ANNUAL RESEARCH MEETING

FOR

GRADUATE STUDENTS

Nagoya, July 1999

Abstracts

SYNERGISM OF PROTEIN KINASES A, C AND MYOSIN LIGHT CHAIN KINASE IN THE SECRETORY CASCADE OF THE PANCREATIC BETA-CELL

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Protein phosphorylation by myosin light chain kinase (MLCK), protein kinases A and C (PKA and PKC, respectively) play positive roles in insulin secretion from the pancreatic beta-cell. To investigate the underlying mechanisms, we examined intracellular immunofluorescence and immunoelectron microscopes, and also investigated intracellular traffic of the granules in cultured beta-cell (MIN6) by video-microscopy. Considerable parts of MLCK immunoreactivity were co-localized with the insulin granules. Subcellular fractionation of MIN6 cell extracts revealed that myosin light chain (MLC) may be distributed with the insulin-rich fractions, and immunofluorescence staining using specific antibodies against mono- and di-phosphorylated MLCs depicted presence of phosphorylated MLCs in cytoplasm, in part, with co-localization with the insulin granules. Activation of PKC by 12-*O*-tetradecanoyl-phorbol 13-acetate (TPA) caused a shift of both insulin granules and MLCK to the cell periphery, which was not reproduced by the adenylate cyclase activator, forskolin. In contrast, forskolin, not TPA, increased the granule movement. Co-stimulation of the beta-cell by TPA and forskolin induced drastic translocation of insulin granules and MLCK to the cell periphery, resulting in enormous potentiation of insulin release. These findings suggest these protein kinases increase insulin granules in the ready-releasable pool via acting on different steps in the secretory cascade.

MIDKINE RESCUES WILMS' TUMOR CELLS FROM CISPLATIN-INDUCED APOPTOSIS: REGULATION OF BCL-2 EXPRESSION BY MIDKINE

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Midkine (MK) is a heparin-binding growth factor that is involved in diverse biological phenomena, *e.g.* neuronal survival, carcinogenesis and tissue repair. MK expression is detected mainly in the kidney in adult mice. In this study, we show that, at a dose that can induce a recoverable renal damage and induce apoptosis, cisplatin (CDDP) transiently suppressed MK expression in mouse kidney. *In vitro*, CDDP suppressed MK expression and induced apoptosis in cultured G401, a Wilms' tumor cell line. Exogenous MK protein partially rescued G401 cells from CDDP-induced apoptosis. MK enhanced the expression of Bcl-2, but not that of Bcl-x_L, in G401 cells in a dose-dependent manner, and prevented the Bcl-2 reduction due to CDDP. Moreover, Bcl-2 expression in mouse kidneys was also transiently suppressed by CDDP

treatment, and its expression profile was similar to that of MK. These results imply that MK exerts a cytoprotective activity from a damaging insult, presumably at least in part through enhancing the expression of Bcl-2.

HYDROCEPHALUS IN MICE FOLLOWING X-IRRADIATION AT EARLY GESTATIONAL STAGE: POSSIBLY DUE TO PERSISTENT DECELERATION OF CELL PROLIFERATION

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Pregnant mice were exposed to at 1.4 Gy X-radiation on gestational day 7 (G7). Cell death was evident in the neuroepithelium 4 hr after exposure. The lamina terminalis of the irradiated brain of G9 was thinner than that of the control. The brain mantle was thinner and lateral ventricles were larger in the irradiated brain than those in controls; the corpus striatum and thalamus did not develop well in the embryonic stage. However, no differences in the external appearance were noted between hydrocephalic and control groups. The cerebral aqueduct was open in the hydrocephalic brains on G15, G17 and G19 when the lateral ventricles were markedly dilated. Stenosis of the cerebral aqueduct occurred postnatally. In proliferating cell nuclear antigen (PCNA)-immunostaining, PCNA-positive cells were few in the irradiated neuroepithelium and mesenchyme at 4 hr after exposure. Even on G11 and G13 the ratio of PCNA-positive cells was lower in the irradiated brain than those of the control, indicating a decelerated proliferation of successive cell generations following exposure. Cell death in the neuroepithelium and persistent deceleration of neural cell proliferation resulting in the underdevelopment of brain parenchyma with compensatory ventricular dilatation might be the cause of hydrocephalus.

DISTURBED MIGRATION OF PURKINJE CELLS AND ABNORMAL FOLIATION OF THE CEREBELLUM OF THE RAT FOLLOWING PRENATAL EXPOSURE TO X-IRRADIATION

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X-irradiation during the latter gestational period in rats causes abnormal foliation with heterotopic Purkinje cells in the cerebellum. Pregnant rats were exposed to 2.5 Gy X-radiation on gestational day 21, and the expressions of Reelin, neural cell adhesion molecule (NCAM), fibronectin and tenascin in the cerebellum were examined by immunohistochemistry. Six hours after exposure, extensive cell death was evident in the external granular layer (EGL). On post-

natal day (P) 9, while Purkinje cells were aligned underneath the EGL and their dendrites had developed in the control, Purkinje cells with shorter and abnormally oriented dendrites failed to align in the irradiated rat. Those remained in the heterotopic location, then abnormal folia developed. Reelin expression in the EGL and internal granular layer was decreased by the exposure and the decrease continued until P9. On the other hand, NCAM, fibronectin and tenascin expressions were not affected by X-radiation. The expression of *reelin* mRNA was examined by Northern blot analysis. X-radiation caused significant decrease in *reelin* mRNA from P0 to P9. Since Reelin is known to play a pivotal role in the migration of neurons, our current results suggest that the lack of Reelin is responsible for the deranged patterning of Purkinje cells.

