ANNUAL RESEARCH MEETING

FOR

GRADUATE STUDENTS

Nagoya, December 1996

Abstracts

CHARACTERISTIC STRUCTURE AND FUNCTIONS OF HEMOLYMPH NODES IN RATS

KENICHI SAKITA

1st. Department of Pathology

The hemolymph nodes (HLs) are characterized by abundant erythrocytes in the lymphatic sinus and medullary cords. These nodes are found in rats, cattles and sheeps. In the present study, the characteristic structure and some immunological functions of rat hemolymph nodes were compared with those of ordinary lymph nodes (OLs) and the spleens. Forty male Wistar rats were used in the experiments. Electron microscopy revealed that, in the medullary cords of HLs, erythrocytes passed through capillary walls via expanded junctions of their endothelial cells, but no direct traffic was found between lymphatic sinuses and blood vessels. A large number of carbon particles appeared in the lymphatic sinus and medullary cords of HLs shortly after intravenous injection of carbon particles, while OLs showed no deposition of carbon particles. Immunohistochemical studies showed that lipopolysaccharide (LPS) reached the lymphatic sinus and medullary cords of HLs 4 hours after intravenous injection, and caused the appearance of more IgM-producing lymphocytes in HLs than in OLs on day 5 after injection. Moreover, the numbers of plaque forming cells (PFCs) in HLs, OLs and the spleen were examined on day 4 after intravenous injection of sheep red blood cells. The number of PFCs in HLs was intermediate between those of OLs and the spleen. In comparison with various HLs, the pancreaticosplenic lymph nodes had the most of PFCs, rather than the ventro-mediastinal or lumbar lymph nodes. These results strongly suggest that HLs have a semi-open structure with respect to microcirculation and immunological functions intermediate between those of OLs and the spleen.

DETECTION AND QUANTIFICATION OF VIRUS DNA IN PLASMA OF PATIENTS WITH EPSTEIN-BARR VIRUS-ASSOCIATED DISEASES

MITSUAKI YAMAMOTO

Department of Pediatrics

Epstein-Barr virus (EBV) causes various diseases, such as infectious mononucleosis (IM), fatal IM, virus associated hemophagocytic syndrome (VAHS), and chronic active EBV infection (CAEBV). In this study, cell-free EBV DNA was detected in plasma of patients with EBV-associated diseases by PCR assay. The patients included 20 cases with IM, 2 cases with fatal IM, 4 cases with EB-VAHS, 4 cases with CAEBV, and 38 healthy children (20 EBV-seropositive and 18 seronegative). In IM, plasma samples were positive for EBV DNA in all patients (100%) in the acute phase and 44% in convalescence, but negative (0%) in the 38 healthy control children. Quantitative PCR assay revealed that the plasma of IM patients contained the highest number of virus DNA within 7 days following disease onset (mean; 6×10^4 copies/ml). The EBV DNA concentration decreased thereafter, as the patients recovered. The plasma of

patients with fatal IM contained over 100 times more copies (3×10^7 copies/ml) than in IM. The plasma of patients with the acute phase of EB-VAHS contained 10 times more copies (5×10^5 copies/ml) than in IM, then similarly decreased in the convalescent phase (2×10^4 copies/ml). Virus DNA in CAEBV (6×10^4 copies/ml) was similar to that noted in IM, however, became higher (1×10^6 copies/ml) when their clinical status deteriorated. These data suggest that the presence of cell-free EBV DNA in plasma is a common phenomenon in EBV-associated diseases. The concentration of plasma EBV DNA seems to be higher in severer clinical categories of EBV diseases.

EVALUATION OF HANDLING METHODS IN THE HISTOLOGICAL DIAGNOSIS OF *HELICOBACTER PYLORI*: THE EFFECT OF FILTER PAPER

TAKIO YOKOI

1st Department of Pathology

Filter paper has been conventionally used as a receptacle for the biopsy specimen prior to fixation. The aim of this study was to determine the presence of any effect caused by the use of filter paper. The study population consisted of 104 consecutive patients undergoing endoscopic examination. Two antral biopsy specimens from the same area were obtained from each patient. One specimen was put onto a piece of filter paper, and the other into a plastic case. The specimens were fixed overnight in buffered formalin, embedded in paraffin, sectioned, and stained with hematoxylin and eosin, and Giemsa. A direct smear was also prepared from 77 patients by vigorously rubbing the filter paper on a glass slide and staining it with Giemsa. The detection rate of *H. pylori* was 47.1% (49/104) for the filter paper method, 56.7% (59/104) for the plastic case method, and 57.7% (60/104) by either of the two handling methods. Of the 60 positive patients, 11 filter-paper specimens were negative, while only one plastic-case specimen was negative. Statistical analysis revealed a significant difference between the two groups ($p<0.01$). On the amount of *H. pylori*, the filter paper method showed a significantly lower grade than the plastic case method ($p<0.05$). In the Giemsa-stained smears, *H. pylori* was identified in 17 (22.1%) of the 77 patients studied. Use of the filter paper may decrease the sensitivity for detection of *H. pylori* infection. We recommend *not* using the filter paper in the histological diagnosis of *H. pylori*.

IMMUNOHISTOCHEMICAL AND IMMUNOGENETIC ANALYSIS OF OCULAR ADNEXAL LYMPHOID PROLIFERATIONS

TOSHINOBU KUBOTA

Department of Ophthalmology

We examined 20 cases (21 specimens) of ocular adnexal lymphoid proliferations, using the histological, immunohistochemical and molecular genetic examination. The latter two methods were performed to detect the light chain restriction of immunoglobulin with PAP methods, and clonality of immunoglobulin heavy chain gene with hemi-nested polymerase chain reaction (PCR) method, respectively. Although 8 cases were morphologically undetermined whether neoplastic or not, clonality was revealed in 1 case with immunohistochemistry and 4 cases with PCR method. Two cases showed discordant results between immunohistochemistry and PCR probably due to somatic mutations of the frame work region of the immunoglobulin heavy chain gene. We, therefore, concluded that examinations with these methods reach the better understanding of the nature of the ocular adnexal lymphoid proliferations. Furthermore, the immunoglobulin gene PCR method is very useful in practical examination, as it can apply with the formalin-fixed paraffin-embedded specimens.

