaluation of tissue segmentation methods for cerebral ^{1}H-magnetic resonance spectroscopic imaging (MRSI) using short-TE PRESS

Kohki Yoshikawa, Yusuke Inoue, Naoki Yoshioka, Tsuyoshi Matsuda[4], Seizo Takahashi[4] and Takashi Ogino[5]:[4]GE Yokogawa Medical Systems, Ltd., and [5]National Institute of Neuroscience

The goal of our study is to evaluate the accuracy of variable tissue segmentation methods for quantitative cerebral proton MRSI. Tissue segmentation was performed on the T1-W, T2-W, PD-W, T1-calculating, and T2-calculating image data sets using own-developed software of SGI-02 and Sun SPARK 20. T1- and T2-images were calculated by using the images of FSE 300/7,500/7,1000/7, 6000/7 and of FSE 6000/7, 6000/100 respectively. Classification of pixels as either CSF, gray, or white matter was performed using histogram analysis of pixel intensities. The 10 or 20 slices corresponding to the thickness of the MRSI slab were extracted from the segmented data sets and averaged to create images of gray matter, white matter, and CSF. The metabolite concentrations were corrected for the partial volume effect due to CSF. Ten healthy control subjects were studied, after informed consent. The 3D-MRSI data were obtained by PRESS sequence (TR/TE=2000msec/30msec, phase steps=16x16, slice thickness=20mm, and acquisition time=19.3minutes). A 1.5 T General Electric (GE, Milwaukee, WI) Signa Lx NV/i was used, running 8.25v software. These techniques were applied to study differences in metabolite concentration between gray and white matter in normal volunteers (n-10). The estimated ratios of metabolite concentration were: NAA/Cr=2.250; Cho/Cre=0.910 for gray matter and NAA/Cr=1.907; Cho/Cre=1.083 for white matter. The correction of inhomogeneous B0 and B1 fields and the automatic phase correction could be done successfully by using the MRSI data without water suppression and chemical shift of the spectral peak of the water respectively.

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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. Establishment of an assay for human hematopoietic stem cells

Hiroshi Yoshino, Takahiro Ueda, Yasuhiro Ebihara, Atsushi Manabe, Ryuhei Tanaka, Kohichiro Tsuji, Shigetaka Asano

Transplantable hematopoietic stem cells (HSC) are characterized by an ability to permanently regenerate the entire blood-forming system. An assay system evaluating human HSC would allow a more rational approach to the development of clinical treatments involving HSC transplantation, *ex vivo* HSC expansion and gene therapy. However, in contrast to mouse *in vivo* assays using syngeneic recipients, until recently, no comparable system has been established in human. To address this problem, several strategies have been pursued to develop an animal recipient for human HSC. Recently, we established a xenotransplatation system for human HSC into NOD/Shi-scid mice, which possess lack of mature lymphocytes, macrophage dysfunction and absence of circulating complements. When treated with anti-asialo GM1 antibody to delete natural killer cells, the NOD/Shi-scid mice revealed efficient engraftment of human cord blood (CB) $CD34^+$ cells. Analysis of recipient bone marrow (BM) cells 10 weeks after the transplantation showed the multilineage reconstitution, and contained a large number of human $CD34^+$ cells and colony-forming cells (CFC). Thus, this mouse model may provide a useful preclinical tool for assaying human HSC.

2. Efficient expansion of human hematopoietic stem cells by a combination of c-Kit, Flt3, c-Mpl and gp130 signals

Takahiro Ueda, Hiroshi Yoshino, Yasuhiro Ebihara, Atsushi Manabe, Ryuhei Tanaka, Kohichiro Tsuji, Shigetaka Asano

There has recently been great interest in the *ex vivo* expansion of human HSC for a variety of developing clinical application including HSC transplantation and gene therapy. To obtain the optimal culture condition for human HSC expansion, many investigators used various combinations of cytokines which have been shown to act on primitive hematopoietic cells, since proliferation and differentiation of HSC are thought to be regulated by interactions of various cytokine receptor signals. In particular, tyrosine kinase receptor signals, such as c-Kit signal by stem cell factor (SCF) and Flt3 signal by Flt3 ligand (FL), play key roles in primitive hematopoiesis. Thrombopoietin (TPO), a ligand for c-Mpl, originally identified as a primary regulator for megakaryopoiesis, has also been shown to stimulate the expansion of primitive hematopoietic cells. In addition, we have demonstrated that gp130 signal activated by a complex of interleukin (IL)-6 and soluble IL-6 receptor (IL-6/sIL-6R) synergizes with c-Kit or Flt3 signal to expand multipotential hematopoietic progenitor cells.

Clinically transplantable HSC should prove to retain the long-term reconstituting ability. Until recently, however, most of human HSC expansion studies aimed at clinical application have used the assays for $CD34^+$ cells, CFC and long-term culture initiating cells (LTC-IC) to optimize the culture conditions. These surrogate assays have been shown not to reflect a stem cell activity. Recent development of an assay measuring the ability to reconstitute hematopoiesis of NOD/Shi-scid mice in our department as shown above enabled us to evaluate the stem cell activity of expanded hematopoietic cells. Then, using the xenotransplantation of human hematopoietic cells into NOD/Shi-scid mice, we established a novel expansion system of human HSC with a combination of SCF, FL, TPO and IL-6/sIL-6R. When CB $CD34^+$ cells were cultured with the cytokine combination for 1 week, analysis of human $CD45^+$ cells in BM of the recipients transplanted with the cultured cells showed significant expansion of human long-term reconstituting HSC in the culture. Our culture system may pave the way for the clinical application of *ex vivo* expansion of human HSC.