β-ADRENERGIC MODULATION OF L-TYPE Ca^{2+}-CHANNEL CURRENTS IN EARLY-STAGE EMBRYONIC MOUSE HEART

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Little information is available concerning the modulation of cardiac function by β-adrenergic agonists in early-stage embryonic mammalian heart. We have examined the effects of isoproterenol (ISO) on the spontaneous beating rate and action potential (AP) configuration in embryonic mouse hearts at 9.5 days postcoitum (dpc), just 1 day after they started to beat. ISO (3 μM) increased the spontaneous beating rate in whole hearts, dissected ventricles, and isolated ventricular myocytes. In ventricular myocytes, ISO also increased the slope of the pacemaker potential and the action potential duration but decreased the maximum upstroke velocity. In whole cell voltage-clamp experiments, the Ca^{2+}-channel currents were measured as Ba^{2+} currents (I_{Ba}). I_{Ba} was significantly enhanced from –4.7 ± 0.9 to –6.7 ± 1.2 pA/pF (by 52.4 ± 14.8%, n = 10) by ISO (3 μM). Propranolol (3 μM) reversed the effect of ISO. Forskolin (FOR, 10 μM) produced an increase in I_{Ba} by 95.5 ± 18.8% (n = 8). To compare the developmental changes in responses to ISO and FOR, we also investigated the effects of ISO and FOR on I_{Ba} in late-stage (18 dpc) ventricular myocytes. ISO (3 μM) caused an appreciably greater increase in I_{Ba} from –6.2 ± 0.5 to –14.5 ± 2.2 pA/pF (by 137.8 ± 33.0%, n = 8). In contrast, the increase in I_{Ba} by 10 μM FOR at 18 dpc (by 120.0 ± 23.0%, n = 7) was comparable to that observed at 9.5 dpc ($P > 0.05$). These results indicate that the L-type Ca^{2+}-channel currents are modulated by β-adrenergic receptors in the embryonic mouse heart as early as 9.5 dpc, probably via a cAMP-dependent pathway.

BAROREFLEX CONTROL OF MUSCLE SYMPATHETIC NERVE ACTIVITY AFTER 120 DAYS OF 6° HEAD-DOWN BED REST

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To examine how long-lasting microgravity simulated by 6° head-down bed rest (HDBR) induces changes in the baroreflex control of muscle sympathetic nerve activity (MSNA) at rest and changes in responses of MSNA to orthostasis, six healthy male volunteers (range, 26–42 years) participated in the Valsalva's maneuver and head-up tilt (HUT) tests before and after 120 days of HDBR. MSNA was directly measured using a microneurographic technique. After long-term HDBR, resting supine MSNA and the heart rate were augmented. The baroreflex slopes for MSNA during the Valsalva's maneuver (in supine position) and during 60° HUT test, determined by least squares linear regression analysis, were significantly steeper after than before HDBR, while the baroreflex slopes for RR interval after HDBR were significantly flatter than before. The increase in MSNA from supine to 60° HUT was similar between before and after HDBR, but the mean blood pressure decreased in 60° HUT after HDBR. In conclusion, the baroreflex control of MSNA was augmented, while the control of RR interval was attenuated after 120 days of HDBR.

VARIED B CELL IMMUNOPHENOTYPES OF HODGKIN/ REED-STERNBERG CELLS IN CLASSIC HODGKIN'S DISEASE

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There is controversy over reports of B cell expression in Hodgkin or Reed-Sternberg (HRS) cells. We studied 51 cases of classic Hodgkin's disease to immunohistochemically characterize HRS cells for pan B cell markers and specific markers for plasma cells. HRS cells expressed CD20 in 35%, CD19 in 18%, CD79a in 25%, CD40 in 67%, CD138 in 45% and PCA-1 in 45%. In 94% cases, HRS cells expressed more than one B cell marker. We then classified cases into those positive for plasma cell markers (n=27) (group 1) and those negative for them (n=24) (group 2). The average age in group 1 (40 years) was younger than in group 2 (54 years) ($P< .05$). The percentage of nodular sclerosis (NS) subtype in group 1 (52%) was 1.5 times greater than group 2 (33%) ($P< .05$). With regard to Epstein-Barr virus encoded small RNA (EBER) in situ hybridization, 14 cases (64%) were positive in group 2, but only 7 cases (31%) were positive in group 1 ($P< .025$). HRS cells in most cases of classic Hodgkin's disease expressed later stage of B cell development. We consider that two different clinicopathological groups may correlate with the two different expressions of B cell markers.

METHYLGLYOXAL INDUCES APOPTOSIS IN JURKAT LEUKEMIA T-CELLS BY ACTIVATING C-JUN N-TERMINAL KINASE

JUN DU

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Methylglyoxal (MG) is a physiological metabolite but is known to be toxic, inducing stress in cells and causing apoptosis. In this study, we examined molecular mechanisms in the MG-induced signal transduction leading to apoptosis, focusing especially on the role of JNK activation. We first confirmed that MG caused apoptosis in Jurkat cells, and that it was cell-type dependent because it failed to induce apoptosis in MOLT4, HeLa, or COS-7 cells. A caspase inhibitor, Z-DEVD-fmk, completely blocked MG-induced PARP cleavage and apoptosis, showing the critical role of caspase activation. Inhibition of JNK activity by a JNK inhibitor, curcumin, reduced MG-induced caspase-3 activation, PARP cleavage and apoptosis. Stable expression of the dominant negative mutant of JNK also protected cells against apoptosis. Loss of the mitochondrial membrane potential induced by MG was decreased by the dominant negative JNK. These results confirmed an essential role of JNK working upstream of caspases, and a possible involvement of JNK in affecting the mitochondrial membrane potential.