ABORTIVE IMMUNOGLOBULIN HEAVY CHAIN TRANSCRIPTS IN EARLY PRE-B ACUTE LYMPHOBLASTIC LEUKEMIA (ALL)

HIROSHI MORI

Department of Pediatrics

Immature B-lineage acute lymphoblastic leukemia (ALL) is divided into two subgroups, pre-B ALL and early pre-B ALL, by the presence or absence of cytoplasmic μ heavy-chain (Cμ), respectively. In this study, we analyzed the relationship between the expression and the sequences of the immunoglobulin heavy chain (IgH) transcripts in ALL cells and normal counterparts ($CD10^{+/-}CD19^{+}$ surface Ig^{-}) sorted from the bone marrow. Northern blot analysis showed that the $C\mu^{+}$ALL samples tended to express greater amounts of μ-chain transcripts than $C\mu^{-}$ALL samples. The IgH transcripts in both ALL and the normal counterparts contained rare somatic mutation in the V_H gene, and represented the similar usage of D and J_H gene segments. The IgH transcripts in the normal counterparts (70/71 clones) were productive. On the other hand, the IgH transcripts in the 7 $C\mu^{+}$ ALL cells were productive, whereas those in 7 $C\mu^{-}$ALL cells had nonsense codons and/or frame shift in each complementarity determining region (CDR)-3 sequence. Our data suggest that the ALL classification of pre-B and early pre-B do not necessarily indicate their cellular origin, which is mainly determined by V_H-D-J_H gene recombinational events. Furthermore, the normal immature B-cells with nonproductive μ-chain transcripts undergo the negative selection, from which $C\mu^{-}$ALL cells might have escaped.

DIFFERENTIAL SENSITIVITIES TO HYPERBARIC OXYGEN ON LYMPHOCYTE SUBPOPULATIONS OF NORMAL AND AUTOIMMUNE MICE

XIAOHE XU

Department of Hyperbaric Medicine

We studied the effect of repeated daily exposure to hyperbaric oxygen (HBO): 2.8 atmosphere absolute 100% oxygen for 4 hours daily, (over 3–7 days), on immune system of normal (BALB/c and MRL-MP-+/+) and autoimmune (MRL-MP-lpr/lpr) mice. HBO-exposed BALB/c mice showed a remarkable decrease in cell population of spleen and thymus. We found that the sensitivity to HBO varied among subpopulations of lymphocytes. For example, total numbers of $CD4^+CD8^+$ double positive cells in the thymus and $B220^+$ B cells in the spleen were more sensitive than $CD4^+$ or and $CD8^+$ single positive T cells in the thymus, and Thy-1^+T cells in the spleen. Accordingly, the proliferative response of T cells to ConA was not impaired in the HBO-exposed mice. Exposure of MRL-Mp-lpr/lpr mice to HBO caused a marked reduction of weight and cell population in the otherwise enlarged spleen and lymph nodes, and amongst others $B220^+$/Thy-1^+ abnormal cells. These results suggest the HBO therapy may be applicable for the treatment of some autoimmune diseases.

CD95 (FAS) MAY CONTROL THE EXPANSION OF ACTIVATED T CELLS AFTER ELIMINATION OF BACTERIA DURING THE COURSE OF MURINE LISTERIOSIS

YOSHIKAZU FUSE

Department of Immunological Medicine

CD95 (Fas) is known to mediate activation-induced T cell death by apoptosis. To understand the role of CD95 during the course of bacterial infection, we examined the kinetics of αβ and γδ T cells in the peritoneal cavity and liver of CD95-defective MRL/lpr mice of 5 weeks-old after intraperitoneal infection with *Listeria monocytogenes*. The number of bacteria decreased to undetectable level in the spleen by day 10 after infection with 7×10^3 *Listeria*, similar to MRL/+/+ mice. The number of αβ T cells expressing CD44 and CD95 reached peak in the peritoneal cavity on day 6 after listerial infection and thereafter decreased gradually in MRL/+/+ mice, whereas $CD44^+$αβ T cells continued to increase throughout the course of listerial infection in MRL/lpr mice. Freshly isolated T cells from MRL/+/+ mice infected with *Listeria* 10 days previously showed DNA fragmentation with apoptosis, whereas such fragmentation was not prominent in T cells from infected MRL/lpr mice. In correlation with increased number of $CD44^+$ αβ T cells, *Listeria*-specific T cell proliferation of the peritoneal exudate cells was significantly greater in MRL/lpr mice than MRL/+/+ mice on day 10 after listerial infection. In

contrast to αβ T cells, γδ T cells increased only temporally in the peritoneal cavity and liver after listerial infection in both MRL/lpr and MRL/+/+ mice. These results suggest that CD95-mediated cell death with apoptosis may be involved in termination of αβ T cell-mediated immuned response after the battle against *Listeria* has been won, whereas γδ T cells may undergo apoptosis independently of CD95 during the course of listerial infection.

CHARACTERISTICS OF ANTIGEN-RECOGNITION SYSTEM AND FUNCTION OF HEAT SHOCK PROTEIN-REACTIVE T CELLS

YUKI KIMURA

Laboratory of Germfree Life, Res. Inst. for Disease Mechanism and Control

In this study, we examined the characteristics of antigen-recognition, function, and role *in vivo* of heat shock protein (HSP)-reactive T cells of rats after infection with *Listeria monocytogenes*. The TCRγδ$^+$ T cells significantly increased in the peritoneal cavity on day 6 and then decreased by day 10 after infection, in parallel with the kinetics of HSP60 expression in the peritoneal macrophages during listeriosis in F344 rats. Most of the early appearing TCR γδ$^+$ T cells were of the CD4$^-$ CD8αβ$^+$ CD5$^+$ LFA-1α^{high} CD45RC$^-$ IL-2Rα$^-$ phenotype and significant fraction of the TCRγδ$^+$ T cells expressed CD8α only. The increase in TCRγδ$^+$ T cells during listeriosis was prominent in F1 (F344 × Lewis) rats but only marginal in Lewis rats, which was correlated with the expression level of HSP60 in the peritoneal macrophages. We also show that CD4$^+$ T cells capable of recognizing mycobacterial HSP70 appear in the peritoneal cavity of F344 rats on day 10 after listerial infection. The HSP70-reactive CD4$^+$ T cells used Vβ 16 gene segment predominantly and required RT1B major histocompatibility complex (MHC) class II molecule for antigen presentation. The culture supernatants of the HSP70-reactive T cells contained transforming growth factor (TGF)-β1 but not interferon (IFN)-γ. Furthermore, the HSP70-reactive T cells expressed mRNA specific for TGF-β1, IL-10, and IL-15 but no mRNA for either T helper cell (Th) 1-type cytokines such as IL-2 and IFN-γ or a Th2-type cytokine such as IL-4. Preimmunization with mycobacterial HSP70 rendered rat susceptible to listerial infection. Collectively, these results imply that the HSP60-reactive TCRγδ$^+$ T cells contribute to the host defense at the early stage after listerial infection and the HSP70-reactive CD4$^+$ T cells play a role in regulating inflammation rather than in protecting against bacteria during rat listeriosis.