3. 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, Ryuhei Tanaka, Kohichiro Tsuji, Shigetaka Asano

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.

4. Impaired Granulopoiesis in the Truncated G-CSFR-Transgenic Mice

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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.

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

Feng Ma, Atsushi Manabe, Miyuki Ito, Kohichiro Tsuji, Shigetaka Asano

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 a 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 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 mathod 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 $IL\text{-}7R^+$ and $IL\text{-}7R^-$ cells into second FTOC resulted in a predominant generation of $IL\text{-}7R^+$ cells from both fractions, while $IL\text{-}7R^-$ cells proliferated more potently in the second FTOC than $IL\text{-}7R^+$ cells did, suggesting that a conversion of $IL\text{-}7R^-$ to $IL\text{-}7R^+$ pathway existed in the proliferational process of T-ALL lymphoblasts. Our current study provide 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.

6. Gene therapy for recurrent neuroblastoma

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Although a notable improvement of cure rate in childhood cancer has been observed owing to development of multiagent chemotherapy, approximately one half of children with cancer cannot 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.

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 neuroblastoma. 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 neuroblastoma cells transduced with IL-2 into patients with relapsed neuroblastoma exerted regression of tumors. It is possible that gene-transduced neuroblastoma 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 neuroblastoma cells. Preclinical experiments are being performed to show the feasibility of this therapy.

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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 2000, approximately 130 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) of the Advanced Clinical Research Center and Department of Clinical Immunology & AIDS Research Center join the clinic. Supported by clinicians of three departments, 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

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a. Treatment of HIV infection in IMSUT hospital

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

On a monthly basis, approximately 130 patients have visited the out-patient clinic this year. 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 increased again, reasons for admission in many cases were adverse effects caused by HAART and the number of seriously ill patients due to immunodeficiency-related disorders was still 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 $\geq$300/mm^3 : Evaluation of Subcutaneous Proleukin in a Randomized International Trial (ESPRIT)

This year we joined international clinical study called "ESPRIT" which is organized by National Cancer Institute in US. 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, in-

creases 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.

1. 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.
2. Whether or not IL-2 can be administered safely over an extended period of time to HIV infection patients.

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 twenty patients are assigned to IMSUT hospital. We already finished necessary paper works, passed site visit evaluation and are going to enroll patients next year.

b. Clinical research on HIV infection

i) Predominance of genotype A HBV in HBV-HIV-1 dually positive population as compared to HIV-1-negative counterpart in Japan

In Annual Report 1999, we described genotypic characterization of hepatitis B virus (HBV) in Japan. We extended this year the clinical research in this field to relationship between HIV and HBV genotypes.

HBVgenotype: HBV DNA fragments including the complete S gene were amplified from 18 Japanese patients with HIV-1-infection. These 18 sequences together with 16 sequences reported in the literature were compared by UPGMA method and a phylogenetic tree was constructed. Thirteen out of 18 S gene sequences were categorized as genotype A, 3 were genotype B and 2 were genotype C. Among the 13 genotype A carriers, 12 (92%) were men who had sex with men (MSM) while the other was a hemophiliac. It is of note that 10 of these 13 genotype A samples were highly homologous to the strains isolated in USA. Two of the 3 genotype B carriers reported heterosexual intercourse as a risk factor for both HIV-1 and HBV infection while the other was a hemophiliac. Two patients were the carriers of genotype C. One patient reported heterosexual intercourse as a risk factor for both HIV-1 and HBV infection. The other was MSM who reported sexual intercourse as a risk factor for HIV-1 infection but vertical transmission for HBV infection.

HIV subtype: The C2-V3 region of HIV-1 *env* gene was amplified by nested PCR in 15 out of 18 patients in whom HBV genotype was studied. One, 12, 1 and 1 HIV-1 were categorized as subtype A, B, C and E, respectively. Nine of the 12 patients with subtype B HIV-1 were MSM while 2 patients were hemophiliac and one reported heterosexual intercourse as a risk factor. Both patients with subtype A and subtype E HIV-1 reported heterosexual intercourse as a risk factor. The patient with subtype C was MSM.

In the present study we found that the genotype A HBV was predominant among HBV-HIV-1 dually-infected Japanese MSM. Among 13 MSM, one (patient 6) with genotype C was presumably infected with HBV perinatally because his mother was HBV-positive and he had been HBV-positive before he became HIV-1-positive. Consequently, all MSM patients who were infected with HBV through sexual intercourse had genotype A HBV. This result disclosed a striking difference from the previous study which showed that genotype C was the most prevalent HBV in the Far East including Japan. Considering the decreasing rate of vertical transmission of genotype C, the genotype map in Japan may look different in the future.