CHARACTERIZATION OF THE HERPES SIMPLEX VIRUS TYPE 2 (HSV-2) US2 GENE PRODUCT AND A US2-DEFICIENT HSV-2 MUTANT

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The herpes simplex virus type 2 (HSV-2) US2 gene product was identified by using a rabbit polyclonal antiserum raised against a recombinant 6 × His-US2 fusion protein expressed in Escherichia coli. The antiserum reacted specifically with a 39 kDa protein in HSV-2 strain 186-infected cell lysates. The protein was not detectable in the presence of the virus DNA synthesis inhibitor phosphonoacetic acid. Indirect immunofluorescence studies localized the US2 protein in the cytoplasm and as discrete granules at late times post-infection within and at the periphery of the nucleus, and nuclear fractionation studies showed that the protein was partially associated with the nuclear matrix of infected cells. The protein was easily detected in purified virions. Also, a US2 insertion mutant was constructed which contained an ICP6-*lacZ* insertion in the US2 gene. This mutant was as virulent as wild-type virus in mice when inoculated by the footpad route. The importance of the US2 protein of HSV-2 in the virus life-cycle may be apparent only in the natural human host.

CIGARETTE SMOKING AS A RISK FACTOR FOR CHRONIC PANCREATITIS: A CASE-CONTROL STUDY IN JAPAN

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We conducted a hospital-based case-control study to examine the association of cigarette smoking with chronic pancreatitis. Ninety one male patients with chronic pancreatitis newly diagnosed from July 1997 to December 1998 were recruited as cases, and 175 controls were individually matched to each case for gender, age (±5 years), hospital, and time of the first visit to a hospital (±1 year). A self-administered questionnaire was used to collect information on use of tobacco and alcohol, diet and other factors. Odds ratios (ORs) and their 95% confidence intervals (CIs) were calculated by multiple conditional logistic models, adjusting for body mass index, education level and alcohol drinking. Compared with non-smokers, the ORs (95% CIs) were 7.8 (2.2–27.3) for all current smokers, and 14.7 (3.1–69.9), 5.5 (1.5–20.1), 12.2 (2.4–71.0) for those consuming <20, 20–39 and ≥40 cigarettes per day, respectively. Risk of chronic pancreatitis significantly increased with increasing cumulative amount of smoking ($p<0.05$). Analysis for the effect of combined use of tobacco and alcohol showed that cigarette smoking was associated with the higher risk in both of the two alcohol consumption level. Our findings indicated that cigarette smoking may be an independent and significant risk factor for chronic pancreatitis.

VEGETABLES, HIGH NITRATE FOODS, INCREASED BREATH NITROUS OXIDE

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We have demonstrated nitrous oxide (N_2O), a metabolite of the reduction of nitrate (NO_3^-) by microflora, in exhaled air. The purpose of this study is to examine the effect of ingesting vegetables, which contain high levels of NO_3^-, on breath N_2O levels. We measured exhaled N_2O in six healthy subjects aged 20–41 years at 15-min intervals after the following: 1) no ingestion; 2) ingestion of 180 g of vegetable juice; and 3) ingestion of 50 g of lettuce. N_2O levels were measured by an infrared-photoacoustic (IR-PAS) analyzer. N_2O was detectable in all subjects regardless of treatment protocol. Lettuce and vegetable juice significantly increased breath N_2O levels. Total excretions in breath N_2O were significantly higher in lettuce group (P=0.028) and vegetable group (P=0.046) than controls. Breath N_2O increases after vegetable ingestion, probably due to denitrification of NO_3^- by normal microflora in the intestinal tract.

SEVERE PURPURA FULMINANS IS CAUSED BY HOMOZYGOUS Δ8857 MUTATION (PROTEIN C-NAGOYA) AND SUCCESSFULLY TREATED WITH ACTIVATED-PROTEIN C CONCENTRATE

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Purpura fulminans is fatal and occurs in patients with homozygous protein C (PC) deficiency. We report a Japanese patient who developed purpura fulminans and DIC with undetectable plasma PC activity and antigen. Oral anticoagulation and injection of FFP were started but had no effects. Subsequently, specific therapy was introduced with intravenous infusions of purified activated-protein C (APC) concentrate, then her DIC and necrotic skin lesions improved markedly. DNA sequence analysis identified a single nucleotide deletion of nt 8857 within the exon IX. This mutation, reported as protein C Nagoya, results in the aberrant polypeptide addition from residue 381 of the mutant protein C. Impaired intracellular transport and protein maturation would be the cause of the complete absence of PC. DNA study of the family member revealed that the patient was homozygous for the mutation, although the heterozygous parents were asymptomatic. This is the seventh case of the mutation that has been exclusively reported in Japan, but is the first report of the homozygous case. These facts may indicate that protein C Nagoya is one of the risk factors of thrombosis in Japanese population.