INTERCELLULAR ADHESION MOLECULE-1 EXPRESSION ON GLIA FOLLOWING BRAIN INJURY: PARTICIPATION OF INTERLEUKIN-1 BETA

MIKINE SHIBAYAMA

Department of Neurosurgery

Interleukin-1 (IL-1), one of the most important inflammatory cytokines, promotes glia to express intercellular adhesion molecule-1 (ICAM-1) *in vitro*. IL-1 is known to be produced *in situ* immediately after brain insults. Now, we have found that glia, including astrocytes, express ICAM-1 *in vivo* following cortical stab wounds. To evaluate the participation of IL-1β in post-traumatic ICAM-1 expression on glia *in vivo*, we performed the following experiments. A cortical stab wound was made in the brain of a mouse. ICAM-1-immunopositive glia begun to emerge around the wound from 6 hrs post-lesioning. The number of cells reached a maximum at 48 hrs and persisted until 7 days post-lesioning. Next, a neutralizing monoclonal antibody against IL-1β was infused into the wound immediately following the injury. This treatment resulted in a significant reduction of ICAM-1-positive glia at 24 and 48 hrs post-lesioning. Therefore, we concluded that IL-1β affects ICAM-1 expression on glia *in vivo* after experimental brain injury, and presumably plays an important role in brain wound repair.

PHOSPHORYLATED RETINOBLASTOMA PROTEIN STIMULATES DNA POLYMERASE α BY PHYSICAL ASSOCIATION

MASAHARU TAKEMURA

Laboratory of Cancer Cell Biology

Human retinoblastoma (Rb) protein, immunopurified from an extract of recombinant baculovirus-infected cells, stimulated 10- to 100-fold the activity of DNA polymerase α from calf thymus or human HeLa cells. Dephosphorylation of Rb protein by either potato acid phosphatase or protein phosphatase 2A largely diminished its stimulatory effect, indicating that the phosphorylation is crucial for the stimulation. Rb protein isolated from human Burkitt lymphoma Raji cells also stimulated DNA polymerase α. On the other hand, Rb protein did not affect DNA primase or Klenow fragment of *Escherichia coli* DNA polymerase I. By immunoprecipitation using anti-DNA polymerase α antibody, phosphorylated Rb protein in the nuclear extract of Raji cells was co-precipitated with DNA polymerase α, indicating the complex formation between DNA polymerase α and Rb protein *in vivo*. DNA polymerase α specifically bound to a purified Rb protein-immobilized Sepharose column, suggesting the direct association of these two proteins. These observations suggest a new function of Rb protein in the regulation of cell cycle: when it is phosphorylated in late G_1 to S phase, the hyperphosphorylated Rb protein may promote DNA replication through its stimulation of DNA polymerase α, besides the well-

known suppression of transcription in G_0 to early G_1 phase in its underphosphorylated form.

MODULATION OF TYROSINE KINASE ACTIVITY HAS MULTIPLE ACTIONS ON INSULIN RELEASE FROM THE PANCREATIC β-CELL; STUDIES WITH LAVENDUSTIN A

MITSUO HISATOMI

2nd Department of Internal Medicine

Possible roles of tyrosine kinases in the regulation of insulin release from a hamster β-cell line (HIT T15) were investigated using selective tyrosine kinase inhibitors. Genistein increased insulin release induced by glucose, while herbimycin A, tyrphostins and the erbstatin analogue failed to change the release. Lavendustin A at 1—1000 nM caused a concave-shaped inhibition of insulin release stimulated by 7 mM glucose. The inhibitory effect of lavendustin A was overcome by higher concentrations of glucose. Lavendustin B, the negative control analogue, had no effect on the release. Lavendustin A within a nanomolar range progressively inhibited insulin release by high K^+-depolarization, whereas the inhibitor did not change insulin release by Ca^{2+}-ionophore (A23187). On the contrary, lavendustin A at 10 nM significantly increased insulin release when glucose-induced insulin release was enhanced by either forskolin or 12-*O*-tetradecanoylphorbol 13-acetate, the activators of adenylate cyclase and protein kinase C, respectively. Lavendustin A failed to influence Ca^{2+}-induced insulin release from HIT cells permeabilized with streptolysin-O. These findings suggest that tyrosine kinases may play versatile roles in the control of insulin release from the pancreatic β-cell.

ANALYSIS OF SIGNALING PATHWAY CRITICAL FOR MMP-2 ACTIVATION IN *src*-TRANSFORMED CELLS BY INTERFERON AND DOMINANT NEGATIVE RAS

AYE AYE THANT

Department of Obstetrics and Gynaecology

A large body of evidence suggests that matrix metallo-proteinases (MMPs) that catalyze degradation of extracellular matrix play a crucial role in tumor invasion and metastasis. Despite their importance, signaling pathways critical for the activation of MMP remains largely unclear. Here, we characterized signaling pathways for MMP-2 activation in chicken embryonic fibroblasts (CEF) transformed with *src* or other oncogenes including *yes, fps, ras* and *mos* by interferon (IFN)-treatment and expression of dominant negative form of Ras in *src*-transformed 3Y1 (SR3Y1). We found that transformation by various oncogenes elevated MMP-2 activity by

two steps, i.e. augmented secretion of proenzyme form of MMP-2 followed by its proteolytic activation. Treatment of these transformed CEF with IFN selectively blocked the proteolytic activation of MMP-2, but had no effect on augmented secretion of MMP-2. However we found IFN-treatment suppressed neither Src protein expression nor its kinase activity. IFN-treatment also strongly inhibited invasiveness, cell growth, DNA synthesis and glucose-uptake of transformed cells. These results suggest that proteolytic activation of MMP-2 is indeed critical for invasion, and that signaling pathways critical for these transformation parameters closely couple with that of MMP-2-activation. In contrast, IFN-treatment had no clear effect on transformed morphology, suggesting that its signaling pathway may segregate in part from that of MMP-2-activation. Expression of MT-MMP that catalyses proteolytic activation of MMP-2 was also examined. We found no difference in MT-MMP-expression between transformation and IFN-treatment, suggesting that unidentified factors sensitive to IFN-treatment regulate MMP-2 activation in *src*-transformed CEF. We next studied the activation of MMP-2 by expressing dominant negative Ras in SR3Y1 cells. Almost all successively transfected cells showed lack of augmented secretion and activation of MMP-2, indicating that Ras is probably required for both augmented secretion and activation of MMP-2 in *src*-transformed cell.