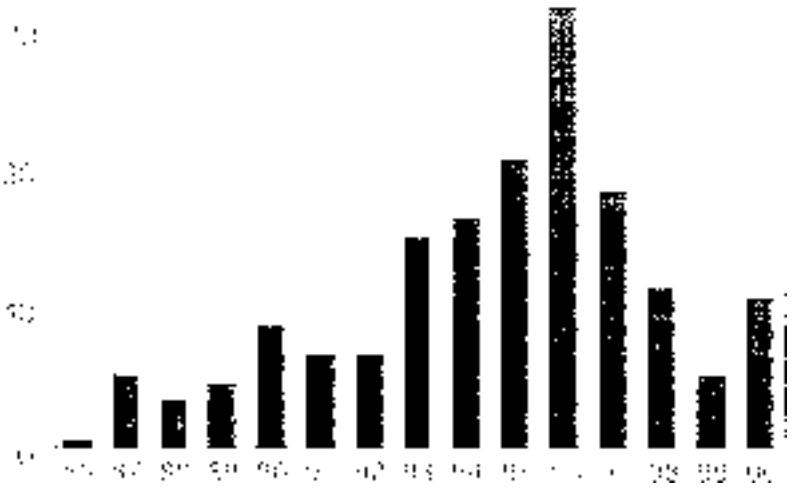

Figure. Number of annual admission of HIV-infected patients

ii) Desensitization of fluconazole hypersensitivity

Amphotericin B and azole antifungal drugs are key agents which are used to treat cryptococcal meningitis associated with AIDS. Fluconazole offers several advantages over amphotericin B since it is available in an oral formulation and has a profile of more favorable adverse effects. We reported this year a case who experienced hypersensitivity reaction to fluconazole but was successfully desensitized to it.

A 53-year-old HIV-positive Japanese man was admitted to our hospital for treatment of cytomegalovirus retinitis on March 29, 2000. Chest X-ray film showed multiple nodular shadows in the right upper lobe which was diagnosed as pulmonary cryptococcosis. He was administered fluconazole 400 mg intravenously on April 22, but developed hypersensitivity reaction such as fever and erythematous rash on his face and body. After obtaining his informed consent, we administered him gradually-increased doses of the drug for 7 days for desensitization purpose. The single oral dosages of fluconazole on days 1, 2, 3, 4, 5, 6 and 7 were 5, 10, 20, 50, 100, 200 and 400 mg, respectively. After that, he was administered 400 mg of fluconazole orally once a day. The desensitization regimen proved successful since the patient did not develop any adverse reactions during those 7 days. Although the desensitization protocol should be evaluated in a clinical trial involving representative number of HIV-infected individuals, it seems that both 7-day and 15-day desensitization regimens can be applied to HIV-infected patients with hypersensitivity to fluconazole under careful observation for the development of a rash and a fever.

2. Diagnosis and Treatment of Tropical Diseases

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This year, we treated 16 patients with malaria among which 10 had falciparum malaria. Three cases with falciparum malaria were severely infected and successfully treated with intravenous quinine and hemodialysis. We also treated two cases with dengue fever, one amebiasis, one cutaneous larva migrans, one ocular larva migrans, two trichuriasis, one schistosomiasis, one giardiasis, and two tapeworm infection.

Publications

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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) gene therapy for GH deficiency, (2) Identification of genes involved in outflow tract formation during embryonic heart development, (3) transformation of grafted muscle by BMP-2, (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. Gene therapy for growth hormone (GH)-deficiency

Inazawa T. et al.

Growth hormone (GH) deficiency causes growth disturbance in children. For treatment of GH deficiency, injection of recombinant human GH (hGH) is undertaken in general. However, the recombinant hGH is so expensive that it is near 40% of total medical cost for chronic diseases of children in Japan. In addition, it is troublesome for children to inject hGH every day. Therefore more convenient and economic therapy for GH deficiency is expected. We intended to treat GH deficiency using *ex vivo* gene therapy. Human 20K GH gene was incorporated into VSV-G pseudotype retrovirus vector, and introduced into human skin fibroblasts, primate skin fibroblasts, or bone marrow stromal cells. Not only production of 20KGH by those cells has been detected *in vitro*, but *in vivo* expression of 20K GH for more than a month was also confirmed in primates which was intravenously injected with transduced bone marrow stromal cells.

We have another project to make a circadian rhythm of 20KGH. Naturally GH is secreted during sleep. We intend to create GH secretion rhythm using endogenous circadian rhythm of glucocorticoids. The 20K GH gene was inserted into downstream of glucocorticoid-sensitive LTR of pMSG plasmid. The obtained 20K GH-pMSG plasmid was lipofected into 3Y1 cells together with puromycin-resistance plasmid. Lots of colonies were picked up after selection with puromycin, and among them some stable GH producing cell lines were obtained. These cells produced 20K GH in response to glucocorticoid stimulation up to 6 fold of the basal level *in vitro*. *In vivo* production of 20K GH responding to glucocorticoid administration was also detected when the cells were microcapsulelated and transplanted into abdominal cavity of rats. Intraabdominal application of dexamethasone increased plasma hGH concentration, indicating that hGH expression is also regulated *in vivo*. We are incorporating hGH gene into an AAV vector, which is expected to express hGH more efficiently.

2. 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 majorities 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 related to birth defects of the heart in humans. In fact, this 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 arises 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. As a result of pursuit of responsible gene, it was found Cspg2 (versican) gene is disrupted in hdf mouse. This information is helpful to understand the importance of extra-cellular matrix in the process of heart formation; however, it is still enigmatic how lack of expression of Cspg2 gene leads to defect of embryonic heart development. In order to address this question, 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 under process of elucidation of truly up-regulated or down-regulated genes among them and involvement of these genes for the embryonic heart development.