METHYLATION STATUS OF THE *p15^{INK4B}* GENE IN HEMATOPOIETIC PROGENITORS AND PERIPHERAL BLOOD CELLS IN MYELODYSPLASTIC SYNDROMES

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To know whether *p15^{INK4B}* gene methylation is restricted to undifferentiated blastic cells, or whether differentiated cells of MDS-origin also harbor this epigenetic alteration, we analyzed the methylation status of the *p15^{INK4B}* gene in MDS by the methylation-specific PCR (MSP) method. The bone marrow mononuclear cells (BM-MNCs) of 23 MDS patients were analyzed, and 6 of them showed *p15^{INK4B}* methylation. In simultaneous progenitor cell assay, 2 of the 6 patients with *p15^{INK4B}*-methylated BM-MNCs, erythroid and/or non-erythroid colonies formed were subjected to molecular analysis. Colonies with and without *p15^{INK4B}* methylation were detected in both patients. Furthermore, X-chromosome inactivation (XCI) pattern of each colony was simultaneously determined by MSP-based *human androgen receptor* gene analysis, and all *p15^{INK4B}*-methylated colonies showed the same XCI pattern, which was dominant among the

colonies, while $p15^{INK4B}$-unmethylated colonies showed both patterns of XCI, in each of the two patients. We then examined the methylation status of the $p15^{INK4B}$ gene of granulocyte (PB-PMN) fractions from 10 patients with available peripheral blood cells. In all four patients with $p15^{INK4B}$-methylated BM-MNCs, their PB-PMNs showed $p15^{INK4B}$ methylation. These results suggest that $p15^{INK4B}$ methylation in hematopoietic cells in MDS patients is restricted to the MDS clone but not necessarily to blast cells.

PROTAMINE AUGMENTS ENDOTHELIAL CALCIUM RESPONSE INDUCED BY MECHANICAL STRETCH

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In response to mechanical stretch, the vascular endothelium exhibits various responses. However, the molecular mechanisms by which the cells sense the mechanical forces remain unknown. One possible candidate of mechanosensors in endothelial cells is a stretch activated cation channel (SACCat) sensitive to micromolar concentrations of Gd^{3+}. We have developed a system for measuring $[Ca^{2+}]_i$ mobilization in cells subjected to controlled uniaxial stretch of their substrate. Using this system, we observed that the $[Ca^{2+}]$: response to stretch was dependent on extracellular Ca^{2+} and it is inhibited by Gd^{3+}. We have thus reconfirmed our previous finding that activation of SACCat is involved in Ca^{2+} mobilization to mechanical stretch in endothelial cells. Furthermore, we have observed that the stretch-induced $[Ca^{2+}]_i$ increase in vascular endothelium is augmented by basic polypeptides such as protamine. This augmented response was not inhibited with Gd^{3+} or by deprivation of extracellular calcium. In contrast, it was significantly inhibited by depletion of intracellular calcium stores with thapsigargin or by pretreatment with phospholipase inhibitors such as U73122 and manoalide. These results suggest the presence of a mechanoreceptor distinct from the SACCat in vascular endothelium. This augmented calcium response may contribute to hypotension induced by application of protamine to neutralize heparin after cardiovascular surgery.

ARE AUTOANTIBODIES AGAINST LEWIS ANTIGENS INVOLVED IN THE PATHOGENESIS OF *HELICOBACTER PYLORI*-INDUCED PEPTIC ULCERS?

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We examined whether anti-Lewis x (Le^x) and y (Le^y) autoantibodies affect the pathogenesis

of *H. pylori*-induced peptic ulcers. Of 11 patients with peptic ulcers, 10 patients had both anti-Le^x and -Le^y immunoglobulin G (IgG) antibodies, and 1 patient had only anti-Le^x antibody. After successful eradication of *H. pylori*, we measured the serum titer of anti-Le^x and -Le^y antibodies. Six patients had reduction of the titers of anti-Le^x and/or -Le^y antibodies, whereas no notable changes were detected in 5 patients in the follow-up. This result suggests that anti-Le^x and -Le^y autoantibodies had no critical role in the development of *H. pylori*-induced peptic ulcer.

ARGININE VASOPRESSIN INHIBITS FLUID SECRETION IN GUINEA-PIG PANCREATIC DUCT CELLS

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The effects of arginine vasopressin (AVP) on pancreatic ductal secretion were studied in guinea-pigs.

In the isolated vascularly-perfused pancreas, AVP reduced secretin-stimulated fluid secretion and increased the vascular resistance when the perfusion rate was held constant.

In the isolated interlobular duct segments, AVP inhibited secretin-stimulated fluid secretion, indicating the direct inhibitory action of AVP on the duct cells. AVP affected neither the basal nor secretin-induced cyclic AMP productions, suggesting that AVP inhibits the fluid secretion at a point distal to the production of cyclic AMP. AVP increased intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in the absence of extracellular Ca^{2+}. When $[Ca^{2+}]_i$ was elevated by the application of thapsigargin, AVP caused a rapid decrease in $[Ca^{2+}]_i$.

AVP seems to activate both Ca^{2+} release from intracellular stores and Ca^{2+} efflux across the plasma membrane, but its relation to the inhibition of fluid secretion remains to be clarified.

It is concluded that AVP directly inhibits secretin-stimulated ductal fluid secretion in the guinea-pig pancreas.