THE EFFECT OF HEAT SHOCK RESPONSE BY DITHIOTHREITOL (DTT)

TOSHIKATSU MORI

Department of Oral Surgery

A series of proteins induced by heat shock response and any other stresses are called heat shock protein (Hsp). It is known that rapid induction of Hsp are caused by the combination of heat shock factor (HSF) and heat shock element (HSE). It was recently reported that DTT was a reducing agent and supressed heat shock response by inhibition of HSF activity. To examine the effect of heat shock response by DTT, we added HeLa cells in the existense of DTT or not to various stresses and detected the expression of Hsp70 and Hsp40 by northern blot and western blot. The expression of Hsp70 and Hsp40 were inhibited by DTT under the almost conditions. These results suggests that because the cells are kept reducing condition by DTT, the cells cannot change oxidative condition and heat shock response is difficult to happen.

DEMONSTRATION OF A CA^{2+}/CALMODULIN DEPENDENT PROTEIN KINASE CASCADE IN THE HOG HEART

ARATA UEMURA

3rd Department of Internal Medicine

Members of the Ca^{2+}/calmodulin dependent protein kinase (CaMK) family such as CaMKI, CaMKII, CaMKIV, and CaMK kinase have been identified in the brain and well characterized, but little is known about their equivalents in the heart. Thus only a CaMKII δ isoform has been reported, in 1995. In the present study, CaMKI, CaMKII, and CaMKK as an activator were purified for the first time from the hog heart. Heart CaMKI and CaMKII have isoforms, with molecular weights of 40 and 41kDa for CaMKI, and 52–55kDa for CaMKII on SDS-PAGE. Northern blot analysis revealed heart CaMKI to be the α isoform of brain CaMKI. Both kinases exhibited autophosphorylation as well as phosphorylation of the synthetic peptide, syntide 2, which is homologous to phosphorylation site 2 in glycogen synthase. However, the protein phosphorylation system was found to differ in essentially between CaMKI and CaMKII, only the former requiring an activator to undergo autophosphorylation. That activator showed a 130kDa band on SDS-PAGE. It did not bind a calmodulin column, but phosphorylated brain recombinant CaMKI, while the brain activator did not phosphorylate heart CaMKI. It means that the heart specific activator phosphorylates heart CaMKI. These data indicate that a heart specific CaM kinase cascade, consisting of CaMKI, CaMKII and an activator, exist along with a heart specific protein phosphorylation system. The presence of this CaMK cascade suggests that the heart possesses the highest order of biochemical organization except for the brain.

EFFECT OF KN-62, Ca^{2+}/CALMODULIN-DEPENDENT PROTEIN KINASE II INHIBITOR, ON ADRIMYCIN RESISTANCE OF HUMAN OVARIAN CANCER CELLS

NAOKO OBATA

Department of Obstetrics and Gynecology

We examined effects of an isoquinolinesulfonamide derivative, KN-62, on human ovarian cancer cells, NOS3 and NOS3AR, the latter is resistant to Adriamycin (ADR). MTT assays revealed that 10 μM KN-62 overcame the resistance. KN-62 had little effect on glutathione-S-transferase (GST) activity. In studies on the intracellular accumulation of ADR, KN-62 increased the ADR content in the resistant cells close to the level seen in the sensitive cells. Recent studies revealed that the intracellular ADR content is regulated by P-glycoprotein. On the other hand, KN-62 is a specific inhibitor of Ca^{2+}/calmodulin-dependent protein kinase II (CaM kinase II) and we detected the kinase in NOS3AR cells. These findings suggest that the reversal of the resistance against ADR by KN-62 is mainly due to higher accumulation of ADR in

NOS3AR cells and that CaM kinase II might phosphorylate P-glycoprotein and activate its extrusive function.

EVIDENCES OF ANTAGONISM BETWEEN AMIODARONE AND TRIIODOTHYRONINE ON THE K^+ CHANNEL ACTIVITIES OF CULTURED RAT CARDIOMYOCYTES

WEINONG GUO

Department of Circulation, Research Institute of Environmental Medicine

Effects of acute and chronic treatments with amiodarone, both in the presence and the absence of exogenous triiodothyronine (T_3), on repolarizing outward K^+ currents were investigated by patch-clamp technique in cultured newborn rat ventricular cells. Acute exposure to amiodarone dose-dependently inhibited the transient outward (I_{to}, IC_{50}=4.9 μM) and the steady-state outward (I_K, IC_{50}=6.3 μM) K^+ currents. The dose-response curve of this acute inhibitory action was unaffected by the presence of T_3. When amiodarone was applied chronically, 72-hour exposure to a low dose of the drug (1 μM) significantly decreased the current densities of I_{to} and I_K for the cells cultured in a serum-supplemented medium containing 0.12 nM T_3. In a serum-free medium without T_3, chronic amiodarone treatment revealed null effect on either I_{to} or I_K. In addition, 72-hour in-vitro treatment with T_3 enhanced the current densities of both I_{to} (EC_{50}=0.13 nM) and I_K (EC_{50}=0.33 nM). Concentration-response analysis indicated that amiodarone (1 μM) showed competitive inhibition towards the action of T_3 on I_{to} but noncompetitive inhibition towards the action of T_3 on I_K. These results suggest that different ionic mechanisms are produced by acute and long-term treatments with amiodarone. The latter showed T_3-dependent inhibition of cardiac I_{to} and I_K. When chronically administered, amiodarone may antagonize T_3 and thereby counteract its hormonal effect on K^+ channels. This implies that, at the myocyte level, antagonism of the action of thyroid hormones in K^+ channel activities may contribute to the cardiac effects of chronic amiodarone therapy.