3. Transformation of grafted muscle by BMP-2

Nakaoka T. et al.

Bone transformation of a grafted skeletal muscle would be very useful for treating impaired regional bone formation, including delayed or non-bone fracture union, congenital pseudoarthrosis occurring either alone or in association with von Recklinghausen's disease, and segmental bone defects after trauma, osteomyelitis, or tumor resection. First to test whether high level of expression of BMP-2 induces bone formation *in vivo*, adenoviruses carrying *BMP-2* gene (AxCABMP2) were directly injected into the soleus muscle of adult rat. The *BMP-2* gene was successfully over-expressed in the target muscle by adenovirus-mediated transfer, whereas bone formation in and around the muscle failed to occur in this case. It was hypothesized that this failure was due to a lack of putative osteoprogenitor cells in the normal muscle. Therefore, in order to recruit putative osteoprogenitor cells, we then induced ischemic degeneration of the target muscle by orthotopically grafting it simultaneously with the gene transfer. The combination of *BMP-2* gene transfer and orthotopic muscle grafting resulted in successful ossification of entire region of the grafted muscle, whereas neither muscle grafting alone nor the combination of muscle grafting and adenovirus-mediated transfer of reporter gene, *LacZ*, induced any bone formation in the muscle. The ossification process was evident by positive von Kossa staining of the histological sections and roentgenographical radio-opacity of the region. It was also found that the *BMP-2* transgene over-expressed in grafted muscles inhibited muscle regeneration, which should otherwise follow the muscle degeneration. It is noteworthy that a thick cortex with vast marrow sinus was formed and bone remained even one year after the gene transfer. In conclusion, over-expression of *BMP-2* gene induced massive heterotopic ossification in skeletal muscles under graft-induced ischemic degeneration, which possibly up-regulates osteoprogenitor cells *in situ*. This modality requires a modification in terms of clinical application, because muscle regeneration differs between human and rodents. We are now under way.

4. Immunotherapy for malignancies using dendritic cells

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 collected 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-α. 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 Laboratory Medicine

Our research interest is divided into two major projects. The one is to understand the molecular mechanisms underlying differentiation of normal granulocytes from multipotent stem cells. And the other is to find single-nucleotide polymorphisms (SNPs) in genes relevant to the progression and drug-sensitivities of chronic myelogenous leukemia.

1. Studies on the nuclear factors participating in the transcriptional regulation of alkaline phosphatase gene in neutrophils

Nozomi Yusa, Satoru Yoshida, Kunihito Watanabe[1], Shigetaka Asano[2], Kenzaburo Tani[2] and Noriharu Sato:[1]Institute of Bio-medical Research, Teijin Ltd. and [2]Department of Hematology-Oncology

Using transient transfection assay, we found that about 150 bp DNA fragment in the promoter region that contained TATA box was essential for its basal promoter activity. Since the fragment also contained 3 GC-boxes, we examined Sp1 family of transcriptional factors if they bound to the DNA. Using gel shift assay and Southwestern method, we found Sp3 could bind to the fragment. When the expression vector with minimal promoter sequence was co-transfected with Sp3 expression vector into Schneider cells, Sp3 was shown to activate basal promoter of alkaline phosphatase gene.

2. 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 neutrophls 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.

3. Molecular diagnosis of chronic myelogenous leukemia and acute promyelocytic leukemia by nested RT-PCR

Mayumi Karikomi, Nozomi Yusa, Satoru Yoshida and Noriharu Sato

Using nested RT-PCR, we are now able to detect minimal residual leukemic cells in patients with CML or APL who were treated by allogeneic stem cell transplantation. We are carefully monitoring these patients after hematopoietic stem cell transplantation.

4. Quantitative HIV-1 RNA testing

Satoru Yoshida, Nozomi Yusa and Noriharu Sato

We have began to quantitate HIV-1 RNA levels since January of 1998 and the number of assayed samples is going to amount to 2,200 as of the end of this year. This test provides reliable data for disease prognosis and important informations for treatment of the disease, since serial monitoring of HIV levels let us know when to start the treatment or to change the drugs. In addition, since HIV-1 RNA subtype A and E have some problems of amplification with cur-

rent assay kits, we have studied the application of improved primers for these subtypes. In order to monitor HIV-1 RNA levels under the current detection limit (<400 copies/ml), we are also using hype-sentitive assay methods of HIV-1 RNA.

5. Assay for phagocytic activity of neutrophils and other hematopoietic cells.

Satoru Yoshida, Mayumi Karikomi, 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-colour analysis with various kinds of antibodies to define the characteristics of individual phagocytozing cells.

6. Functional role of Fas and Fas-ligand in glioma cell lines

Hisaaki Shinihara[3], Hideo Yagita[4], Yoji Ikawa[3] and Naoki Oyaizu:[3]Tokyo Medical and Dental Universit and [4]Juntendo University School of Medicine

We found that Fas transduce signals leading to apoptosis and, to our surprise, cell growth as well in gliomas. All the glioma cell lines examined were found to express functional FasL and blocking endogenous Fas/FasL interaction resulted in reduced cell growth. Further, we show evidence that Fas-mediated growth signal was closely linked to extracellular signal regulated kinase (ERK) activation. Regarding the role of Fas/FasL system in tumorigenesis, our present study provide an important implication that some tumors may make use of this system for further progression not only by evading immune surveillance but also by promoting its own growth.