LEVELS OF EXPRESSION OF PLEIOTROPHIN AND PROTEIN TYROSINE PHOSPHATASE ζ ARE DECREASED IN HUMAN COLORECTAL CANCERS

TAISHI YAMAKAWA

3rd Department of Internal Medcine

Pleiotrophin (PTN) and midkine (MK) form a distinct family of heparin binding growth factors. In a variety of human cancers, MK mRNA levels have been found to be increased as

compared to adjacent non-cancerous tissues. We examined the expression of PTN, its putative receptor, namely protein tyrosine phosphatase ζ (PTP ζ), and a related protein, receptor-type protein tyrosine phosphatase γ (RPTP γ), in human colorectal cancers and the adjacent normal mucosae. PTN and PTP ζ mRNA levels were generally decreased in colorectal cancers as compared to those in adjacent normal mucosae, while the RPTP γ level was not significantly different between them.

HIGH-RESOLUTION MR CISTERNOGRAPHY OF THE CEREBELLOPONTINE ANGLE: 2D VERSUS 3D FAST SPIN-ECHO SEQUENCES

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Our purpose was to investigate the cause of signal loss in cerebrospinal fluid (CSF) in the prepontine or cerebellopontine angle cistern on 2D fast spin-echo sequences (FSE) MR images and to compare the cisternographic effects of 2D and 3D FSE sequences. Comparisons were made to assess the effects of intravoxel dephasing, amplitude of the section-selecting gradient, wash-out phenomenon, echo time, and section thickness. The signal loss in CSF in thin-section 2D MR cisternography was mainly attributable to the wash-out phenomenon. Four healthy subjects and 13 patients with ear symptoms were examined, and multisection 3-mm-thick 2D images and 30-mm-slab, 1-mm-section 3D images were compared qualitatively and quantitatively. The contrast-to-noise ratio between CSF and the cerebellar peduncle, and the visibility of cranial nerves and the vertebrobasilar artery were significantly improved on 3D images in 17 subjects. In images from 400 patients, no significant signal loss in the cistern was observed using 3D FSE. We conclude that MR cisternography should be performed using a 3D acquisition.

EVALUATION OF SLEEP APNEA SYNDROME WITH LOW-FIELD MAGNETIC RESONANCE FLUOROSCOPY

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We tried to assess the upper airway status of the sleep apnea syndrome (SAS) patients with low-field MR fluoroscopy. Twenty patients with clinically diagnosed SAS underwent the upper airway monitoring using MR fluoroscopy for 5 minutes while awake and for 30 minutes while asleep. No patients required any sedative drugs because of the very small gradient noise except one case. No occlusion was observed while patients were awake. Nine showed repeated occlu-

sion at retropalatal (Rp) pharynx, while 11 demonstrated both simple Rp occlusion and combined retropalato-retroglossal (Rp+Rg) occlusions (complex occlusion). The mean frequency of occlusion in complex cases was significantly higher than that in simple Rp cases ($p<0.05$).

Low-field MR fluoroscopy was useful in determining the occlusion level while asleep in patient with SAS because of its quiet gantry and long-term monitoring capability. The MR fluoroscopy technique should prove to be a valuable clinical tool for the diagnosis and for determining the appropriate therapy in patients with SAS.

FIBRIN GEL INDUCES THE MIGATION OF SMOOTH MUSCLE CELLS FROM RABBIT AORTIC EXPLANTS

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A major step in the pathogenesis of atherosclerosis is the vectorial migration of smooth muscle cells (SMCs) from the arterial media into the intima. In the present study, we utilized an *in vitro* assay system to evaluate the effects of fibrin gels on the migration of SMCs from explants taken from rabbit aorta. After cultured for 5–7 days in a serum-free condition, the first SMC(s) appeared from explants covered with a fibrin gel. No migration of SMCs from the control explants without fibrin gel was observed. Then the number of migrating cells increased with time. The migration of SMCs into fibrin gels was not dependent on the thrombin concentration in the range of 0.25–1.25 U/ml. A monoclonal antibody to $\alpha v\beta 3$, LM609, completely inhibited the migration of SMCs from the explants, suggesting that $\alpha v\beta 3$ integrin is involved in the migration of SMCs into fibrin gels. SMCs which migrated from the explants showed the positive staining with monoclonal anti-SMC myosin heavy-chain isoform antibodies, SMemb, SM1 and SM2, suggesting that they are in an intermediate state between contractile and synthetic states. In conclusion, the present study showed that fibrin gel itself induces the migration of SMCs from explants.

ZEP: A NOVEL ZINC FINGER PROTEIN CONTAINING A LARGE PROLINE-RICH DOMAIN

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Zep, a novel 49 kDa zinc finger protein, was found in the brain of day-13 mouse embryos and cloned. Zep contains two C2H2-type zinc finger motifs close to the N-terminal region. The majority of the molecule is composed of a proline-rich domain showing similarity to proline-rich domains in transcription factors and a salivary proline-rich protein. In addition to the pro-

line-rich domain, Zep has an acidic domain and a serine/threonine-rich domain, all of which are frequently found in many transcription factors. The overall organization of Zep shows no similarity to any other proteins. There is a nuclear localization signal in Zep, and the Zep-GFP (green fluorescent protein) fusion protein is located predominantly in the nucleus. In the day-13 mouse embryo, Zep is strongly expressed in the nervous system, i.e. brain, spinal cord, and dorsal root ganglia, with strong to weak expression observed in other regions. Zep continues to be strongly expressed in the neonatal brain; however, its expression is weak in the brain and spinal cord of adult mice. *In situ* hybridization reveals strong signals for Zep mRNA in the cerebellum and olfactory bulb with moderate signals detected in the hippocampus and cortex. Strong Zep expression is observed in adult thymus, lung, spleen, testis, and ovary. Zep may be involved in the formation and remodeling of various tissues including nervous tissue, probably through transcriptional regulation.