SUSTAINED CONTRACTION PRODUCED BY CAFFEINE AFTER RYANODINE TREATMENT IN THE CIRCULAR MUSCLE OF THE GUINEA-PIG GASTRIC ANTRUM AND RABBIT PORTAL VEIN

JALAL UDDIN CHOWDHURY

1st Department of Physiology

In smooth muscle, caffeine is known to release Ca^{2+} from the sarcoplasmic reticulum (SR) and ryanodine to deplete this store. In the circular muscle of the guinea-pig gastric antrum and

the rabbit portal vein, however, caffeine (10 mM) was found to produce a sustained contraction after its removal when the preparations were pretreated with ryanodine (1μM). The sustained contraction was not affected by Ca^{2+} channel blockers, nicardipine (3μM) or verapamil (3μM), but readily abolished by removal of the external Ca^{2+} or by addition of cobalt (1mM). The caffeine-induced sustained contraction was accompanied by a maintained increase in intracellular Ca^{2+} concentration measured with fura-2, a Ca^{2+}-sensitive fluorescent dye. It is concluded that caffeine can activate a Ca^{2+} influx pathway insensitive to organic Ca^{2+} channel blockers after ryanodine treatment. The plasma membrane of these smooth muscles may have the property similar to the SR membrane, the Ca^{2+} channel of which can be activated by a combination of caffeine and ryanodine.

A CASE-CONTROL STUDY OF ORAL CANCER IN SHENYANG, NORTH-EAST OF CHINA

WEI-ZHONG SU

Department of Oral Surgery

To investigate the risk factors of oral cancer, a hospital-based case-control study was conducted from March 1989 to September 1995 in Shenyang, Liaoning province of China. One hundred one cases (62 males and 39 females) with oral cancer and 101 age-, gender-matched controls without cancer living in Liaoning province were interviewed. The relationships of educational level, living environment, cigarette smoking and alcohol consumption to the risk of oral cancer were estimated in this study. The risk was increased with low educational level and decreased with a higher educational level. People who living in industrial areas appeared a significantly high risk in females. The risks of oral cancer among smokers and drinkers were significantly high relative to non-smoker and non-drinker, respectively. In the analyses for males, the risks increased with increasing consumption of tobacco and alcoholic beverages; non-filtered cigarettes and hand-rolled cigarettes caused higher risks than filter cigarette, and moreover, the risk caused by Baijiu (a kind of Chinese spirit) was significantly higher than that of all other alcoholic beverages; the risk of combining tobacco and alcoholic beverage was found to be significantly higher than smoking or drinking alone.

EXPERIMENTAL STUDY OF DISTRACTION OSTEOGENESIS IN MAXILLOFACIAL REGION

HAJIME OHKUBO

Department of Oral Surgery

The purpose of this study was to clarify whether vertical bone distraction in tabular bone was possible or not using osseointegrated implants as supports for distraction device.

Adult mongrel dogs were used as experimental subjects. Three pure titanium implants were placed along the sagital suture of calvarial bone. After ten weeks, originally fabricated distraction device was attached and then round shaped osteotomy was performed. After healing period, vertical distraction of calvarial bone was performed. Calvarial bone was distracted vertically at a speed of 0.5mm per day, making enlargement of the cranium possible. The animals were sacrificed at 2,4,6 weeks after the completion of distraction. Gross examination, radiographical and histological examination were done.

The gap that resulted from distraction was closed with newly-formed bone. The implants remained stable during experimental period.

The above results show that vertical distraction of thin calvarial bone can be done using osseointegrated implants as supports. This indicates the possibility of broadening the applications of this method to plastic correction of craniomaxillofacial bone.

EFFECTS OF HIGH-INTENSITY RESISTANCE TRAINING ON BONE MINERAL DENSITY IN MALE POWERLIFTERS

SHIGEKI TSUZUKU

Department of Health Promotion Science II

The purpose of this study was to investigate the effects of high-intensity resistance training on bone mineral density (BMD). Ten powerlifters (mean 20.7 ± 1.3 yr, 167.5 ± 6.0cm, 70.6 ± 10.8kg) and eleven controls (mean 18.4 ± 0.7 yr, 168.5 ± 4.8cm, 64.5 ± 9.4kg) volunteered for this study. Isokinetic muscular strength of the upper and lower limbs were measured with the Cybex II isokinetic dynamometer. BMD of lumbar spine (L2-4), femoral neck, Trochanter region, Ward's triangle and whole body was measured using dual-energy x-ray absorptiometry (DEXA). The muscular strength of the upper and lower limbs and the BMD of the lumbar spine and whole body of the powerlifters were found to be significantly greater when compared to the controls. But there were no differences at femoral neck. Trochanter region and Ward's triangle BMD between powerlifters and controls. The positive linear correlation was found between lumbar spine BMD and powerlifting performance. These findings suggest that high-intensity resistance training increase muscular strength and lumbar spine and whole body BMD, but not proximal femur BMD in young male.

EFFECT OF HEAT STRESS ON MUSCLE SYMPATHETIC NERVE ACTIVITY IN HUMANS

YUKI NIIMI

Department of Neurology

To elucidate the effect of heat stress on the sympathetic nervous system, we evaluated changes in muscle sympathetic nerve activity (MSNA), plasma arginine vasopressin (AVP), tympanic temperature, skin blood flow, cardiac output, mean blood pressure, and heart rate in 9 subjects in response to acute heat stress induced by raising the ambient temperature from 29°C to 34°C and then to 40°C. With the heat exposure, MSNA was significantly increased with a significant increase in tympanic temperature. Skin blood flow and heart rate were also significantly increased, while mean blood pressure tended to decline and cardiac output tended to increase. The combination of the increased MSNA and skin blood flow may have caused the redistribution of the circulatory blood volume from the muscles to the skin, facilitating convection heat loss. The increases in MSNA counteracted the lowered blood pressure during heat exposure. Thus, the increased MSNA may play an important role both in thermoregulation and in the maintenance of blood pressure against heat stress.