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佐藤典治：造血幹細胞移植．臨床病理.(2001年、印刷中)

刈込真由美，吉田　悟．遊佐　希，佐藤典治：フローサイトメトリーを用いた血液細胞の貪食能検査法の確立．日本検査血液学会雑誌．（2001 年、印刷中）

Research Hospital

Department of Clinical AIDS Research

Department of Clinical Immunology & AIDS Research was founded in 1995 to provide medical care for patients with human immunodeficiency virus (HIV) infection and HIV-related opportunistic disorders. In 2000, approximately 130 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. In collaboration with Department of Infectious Diseases & Applied Immunology and Division of Infectious Diseases (DID) of the Advanced Clinical Research Center, our department contributes medical management of HIV-infected patients in IMSUT hospital.

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

Tetsuya Nakamura, Takashi Takahashi[1], Hitomi Nakamura[1], Tomohiko Koibuchi[1], Toshiyuki Miura1, Tokiomi Endoh[1], Miou Sato[1], Akihiro Hitani[1], Mieko Goto[1] and Aikichi Iwamoto[1]:[1]Division of Infectious Diseases

a. Treatment of HIV infection in IMSUT hospital

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

On a monthly basis, approximately 130 patients have visited the out-patient clinic this year. 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 increased again, reasons for admission in many cases were adverse effects caused by HAART and the number of seriously ill patients due to immunodeficiency-related disorders was still 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 $\geq$300/mm^3 : Evaluation of Subcutaneous Proleukin in a Randomized International Trial (ESPRIT)

This year we joined international clinical study called "ESPRIT" which is organized by National Cancer Institute in US. 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.

1. 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.
2. Whether or not IL-2 can be administered safely over an extended period of time to HIV infection patients.

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 twenty patients are assigned to IMSUT hospital. We already finished necessary paper works, passed site visit evaluation and are going to enroll patients next year.

b. Clinical research on HIV infection

i) Predominance of genotype A HBV in HBV-HIV-1 dually positive population as compared to HIV-1-negative counterpart in Japan

In Annual Report 1999, we described genotypic characterization of hepatitis B virus (HBV) in Japan. We extended this year the clinical research in this field to relationship between HIV and HBV genotypes.

HBVgenotype: HBV DNA fragments including the complete S gene were amplified from 18 Japanese patients with HIV-1-infection. These 18 sequences together with 16 sequences reported in the literature were compared by UPGMA method and a phylogenetic tree was constructed. Thirteen out of 18 S gene sequences were categorized as genotype A, 3 were genotype B and 2 were genotype C. Among the 13 genotype A carriers, 12 (92%) were men who had sex with men (MSM) while the other was a hemophiliac. It is of note that 10 of these 13 genotype A samples were highly homologous to the strains isolated in USA. Two of the 3 genotype B carriers reported heterosexual intercourse as a risk factor for both HIV-1 and HBV infection while the other was a hemophiliac. Two patients were the carriers of genotype C. One patient reported heterosexual intercourse as a risk factor for both HIV-1 and HBV infection. The other was MSM who reported sexual intercourse as a risk factor for HIV-1 infection but vertical transmission for HBV infection.

HIV subtype: The C2-V3 region of HIV-1 *env* gene was amplified by nested PCR in 15 out of 18 patients in whom HBV genotype was studied. One, 12, 1 and 1 HIV-1 were categorized as subtype A, B, C and E, respectively. Nine of the 12 patients with subtype B HIV-1 were MSM while 2 patients were hemophiliac and one reported heterosexual intercourse as a risk factor. Both patients with subtype A and subtype E HIV-1 reported heterosexual intercourse as a risk factor. The patient with subtype C was MSM.

In the present study we found that the genotype A HBV was predominant among HBV-HIV-1 dually-infected Japanese MSM. Among 13 MSM, one (patient 6) with genotype C was presumably infected with HBV perinatally because his mother was HBV-positive and he had been HBV-positive before he became HIV-1-positive. Consequently, all MSM patients who were infected with HBV through sexual intercourse had genotype A HBV. This result disclosed a striking difference from the previous study which showed that genotype C was the most prevalent HBV in the Far East including Japan. Considering the decreasing rate of vertical transmission of genotype C, the genotype map in Japan may look different in the future.

ii) Desensitization of fluconazole hypersensitivity

Amphotericin B and azole antifungal drugs are key agents which are used to treat cryptococcal meningitis associated with AIDS. Fluconazole offers several advantages over amphotericin B since it is available in an oral formulation and has a profile of more favorable adverse effects. We reported this year a case who experienced hypersensitivity reaction to fluconazole but was successfully desensitized to it.

A 53-year-old HIV-positive Japanese man was admitted to our hospital for treatment of cytomegalovirus retinitis on March 29, 2000. Chest X-ray film showed multiple nodular shadows in the right upper lobe which was diagnosed as pulmonary cryptococcosis. He was administered fluconazole 400 mg intravenously on April 22, but developed hypersensitivity reaction such as fever and erythematous rash on his face and body. After obtaining his informed consent, we administered him gradually-increased doses of the drug for 7 days for desensitization purpose. The single oral dosages of fluconazole on days 1, 2, 3, 4, 5, 6 and 7 were 5, 10, 20, 50, 100, 200 and 400 mg, respectively. After that, he was administered 400 mg of fluconazole orally once a day. The desensitization regimen proved successful since the patient did not develop any adverse reactions during those 7 days. Although the desensitization protocol should be evaluated in a clinical trial involving representative number of HIV-infected individuals, it seems that both 7-day and 15-day desensitization regimens can be applied to HIV-infected patients with hypersensitivity to fluconazole under careful observation for the development of a rash and a fever.