EXPRESSION OF GDNF RECEPTOR (RET AND GDNFR-α) MRNAS IN THE SPINAL CORD OF PATIENTS WITH AMYOTROPHIC LATERAL SCLEROSIS

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The mRNA levels of RET and GDNFR-α were studied in the spinal cord of patients with amyotrophic lateral sclerosis (ALS) by reverse transcription followed by polymerase chain reaction (RT-PCR) and in situ hybridization (ISH). Semiquantitative RT-PCR analysis revealed that RET mRNA levels in the ALS spinal cord anterior horn were reduced to one fifth of controls in proportion to motor neuron loss, whereas GDNFR-α mRNA was unchanged. ISH analysis showed that RET mRNA was expressed in the anterior horn motor neurons of the spinal cord, but GDNFR-α mRNA was expressed widely in the spinal cord neurons and glial cells. The RET mRNA levels, measured using a CCD image analyzer, were substantially preserved in individual motor neurons of ALS, but varied among those neurons. Relatively high levels of RET mRNA were observed in a certain population of atrophic neurons. On the other hand, the GDNFR-α mRNA levels in the motor neurons were similar in ALS and controls. In addition, the RET protein was also well expressed in individual motor neurons in ALS. These results indicate that GDNF receptor expression persists at mRNA and protein levels in the degenerating motor neurons in ALS, supporting the view that GDNF is a candidate for therapeutic approach to ALS.

AGE-RELATED MORPHOLOGIC CHANGES OF THE CENTRAL CANAL OF THE HUMAN AND RATS SPINAL CORD

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Department of Neurology

We analyzed the age-related morphologic changes in the normal human and aged rats' central canal of the spinal cord. The subjects were 158 autopsied spinal cords (0 to 116 years of age), 235 autopsied brain stem (0 to 94 years of age), and 10 cases of 30-month-old super aged rats. Each segment in each case was investigated from the dorsal column nuclei to S3 levels. The microscopic pictures of the central canal were classified as patent or occluded at each level for each age decade. The patency rate under one year of age was 100% in almost all the segments, which markedly decreased in the second decade, and the canals were occluded in all the segments with advancing age. The occlusion of the central canal started by 10 years of age at the T6 and L5 to S2 levels and ended at the pyramidal decussation levels. We suggest that the central canal does not function after infancy because of its occlusion, and it is not involved in the development of syringomyelia in adult patients. We have to take the different obstructive changes of human and rats' central canal into consideration in estimating the experimental data.

THE ROLE OF LYMPHOTOXIN IN PATHOGENESIS OF POLYMYOSITIS

YIDENG LIANG

Department of Neurology

Polymyositis (PM) is a cell-mediated autoimmune disease. To determine how LT, PF and Fas L are involved in the pathogenesis of PM, we used immunohistochemical staining (IHC), reverse transcriptional polymerase chain reaction (RT-PCR), and *in situ* hybridization (ISH) on muscle specimens from patients with PM, amyotrophic lateral sclerosis (ALS), myotonic dystrophy (MyD) and controls (NC), respectively. There were many mononuclear cells (MNCs) immunoreactive for LT and some for PF and Fas L within the fasciculus in PM muscles. On the other hand, LT-, PF- and Fas L-positive cells were only few or undetected in MyD, ALS and NC muscles. The results of mRNA expression of these three molecules with RT-PCR were consistent with those using IHC methods. The number of MNCs positive for LT with ISH was far higher in PM compared to MyD, ALS and NC ($P<0.05$ or 0.01). The MNCs located in the connective tissue or in the vicinity of necrotizing or nonnecrotizing muscles were mainly LT mRNA- and CD4-positive, while MNCs invading the nonnecrotic fiber were mainly LT mRNA- and CD8-positive. Our results indicated that the expression of LT was upregulated in PM, and LT plays an important role in muscle injury and orchestrating the inflammatory reaction in PM.

INVOLVEMENT OF INTERLEUKIN-8 IN DIALYSIS-RELATED ARTHRITIS

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To elucidate the role of interleukin (IL)-8, a chemotactic factor for neutrophils, in dialysis-related arthritis (DRA) of patients on long-term hemodialysis, the concentration of IL-8 was measured in the synovial fluids of DRA patients with acute arthralgia and joint swelling, and was compared with those in patients with rheumatoid arthritis (RA) or osteoarthritis (OA). We noted marked elevation of IL-8 in the joint fluids of patients with DRA and RA as compared with OA. Furthermore, to determine the role of IL-8 in synovitis, we examined the *in vivo* effect of an intra-articular injection of human recombinant IL-8 on leukocyte infiltration into joint space of rabbits. A single injection of IL-8 to the joints of rabbits induced rapid infiltration of neutrophils into the joint space and synovial tissues, which reached a maximum in 4 hours. The oral administration of indometacin farnesil before the injection of IL-8 alleviated the infiltration of neutrophils. When human synovial cells were incubated with tumor necrosis factor (TNF)-α, the expression of IL-8 mRNA and IL-8 production in the cultured synovial cells were increased. The TNF-α-stimulated expression of IL-8 mRNA and IL-8 production in the cultured synovial cells were markedly inhibited by dexamethasone. In conclusion, IL-8 levels were markedly elevated in the joint fluids of patients with DRA. IL-8 released from synovial cells may be an important factor to induce acute inflammation in DRA. Dexamethasone and indomethacin may be effective for DRA by inhibiting production and chemotactic action of IL-8, respectively.