DYSFUNCTION OF CHOLINERGIC AND DOPAMINERGIC NEURONAL SYSTEMS IN β-AMYLOID PROTEIN-INFUSED RATS

AKIO ITOH

Department of Medical Pharmacy

Accumulations of β-amyloid protein are characteristic and diagnostic features of the brain of Alzheimer's disease patients; however, physiological role of this protein in CNS is unknown. We have previously reported that continuous infusion of β-amyloid protein into rat's cerebral ventricle impairs learning ability and decreases choline acetyltransferase activity, a marker enzyme of cholinergic neuron. In this study, the effects of β-amyloid protein infusion on the release of neurotransmitters in cholinergic and dopaminergic neuronal systems were investigated by using in vivo brain microdialysis method. Nicotine-stimulated release of acetylcholine and dopamine in these animals was significantly lower than that of in vehicle-infused rats. Further, dopamine release induced by high K-stimulation was decreased in β-amyloid protein-infused rats compared to vehicle-infused rats. These results suggest that the release of the two transmitters, acetylcholine and dopamine, was decreased by β-amyloid protein and that learning deficits observed in the β-amyloid protein-infused rats are partly due to the impairment of neurotransmitter release, further, continuous infusion of β-amyloid protein may be a useful method to produce the animal model of Alzheimer's disease.

THE CIRCADIAN RHYTHMS OF CARDIOVASCULAR FUNCTIONS ARE MODULATED BY THE BAROREFLEX AND THE AUTONOMIC NERVOUS SYSTEM IN THE RAT

MITSUTAKA MAKINO

1st Department of Internal Medicine

We assessed the involvement of the baroreflex and the autonomic nervous system in the control of the circadian rhythms of cardiovascular functions by continuously measuring blood pressure (BP), heart rate (HR), and locomotor activity in sinoaortic denervated (SAD), sympathectomized and atropine-injected rats by using a radiotelemetry system. The circadian rhythm of mean blood pressure (MBP) was selectively disrupted in SAD rats under light-dark (LD12:12) cycles as a result of an increase in MBP during the light period, and disappeared under constant darkness (DD). The locomotor activity and HR were not remarkably affected by SAD. The circadian rhythm of MBP was suppressed in sympathectomized rats by decreasing the MBP during the dark period, and the abrupt changes in MBP when the lighting was altered were not seen under LD. Under DD the MBP rhythm similar to that observed under LD was obtained. Sympathectomized rats also showed lower HR levels during the dark period than intact rats under LD cycles. In atropine-injected rats, the MBP and HR increased, especially during the light period, resulting in a reduction of light-dark differences in MBP and HR. The locomotor activity showed an apparent 24-hour variation in the sympathectomized and atropine-injected rats. Findings indicate that the disruption of the baroreflex selectively eliminates the circadian rhythm of BP, and that the circadian rhythms of BP and HR are modulated by the autonomic nervous system in rats. The circadian rhythms of BP and HR are regulated by different mechanisms involving the autonomic nervous system.

THE EFFECT OF THE LOSS OF MOLAR TEETH ON SPATIAL MEMORY AND ACETYLCHOLINE RELEASE FROM THE PARIETAL CORTEX IN AGED RATS

TAKESHI KATO

Department of Oral Surgery

It has been demonstrated that a loss of teeth is a troublesome problem among age-related pathological phenomena of the oral cavity, which influences the entire body, due to the impairment of mastication. The present studies investigated the abilities of learning and memory and acetylcholine (ACh) release in the parietal cortex in aged rats without molar teeth. After the molar teeth of aged rats were extracted, the rats were fed with powdered food for 135 weeks. Although the performance in the radial arm maze was progressively acquired by daily training, an increase number of errors and the decrease initial correct responses were observed in the

aged rats without molar teeth compared to the control aged rats, indicating impaired acquisition of spatial memory in the teethless aged rats. The basal level of extracellular ACh in the parietal cortex was not different between the molar teethless aged rats and the control aged rats. However, the extracellular ACh level of aged teethless rats under high-concentration of K^+ and atropine stimulation was significantly low compared to that of the control aged rats. These results suggest that the impairment of spatial memory in the teethless aged rats may be due to the functional deterioration of the cholinergic neuronal system induced by tooth loss and that there is a possibility that the loss of teeth may be one of the risk factors for senile dementia.

THE MECHANISM OF INTRACELLULAR Ca^{2+} INCREASE BY EXTRACELLULAR ATP IN ISOLATED RABBIT RENAL PROXIMAL TUBULES

HIDEOMI YAMADA

3rd Department of Internal Medicine

The effects of extracellular ATP on cytosolic calcium concentration ($[Ca^{2+}]_i$) and cell membrane potential (Vb) of rabbit renal proximal tubules were investigated using fura-2 and microelectrodes. ATP transiently increased $[Ca^{2+}]_i$ without an apparent sustained phase, and the maximum effect was obtained at 10 μM. ADP, adenosine 5′-*O*-3-thiotriphoshate, and 2-methylthioadenosine 5′-triphosphate were equally effective as ATP, while UTP and adenosine were far less effective. The $[Ca^{2+}]_i$ responses to ATP were strongly inhibited by reactive blue 2, a P2-purinergic receptor antagonist. The removal of extracellular Ca^{2+} as well as the addition of thapsigargin also markedly attenuated the responses to ATP through the depletion of intracellular Ca^{2+} stores. In addition ATP had virtually no effect on Vb except for the occasional small depolarization by 300 μM ATP. These results indicate that extracellular ATP increases $[Ca^{2+}]_i$ through the P2y-purinergic receptor, which primarily mobilizes intracellular Ca^{2+}.

EFFECTS OF NKH477, A NEW WATER-SOLUBLE FORSKOLIN DERIVATIVE, ON HISTAMINE-INDUCED CONTRACTION OF GUINEA PIG AIRWAY SMOOTH MUSCLE

KOHJI SATAKE

2nd Department of Internal Medicine

The bronchorelaxant action of NKH477, a newly developed water-soluble forskolin derivative, was investigated in isolated guinea pig tracheal smooth muscle by simultaneous measurement of isometric tension and the ratio of fura-2 fluorescence signals (R340/380), an index of

intracellular Ca^{2+} concentration ($[Ca^{2+}]$). The tension and R340/380, both of which had elevated by 3 μM histamine, were decreased by 0.1 μM NKH477. These effects of NKH477 were attenuated by pretreatment of the muscle with iberiotoxin (IbTX), a specific blocker of large conductance Ca^{2+} activated K^+ (BK_{Ca}) channels. In the muscle pretreated with 1 μM nifedipine, a blocker of L-type Ca^{2+} channels, the effects of NKH477 to decrease the histamine-induced contraction and elevation of R340/380 were reduced moderately. In the presence of nifedipine, however, the NKH477 actions on the histamine-induced contraction and elevation of R340/380 were not inhibited by IbTX.