2. Diagnosis and Treatment of Tropical Diseases

Tetsuya Nakamura, Akihiro Hitani[1], Mikio Kimura[2] and Aikichi Iwamoto1:[2]Infectious Disease Surveillance Center, National Institute of Infectious Disease

This year, we treated 16 patients with malaria among which 10 had falciparum malaria. Three cases with falciparum malaria were severely infected and successfully treated with intravenous quinine and hemodialysis. We also treated two cases with dengue fever, one amebiasis, one cutaneous larva migrans, one ocular larva migrans, two trichuriasis, one schistosomiasis, one giardiasis, and two tapeworm infection.

Publications

Katano, H., Suda, T., Morishita, Y., Yamamoto, K., Hoshino, Y., Nakamura, K., Tachikawa, N., Sata, T., Hamaguchi, H., Iwamoto, A., and Mori, S. Human herpesvirus 8-assosiated solid lymphomas that occur in AIDS patients take anaplastic large cell morphology. Modern Pathology 13:77-85, 2000.

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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. 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]: [1]Division of Cell Processing, [2]Department of Hematology-Oncology, and [3]Pediatric Hematology-Oncology, 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. Functional expression of chemokine receptor CXCR4 for CB cells differ from adult BM and PB cells in culture

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Pre-B-cell growth-stimulating factor/stromal cell derived factor 1 (PBSF/SDF-1) is a member of CXC chemokines, and its receptor CXCR4 has also been revealed to to be a coreceptor for T-tropic HIV-1 entry. The physiological roles of PBSF/SDF-1 and CXCR4 were studied using mutant mice with a targeted these genes, showing that they are responsible for B-lymphopoiesis and marrow myelopoiesis. Recently, it has been reported that the expression of CXCR4 is critical for the engraftment of CB cells into the murine BM. However, we found that highly purified $CD34^+CXCR4^+$ BM cells lack myeloid progenitors, but give to lymphoid progenitors, wheres $CD34^+CXCR4^-$ BM cells can generate both of these. To address these questions, we have studied the proliferative and diffentiative potentials of CB and PB (G-CSF mobilized) $CD34^+$ cells along the myeloid and lymphoid pathway, and compare these results to those with BM cells. The myeloid colony frming potentials were as follows;

Unlike BM and PB cells, both $CD34^+CXCR4^+$ and $CD34^+CXCR4^-$ CB cells similarily had myeloid colony forming potentials. Concerning lymphoid progenitors, $CD34^+CXCR4^-$ BM cells could generate pre-B cells more abundantly than $CD34^+CXCR4^+$ BM cells. In contrast, both $CD34^+CXCR4^+$ and $CD34^+CXCR4^-$ CB cells have the similar potentials in producing pre-B cells. these results suggest that the requirement of the functional expression of CXCR4 for CB cells may differ from that for adult BM and PB cells. Further studies were needed to understand the significance of expression in the homing, engraftment, and repopulation of hematopoietic stem cells in the different organ.

4. Erythroid progenitos differentiate and mature in response to endogenous erythropoietin

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It has been reported that stimulation of glycoprotein 130 (gp130) by a combination of human soluble

	Number of Colonies / 300 cells					
	G	M	GM	B	Mix	Total
$CD34^+CXCR4^+$						
BM	0	0.3±0.6	1.3±1.2	2.3±1.2	0	4.0±1.0
PB	0	0	0	0	0	0
CB	0	4.3±1.5	24.6±5.1	41.7±1.5	0.6±1.2	71.0±5.0
$CD34^+CXCR4^-$						
BM	2.0±1.0	8.3±1.5	46.0±4.62	53.3±3.5	1.7±1.2	111.3±4.0
PB	1.3±0.5	3.0±5.0	35.7±2.0	12.0±2.0	0	52.7±3.1
CB	0	9.3±4.0	41.3±6.1	59.7±4.5	0	109.3±4.5

IL-6R (sIL-6R) and IL-6 could support proliferation, differentiation and terminal maturation of erythroid cells in the absence of erythropoietin (EPO) from human CD34+ cells in culture with SCF. This observation suggested that differentiation of hematopoietic stem/progenitor cells to erythroid cells was progressed along an intrinsic program, and that EPOR could be replaced by other cytokine receptors and was dispensable for erythropoiesis. We examined the role of EPOR in erythropoiesis stimulated by SCF, sIL-6R, and IL-6. Surprisingly, elimination of EPOR expression by antisense oligodeoxynucleotide (AS ODN) suppressed erythropoiesis in the absence of EPO. EPO mRNA was detected in the erythroid cells but not myeloid cells cultured in the presence of SCF, sIL-6R, and IL-6. Furthermore, high concentrations of anti-EPO neutralizing antibody abrogated erythropoiesis in cultures without extrinsic EPO. Based on these results, we suggest that erythroid progenitors per se secrete EPO and have the potential to differentiate and maturate in an autocrine manner by endogenous EPO.

5. 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 non-specific 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.