INTRAPERITONEAL DELIVERY OF hrR3 AND GANCICLOVIR PROLONGS SURVIVAL IN MICE WITH DISSEMINATED PANCREATIC CANCER

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We have investigated a novel therapeutic strategy using the vector hrR3 based on the herpes simplex virus type 1 thymidine kinase (HSV-tk)/ganciclovir (GCV) paradigm in a mouse abdominal tumor model. hrR3 lacks the ribonucleotide reductase (RR) gene, which is a key enzyme in the biosynthesis of DNA in all prokaryotic and eukaryotic cells. This deficiency renders the mutant virus replication-competent only in dividing cells. Therefore, replication of the virus is restricted largely to tumors, and normal organs are protected from the adverse effect of treatment. hrR3 possesses an intact HSV-tk gene that can be used for metabolic activation of GCV, which acts to disrupt cellular and viral DNA replication. The phenomenon that HSV-tk-

expressing cells also can induce cell death in neighboring cells, which do not express HSV-tk, has been called the bystander effect, and it may be due to induction of apoptosis. We hypothesized that the combination of hrR3 and GCV would improve outcome when used to treat intraperitoneal dissemination of pancreatic cancer. In this study, we thus investigated its effect in a mouse model. Intraperitoneal delivery of hrR3 and ganciclovir improves survival in this murine model of peritoneal dissemination of pancreatic cancer.

HUMAN LIVERS WITH CIRRHOSIS AND HEPATOCELLULAR CARCINOMA HAVE LESS MITOCHONDRIAL DNA DELETION THAN NORMAL HUMAN LIVERS

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We measured the populations of mutated mitochondrial DNAs with the 7,436 bp or the 4,977 bp deletion from apparently normal human liver and human livers with chronic hepatitis, cirrhosis, and hepatocellular carcinoma. The amount of the mutated mitochondrial DNA was at the same level between normal and chronically hepatitic livers but was significantly lower in human livers with cirrhosis and hepatocellular carcinoma, especially the latter, suggesting that the mutated mitochondrial DNAs may be decreased with the progress of liver disease from chronic hepatitis to cirrhosis and hepatocellular carcinoma. This phenomenon is opposite to that occurring in the ageing process. The fact that the amount of the mutated mtDNA decreased with the progress of liver disease from chronic hepatitis to cirrhosis and hepatocellular carcinoma could be attributed to the liver regeneration and repetitive mitosis.

THE EFFECT OF DA FANG FENG TANG ON THE TREATMENT OF TYPE II COLLAGEN-INDUCED ARTHRITIS IN DBA/1 MICE

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We investigated the effect of Da Fang Feng Tang (DFFT), one herbal medicine which is believed to be effective for treating human rheumatoid arthritis (RA), was given to DBA/1 mice at the onset of type II collagen-induced arthritis (CIA). Granules of the crude DFFT extract were administrated by gastric gavage at a dose of 1.6g/kg/d for 12 weeks, starting the day CIA began. The levels of anticollagen IgG antibody were significantly decreased in the sera of the DFFT-treated group compared with the control group from weeks 2 to 7 after the onset of CIA. The severity of arthritis in the DFFT-treated group was markedly alleviated when com-

pared with the control group. In addition, the histological examination of the DFFT-treated group showed less cartilage and bone erosion. These results suggest that the administration of DFFT suppressed the development of CIA in mice and support the belief that DFFT is effective in treating human RA.

QUANTITATION ANALYSIS OF MATRIX METALLOPROTEINASES-2 AND -9, AND THEIR TISSUE INHIBITORS-1 AND -2 IN HUMAN PLACENTA THROUGHOUT GESTATION

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To elucidate the implication of type IV collagenases (MMP-2 AND MMP-9) and their tissue inhibitors (TIMP-1 and TIMP-2) for placental development, we quantified their levels in the conditioned media of placental organ culture and primary culture of the trophoblast as well as in the tissue extracts of placentas from different stages of gestation using specific enzyme-linked immunosorbent assays. First trimester villous tissue secreted about 10 times more pro-MMP-2 than pro-MMP-9, and pro-MMP-2 levels dramatically decreased in the second trimester. ON the other hand, pro-MMP-9 levels were more than 10 times higher than those of pro-MMP-2 in the primary culture of the first trimester trophoblast, indicating the involvement of stromal cells for prominent pro-MMP-2 secretion from first trimester villous tissue described above. Levels of TIMPs, especially those of TIMP-2, remained constant throughout gestation both in the culture media and tissue extracts. Gelatin zymography revealed abundant secretion of the active form of MMP-2 as well as pro-MMP-2 from first trimester villous tissue. Western immunoblot analysis confirmed the presence of both TIMP-1 and TIMP-2 in placental tissue. These results suggest that active secretion of MMP-2 from villous tissue in the first trimester and constant production of TIMPs throughout gestation are characteristic of placental development.