These results suggest that the bronchorelaxant action of NKH477 may result, at least in part, from an activation of BK_{Ca} channels, which causes a hyperpolization of smooth muscle cell membrane and secondary Ca^{2+} influx through L-type Ca^{2+} channels leading to a decrease of $[Ca^{2+}]_i$.

FEMALE STERILITY IN BASIGIN NULL MUTANT MICE

NAOHIKO KUNO

Department of Obstetrics and Gynecology

Basigin (Bsg) is a transmembrane glycoprotein belonging to the immunoglobulin superfamily. In contrast to the proposed functions of Bsg, e.g. construction of the blood-brain barrier and induction of matrix metalloproteases, targeted disruption of mouse Bsg gene resulted in loss of the most of null mutant embryos around implantation, both male and female sterility, and neurological abnormalities. Among these phenotypes, the cause of female sterility had been uncertain, which prompted us to perform the present study. Follicles and corpora lutea are observed and spontaneous ovulation is considered to take place in the null mutants, although the intervals of estrous cycle were prolonged and irregular, and the frequency of coitus was reduced. The genital tract and oviduct were not obstructed. In vitro fertilization showed the ability of fertilization of the oocytes of null mutant mice. However, a study of transfer of normal blastocysts into the null mutant uteri exhibited no sign of pregnancy. Expression of basigin in wild type uterus became more intense at the sites of embryo apposition in 4.25-day p.c.. These findings indicate that uterine Bsg is a crucial molecule for pregnancy and suggest that it plays an important role in implantation.

THE *IN VITRO* EXPANSION OF TRIPLET REPEAT SEQUENCES OF GENETIC DISEASES

MIKIYA NAKAYABU

Department of Neurology

The expansion of the triplet repeats is the causation of several human genetic diseases such as myotonic dystrophy, spinobulbar muscular atrophy, spinocerebellar ataxia 1 etc., i.e., novel mutations with nonmendelian genetic properties. We studied the mechanism of expansion of the triplet repeat by *in vitro* DNA synthesis reaction using purfied DNA polymerase α & β, human immunodeficiency virus type 1 reverse transcriptase, large fragment of *E. coli* DNA polymerase 1 with synthetic DNA oligomers. Product DNA analysis revealed that $(CAG)_5/(CTG)_5$ duplex was expanded by these enzymes. In contrast, the triplet repeats duplex with flanking sequences was hardly expanded. When two or five mismatch pairs were introduced into this duplex, a remarkable expansion was observed. These results suggest that the triplet repeat is expanded due to a slippage between template and product during DNA polymerase reaction. The existence of mismatch base pairs greatly enhances the probability of the expansion presumably by the formation of loop or hairpin structure in the product strand. Since mismatched base pairs are created by misincorporation, the fidelity of replication of triplet repeat DNA may be an important factor in the expansion of triplet repeat sequences.

TRANSACTIVATINAL REGURATION OF ANDROGEN RECEPTOR IN X-LINKED SPINAL BULBAR MUSCULAR ATROPHY

SHIGERU MIWA

Department of Neurology

X-linked spinal bulbar muscular atrophy (SBMA) is an adult form of hereditary motor neuropathy, which genetic mutation is an amplification of a polymorphic tandem CAG repeat in the androgen receptor (AR) gene (CAG; the codon of glutamine, polyglutamine stretch in AR molecule). It is important to know the relationship between the pathogenesis of this disease and the CAG expansion in the AR gene. In this study, we examined if any difference was present in AR binding to the androgen responsive DNA fragment between SBMA and control subjects. We extracted nuclear and cytosol proteins from cultured scrotal skin fibroblasts derived from ten SBMA patients and ten control subjects. Western blotting indicated that the amount of nuclear AR molecules was decreased and its reactive band size was large in SBMA, compared with control. Nuclear extract applied on the gel-mobility shift assay showed increased signals of the shifted band in SBMA, furthermore, the bound activity correlated with the number of CAG repeat. This finding suggests that the increased interaction between the androgen responsive element and AR molecules in SBMA subjects might change the transactivational regulation of AR.

DETECTION OF *ras* GENE MUTATIONS IN PERIOPERATIVE PERIPHERAL BLOOD WITH PANCREATIC ADENOCARCINOMA

SHUJI NOMOTO

2nd Department of Surgery

Surgeons wish to know of any correlation between an operation and incidence of metastasis. In perioperative periods, pancreatic cancer cells were identified by detecting mutant K-*ras* gene by two-step PCR and RFLP analysis in blood samples taken from peripheral blood. In no case was K-*ras* point mutation detected in blood before operation, although mutant bands were observed in all cases at the time lesions were resected. Surprisingly, in five of ten cases, the mutant bands were identified just after laparotomy, before we had reached the primary lesion. In almost all cases, mutant K-*ras* was detected until the fourteen postoperative day. These findings suggest that cancer cells exist in the circulation, and have a potential for hematogenous metastasis during the perioperative period. In conclusion, surgical stress causes hematogenous dissemination of pancreatic cancer cells, and surgeons should employ the appropriate anti-metastasis therapy in the perioperative period.

TWO NOVEL MUTATIONS IN THE CODING REGION FOR NEUROPHYSIN II ASSOCIATED WITH FAMILIAL CENTRAL DIABETES INSIPIDUS

HIROSHI NAGASAKI

First Department of Internal Medicine

Familial central diabetes insipidus (FDI) is an autosomal dominant disease caused by a deficiency of arginine vasopressin (AVP). We have previously reported 3 distinct mutations in the AVP gene in Japanese FDI pedigrees, which result in a substitution of Ser for Gly 57 in the neurophysin II (NPII) moiety of the AVP precursor, a substitution of Thr for Ala at the COOH terminus of the signal peptide and a deletion of Glu 47 in the NPII moiety. In this study, we analyzed the AVP gene in two pedigrees by direct sequencing of the PCR-amplified DNA and found two novel mutations in exon 2 which encodes the central part of the NPII moiety of the precursor. The mutation in one pedigree was a C to A transition at nucleotide position 1891, which replaces Cys 67 (TGC) with stop codon (TGA). As the premature termination eliminates part of the COOH domain of the NPII moiety and the glycoprotein moiety, the conformation of the truncated protein is likely to be markedly different from that of normal precursor. In another pedigree, a G to T transversion was detected at nucleotide position 1874 which substitutes polar Trp (TGG) for hydrophobic Gly 62 (GGG). It is possible that mutated NPII molecules, as a consequence of a conformational change, can not bind AVP or self-associate to form higher oligomer complexes. Interestingly, all mutations we have identified to date, with the exception of

the signal peptide mutation, are located in exon 2, suggesting the importance of the highly conserved central part of the NPII molecules and/or the NPII moiety in the precursor for the AVP synthesis.