6. 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.*

7. 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 insertion 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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Basically our purpose is to improve quality of life of the patients in surgery or in intensive care. In that real direction of clinically based advanced medicine, especially we have investigated recently 1) mechanisms of pain and better analgesic regimen, and 2) coagulation, fibrinolysis, and hemolysis in various conditions. In addition, we support some investigation and clinical reports in other institutions.

1. Tomoki Nishiyama. Interaction between intrathecal morphine and glutamate receptor antagonists in formalin test

The analgesic interaction between intrathecally administered morphine and the NMDA receptor antagonist, ((±)-2-amino-5-phosphonopentanoic acid; AP-5), the NMDA receptor glycine site antagonist ,(5-nitro-6,7-dichloro-2,3-quinoxaline dion; ACEA 1021), or the AMPA (α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid) receptor antagonist (ACEA 2752) in the formalin test was investigated with a rat model of chronic lumbar intrathecal catheterization. After obtaining dose-response curves for each agent, combinations of morphine and AP-5, ACEA 1021 or ACEA 2752 were tested for their effect on the number of flinches in the formalin test and for associated side-effects, such as motor disturbances, flaccidity, and agitation/allodynia. Using isobolographic analyses, a potent analgesic synergy was observed with decreased side-effects between morphine and ACEA 2752 or AP-5. ACEA 1021 increased the analgesic effect of low-dose morphine. Spinal μ-opioid receptor activation and NMDA or AMPA receptor antagonism showed a synergistic antinociception against tonic pain. These results suggest an important direction in the management of inflammatory pain.

2. Tomoki Nishiyama. Interaction among NMDA receptor-, NMDA glycine site-, andAMPA receptor antagonists in spinally mediated analgesia

The NMDA (N-methyl-D-aspartate) receptor antagonists and the NMDA glycine site antagonists given alone have minimal effects on acute nociception. In contrast, the AMPA (alpha-amino-3-hydroxy-5-methylisoxazole-4-propionic acid) receptor antagonists have a great role in acute nociception. We investigated the interactions among these three antagonists in acute nociception. Sprague-Dawley rats (250-300 g) were implanted with chronic lumbar intrathecal catheters and were tested for their thermal withdrawal response using the hot plate test after intrathecal administration of AP-5 (NMDA receptor antagonist), ACEA 1021 (NMDA glycine site antagonist), or ACEA 2085 (AMPA receptor antagonist). The combinations of these three agents were also tested. Intrathecal administration of ACEA 2085 had a dose dependent analgesic effect while intrathecal AP-5 or ACEA 1021 could not induce dose dependent effect. Coadministration of AP-5 10 μg and ACEA 2085 intrathecally showed no changes in the thermal response latency compared to ACEA 2085 alone. ACEA 1021, 12 μg, and AP-5 showed the left ward shift of the dose effect curve only with small doses of AP-5 (1 μg and 3 μg). Only the smallest dose of ACEA 2085 (0.1 ng) with ACEA 1021 12 μg induced antinociception compared with that of ACEA 2085 alone. The combination of the NMDA glycine site antagonist and low doses of the NMDA receptor antagonist or the AMPA receptor antagonist increased the analgesic effect on acute thermal nociception with increased side effects, while the NMDA receptor antagonist and the AMPA receptor antagonist had no such interaction.

3. Tomoki Nishiyama, Rodney JY Ho, Danny D Shen, Tony L Yaksh. The effects of intrathecal morphine encapsulated in L- and D-dipalmitoylphosphatidyl choline liposomes on acute nociception in rats

Liposomes can serve as a sustained-release carrier system permitting the spinal delivery of large opioid doses, while restricting the dose for acute systemic uptake. The purpose of this study was to evaluate the antinociceptive effects of morphine encapsulated in liposomes of two isomeric phospholipids, L-DPPC (L-dipalmitoylphosphatidyl choline) and D-DPPC, in comparison with morphine in saline. Sprague-Dawley rats with chronic lumbar intrathecal catheters were tested for their acute nociceptive response using a hind paw thermal escape test. Their general behavior, motor function, pinna reflex, and corneal reflex were also examined. The duration of antinociception was longer in both liposomal morphine groups than in the free morphine group. The peak antinociceptive effects were observed within 30 minutes after intrathecal morphine, L-DPPC or D-DPPC morphine injection. The rank order of the area under the effect-time curve for antinociception was L-DPPC morphine > D-DPPC morphine > morphine. The ED_{50} was: 2.7 μg (morphine), 4.6 μg (L-DPPC morphine) and 6.4 μg (D-DPPC morphine). D-DPPC morphine had less side effects for a given antinociceptive AUC than morphine. In conclusion, L- and D-DPPC liposome encapsulation of morphine prolonged antinociceptive effect on acute thermal stimulation and could decrease side effects compared to morphine alone.