EFFECT OF DIFFERENT REGIONS OF THE VISUAL FIELD IN INDUCING BODY SWAY

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We analyzed the role of the central and peripheral visual fields in inducing vection and body sway. Ten, healthy volunteer students served as subjects. A depth optokinetic stimulus (DOKS) was projected onto a head-mounted display (HMD) and was perceived to move in

depth. Different amounts of the central or peripheral visual field were masked independently, and the magnitude of the linear vection was evaluated by verbal assessment and by the magnitude of induced body sway. Body sway was monitored by a video-motion-analyzer that recorded the movement of the head, shoulder, hip, knee and ankle. The results showed that the magnitude of vection was correlated with the amplitude of the body sway ($r = 0.57$). When the central visual field was restricted by 10 to 30%, there was almost no change in the induced body sway and vection. However, when central occlusion was greater than 40%, depth perception and induced body movement were greatly reduced. With increasing amounts of peripheral field occlusion from 50 to 90%, there was a greater reduction of both vection and body sway. Vection is strongly correlated with body movement, and vection and body sway were more dependent on stimulation of the peripheral visual field.

SUCCESSFUL CULTURE AND SUSTAINABILITY *IN VIVO* OF GENE-MODIFIED HUMAN ORAL MUCOSAL EPITHELIUM

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Human oral mucosal cells are an attractive site for tissue engineering because they are the most accessible cells in the body and easy to manipulate *in vitro*. They thus have possibilities for targeting by somatic gene therapy. We examined the efficiency of retrovirus-mediated gene transfer and the construction of mucosal epithelium *in vivo*. Human oral mucosal cells were transduced with a retroviral vector carrying the LacZ gene at high efficiency and formed epithelium after G418 selection with 3T3 cells *in vitro*. The cultured oral mucosal epithelium membrane was then grafted onto immunodeficient mice. β-Gal expression was detected histochemically *in vivo* 5 weeks after grafting. Furthermore, we transduced factor IX cDNA into the mucosal epithelium membrane, and it was then transplanted into nude mice. Between 0.6 and 1.8 ng of human factor IX per milliliter was found in mouse plasma, and the production was continued for 23 days *in vivo*. These results confirmed that the oral mucosal epithelium is an ideal target tissue for gene therapy or tissue engineering.

IFN-β GENE THERAPY INDUCES EFFECTIVE ANTITUMOR IMMUNITY TO MALIGNANT GLIOMA

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Although central nervous system is regarded as an immunologically privileged site, we have speculated that cationic liposomes containing the interferon-β (IFN-β) gene, but not IFN-β itself

suppress tumor growth by inducing effective antitumor immunity and apoptosis to malignant glioma. To clarify this hypothesis, the present study aimed to investigate antitumor effects and immune response activation by murine IFN-β gene transfer into GL261 (H-2^b) glioma established in brains of syngeneic C57BL/6 mice. In in vivo experiments, intratumoral administration of liposomes containing the murine IFN-β gene resulted in an 16-fold reduction in the mean volume of residual gliomas in the brains, and massive infiltration of lymphocytes was observed within the residual tumor. More importantly, 40–60% of the treated mice survived with no neurologic symptoms on day 100 after treatment. These surviving animals were rechallenged with either subcutaneous or intracranial injection of GL261 cells on day 100. No tumors developed in these animals over a 50-day period. Specific cytotoxic T cells against GL261 were generated from tumor-infiltrating lymphocytes or spleen cells of these treated mice. These findings suggest that activation of cellular immunity strongly participates in antitumor effects in vivo, together with direct effects of the IFN-β gene on the tumor cells.

CHARACTERIZATION OF $CD4^+CD8\alpha\alpha^+$ AND $CD4^-CD8\alpha\alpha^+$ INTESTINAL INTRAEPITHELIAL LYMPHOCYTES IN RATS

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Intestinal intraepithelial lymphocytes (i-IEL) of aged rats comprise $CD4^+$ $CD8\alpha\alpha^+$ and $CD4^-$ $CD8\alpha\alpha^+$ T cells expressing TCR αβ. We compared characteristics of $CD4^+$ $CD8\alpha\alpha^+$ and $CD4^-$ $CD8\alpha\alpha^+$ i-IEL, which were purified by a cell sorter from the i-IEL of 6-month-old Lewis rats. Most of $CD4^+$ $CD8\alpha\alpha^+$ i-IEL were of the $CD44^{high}$ phenotype, while approximately 80% of $CD4^-$ $CD8\alpha\alpha^+$ i-IEL were $CD44^-$. Vβ usage in the $CD4^-$ $CD8\alpha\alpha^+$ i-IEL was much diversified, while $CD4^+$ $CD8\alpha\alpha^+$ i-IEL showed a skewed Vβ repertoire. The $CD4^+$ $CD8\alpha\alpha^+$ i-IEL but not the $CD4^-$ $CD8\alpha\alpha^+$ i-IEL proliferated in response to syngeneic spleen cells, which was partially inhibited by addition of anti-MHC class I mAb. The $CD4^+$ $CD8\alpha\alpha^+$ i-IEL produced IL-2 and IFN-γ but no IL-4 or TGF-β in response to syngeneic spleen cells, while $CD4^-$ $CD8\alpha\alpha^+$ i-IEL produced abundant levels of TGF-β but no IL-2, IFN-γ, or IL-4. $CD4^+$ $CD8\alpha\alpha^+$ i-IEL proliferated in response to exogenous IL-2 but not to IL-15, while $CD4^-$ $CD8\alpha\alpha^+$ i-IEL could respond to IL-15 as well as IL-2. These results suggest that a significant fraction of $CD4^+$ $CD8\alpha\alpha^+$ i-IEL belongs to Th1 type T cells capable of responding to self-MHC class I, while $CD4^-$ $CD8\alpha\alpha^+$ i-IEL are a unique population with diversified Vβ repertoire that respond to IL-15 in rats.