RECOMBINANT ADENO-ASSOCIATED VIRUS MEDIATED GENE TRANSFER INTO HUMAN LEUKEMIA CELL LINES

TOSHIHIDE ITOU

1st Department of Internal Medicine

Adeno-associated virus (AAV) based vector is one of the promising gene transfer vehicles by virtue of the characteristics of wild-type AAV: tropism to a wide range of human tissues and locus specific integration at chromosome 19q13.3. To elucidate the nature of the recombinant AAV, transduction of neomycin phosphotransferase enzyme gene (NeoR gene) into seven human leukemia cell lines was performed. Transduction efficiencies were assessed by the colony formation assay and by the limiting dilution assay. The results from both assays are highly comparable. Transduction efficiencies of the NeoR gene into K-562, MEG-O1, Raji, MOLT-3, HL-60, U937 and NKM-1 at an MOI of 1 were 2.7%, 2.5%, 0.15%, 0.09%, 0.09%, $<0.025\%$ and $<0.025\%$, respectively. Integration of the NeoR gene into the host genome was detected by Southern blotting analysis. Various sizes of restriction fragments suggested random integration. Fluorescent in-situ hybridization (FISH) study was carried out in four MEG-O1 clones. The NeoR gene was found to be integrated into the host genome (chromosome 1q, 2q and 13) at sites other than chromosome 19q13.3.

MURINE MODEL OF CANCER GENE THERAPY USING ADENOVIRUS VECTORS

HONG YI

Department of Immunology

In human, adenovirus is a very common infectious virus. Adenoviral infection usually induces anti-adenoviral humoral and cellular immunity. Recently adenovirus vector was invented for gene therapy, which was very efficient for infection to human cells. We planned to use this vector for cancer treatment in murine model. We first administered adenovirus carrying LacZ gene (AxCALacZ) to DBA/2 mice intraperitoneally, then two weeks later, P815 tumor cells infected with AxCALacZ were inoculated intraperitoneally to the mice immunized by AxCALacZ. Mice immunized with AxCALacZ rejected the P815 tumor infected with AxCALacZ and survived for long periods. The peritoneal T cells in adenovirus-immune mice had strong killing of adenovirus-infected P815 cells. Further, the mice, which rejected P815 tumor infected

with AxCALacZ, were challenged with parental P815 tumor cells. All mice rejected P815 tumor cells and survived for long periods. These mice developed P815 specific tumor immunity, which was confirmed by cytotoxicity assay.

RETROVIRAL INTRODUCTION OF THE P16 GENE INTO MURINE CELL LINES TO ELICIT MARKED ANTIPROLIFERATIVE EFFECTS

HIROSHI IKEDA

Department of Neurosurgery

In the past few years, several cyclin-dependent kinase inhibitors involved in the negative regulation of the cell cycle have been cloned. The p16 gene is one of these members and is mutated in many transformed cell lines and some primary tumor tissues. Therefore, it is expected to be a candidate for tumor suppressors. We have examined this possibility in murine cell lines (NIH3T3 and RSV-M) which were proved to lack a p16 gene expression. Full-length human p16 cDNA was obtained from a HeLa cell line using PCR amplification. We constructed two separate retrovirus vectors carrying this p16 cDNA. First, we transduced the p16 cDNA into murine cell lines by the retrovirus vector harboring the neomycine-resistance gene. The p16 gene-transduced cells formed no colonies after selection with G418, as compared to the vector-transduced cells. Second, we used another retrovirus vector that expresses both the p16 cDNA and the Lac Z gene, which enables us to distinguish the affected cells from the unaffected ones. Proliferation of the p16 gene-transduced cells was markedly inhibited and their morphological change was also observed. Thus, we concluded that the p16 gene has a high potential as an anti-proliferative agent of the cell cycle and that the loss of its function may play a role in dysregulated proliferation of the cells.

AMINO ACID SUBSTITUTIONS OF THYROID HORMONE RECEPTOR β AT CODON 435 WITH RESISTANCE TO THYROID HORMONE SELECTIVELY ALTER HOMODIMER FORMATION

YOSHIO NOMURA

Department of Endocrinology and Metabolism, Division of Molecular and Cellular Adaptation, Research Institute of Environmental Medicine

Thyroid hormone action is mediated through its nuclear receptors (TRs), which bind to target DNA sequences (TRE) as a homodimer or a heterodimer with 9-cis retinoic acid receptors (RXRs). Mutations of TRβ identified in patients with resistance to thyroid hormone (RTH) cluster primarily at two areas separated by the putative dimerization region. Two TRβ mutations

were newly found in patients with RTH at codon 435 histidine (H435L, H435Q) close to the dimerization region. To study how the side chain charge of amino acids at this position affect receptor characteristics, T_3 binding activity, receptor dimerization, transcriptional activity, and dominant negative action were analyzed in two RTH mutants and two additional artificial mutants (H435R, H435E). T_3 binding affinities of all four mutants were below detection. In electrophoretic mobility shift assay (EMSA) using TRE-DR4 or the inverted palindrome (Lap), heterodimer formation of mutant receptors with RXRα was similar to wild type. However, homodimer formation varied among mutant receptors, especially using TRE-DR4, with a rank order of wild type=H435R > H435Q > H435L ≫ H435E. In the presence of a basic amino acid at codon 435, homodimer formation was preserved, while substitution to neutral or acidic amino acids resulted in decreased homodimer formation. In transient transfection assays using reporter genes under the control of either 2xPal-TK, DR4-TK, Lap-TK or TSHα promoter, these four mutants were inactive in T_3 dependent transcriptional activation. Dominant negative inhibition was similar for all four mutants. These results indicate that 1) newly found TRβ mutations at codon 435 are responsible for RTH and 2) codon 435 in TRβ is located at a position which can predominantly alter homodimer formation on certain TREs such as DR4.