4. Tomoki Nishiyama, Kazuo Hanaoka. The effects of epidural bupivacaine, morphine, and their combination on thermal nociception with different stimulus intensity in rats

The analgesic effect of the drugs depends on the stimulus intensity as well as the potency of the drugs. We investigated the effects of stimulus intensity on antinociceptive potencies of epidural bupivacaine + morphine. Sprague-Dawley rats implanted with chronic lumbar epidural catheters were tested for paw withdrawal response to thermal stimulation after epidural injection of bupivacaine, morphine, or bupivacaine + morphine Two stimulation currents were employed; 5.1 and 4.6 A to provide baseline response latency of approximately 5.0 sec (high intensity) and 10.0 sec (low intensity), respectively. Increasing the dose of epidural morphine in a dose range, which had a maximum effect on low intensity stimulation, was not effective for high intensity stimulation. Bupivacaine, which alone had no effect, potentiated the antinociceptive effect of epidural morphine at both high and low intensity stimuli similarly. We concluded that bupivacaine potentiated the analgesic effect of epidural morphine at both weak and strong nociceptive stimuli similarly, while increasing the dose of epidural morphine was not as effective for strong nociceptive stimulation. Therefore, adding bupivacaine might be more effective than increasing the dose of epidural morphine for strong nociception.

5. Tomoki Nishiyama, Kazuo Hanaoka. Free hemoglobin concentrations in patients receiving massive blood transfusion during emergency surgery for trauma.

To investigate free hemoglobin concentration in patients who received massive blood transfusion during emergency surgery for trauma with consideration of the storage of the transfused blood. Fifteen patients undergoing emergency surgery for multiple trauma and who received blood transfusion of more than 5,000 mL were studied. Transfusion of the stored whole blood in citrate-phosphate glucose solution using a micropore filter was started before surgery. Serum concentrations of hemoglobin (total:THb and free:fHb) and total haptoglobin (THp) were measured until 5,000 mL of blood had been transfused. Serum free haptoglobin (fHp) concentration was calculated. The correlation between the changes in hemoglobin or haptoglobin concentrations and total storage days of the transfused blood was analyzed by a simple regression analysis. Free hemoglobin was detected after 2,000 mL transfusion. THp and fHp decreased after 1,000 mL of transfusion. Total storage time (days) of transfused blood had correlated with the changes of THp ($P < 0.0001$) and fHp ($P = 0.0027$) but not with the changes of THb ($P = 0.984$) and fHb ($P = 0.834$). After blood transfusion during surgery for trauma, serum haptoglobin concentration decreased with transfusion of $\geqq$ 1,000 mL of whole blood with mean storage time of 12.2 days. Free hemoglobin was detected after 2,000 mL transfusion when THp decreased to 1,000 mg/L. Serum haptoglobin concentrations correlated negatively with storage time (days) of transfused blood.

6. Tomoki Nishiyama, Takashi Matsukawa, Kazuo Hanaoka. Is protease inhibitor a choice for the treatment of pre- or mild dissected intravascular coagulation ?

To investigate the effect of a protease inhibitor, gabexate mesilate (FOY) on pre- disseminated intravascular coagulation (DIC) or mild DIC in comparison with the control without any anticoagulation therapy, 40 adult patients with DIC score between 6 and 8 (pre- or mild DIC) were divided into two groups. In 20 patients FOY 2 mg/kg/h was infused with 2 ml/h (FOY group) and in other 20

patients saline 2 ml/h (Control group) was infused during the study (seven days). The following parameters were determined at the time of admission to the intensive care unit (before treatment), and 1, 3, 5, and 7 days thereafter: platelet count, AT III activity, serum or plasma levels of fibrinogen, FDP, D-dimer, fibrin monomer (FM), thrombin-antithrombin III complex (TAT), and plasmin-plasmin inhibitor complex (PIC), prothrombin time (PT) ratio, and DIC score. Two patients in the FOY group and four in the Control group were excluded from the study because they expired during the study, therefore 34 patients were analyzed. The measured variables of coagulation and fibrinolysis were not significantly different between the two groups except for the D-dimer on the day 3 (FOY group showed higher level). D-dimer level and DIC score went down more quickly in the Control group than the FOY group, but not significantly. Mortality in one month was 40% (8/20) in the FOY group and 35% (7/20) in the Control group without any differences between the two groups. In a limited number of patients (N=34), FOY 2mg/kg/h could not inhibit coagulation or fibrinolysis and FOY could not improve DIC score or mortality in pre-DIC or mild DIC.

7. Tomoki Nishiyama, Kazuo Hanaoka. Nicardipine did not activate renin-angiotensin-aldosterone system during isoflurane or sevoflurane anesthesia

To investigate the changes of renin-angiotensin-aldosterone system by nicardipine administration during isoflurane or sevoflurane anesthesia, 20 patients aged 40 to 70 for elective neurosurgery were studied. Anesthesia was induced with thiopental, midazolam and fentanyl and was maintained with nitrous oxide in oxygen and isoflurane or sevoflurane. When blood pressure was constant, 0.017 mg/kg nicardipine was administered as a bolus. Blood pressure, heart rate, and plasma concentrations of nicardipine, angiotensin I and II, aldosterone and renin activity were measured for 30 min. after nicardipine administration. Blood pressure decreased significantly for 30 min. after nicardipine administration in both groups with lower values in sevoflurane anesthesia. Heart rate increased with significance only in isoflurane group. Plasma nicardipine concentration did not differ between isoflurane and sevoflurane groups. Plasma renin activity and concentrations of angiotensin II and aldosterone did not change in both groups and had no differences between the groups. Plasma concentration of angiotensin I increased at 20 and 30 min. after nicardipine administration in isoflurane group but did not increase in sevoflurane group. The activity of renin-angiotensin-aldosterone system did not increase by a single shot administration of nicardipine in isoflurane or sevoflurane anesthesia.

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