ANNUAL RESEARCH MEETING

FOR

GRADUATE STUDENTS

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Abstracts

PROTECTIVE EFFECT OF INTERLEUKIN-6 AGAINST THE DEATH OF PC12 CELLS CAUSED BY SERUM DEPRIVATION OR BY THE ADDITION OF A CALCIUM IONOPHORE

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Interleukin-6 (IL-6) is known to differentiate the rat pheochromocytoma cell line PC12 to neuron-like cells. We examined the effect of IL-6 on the death of PC12 cells. IL-6 significantly blocked the death of PC12 cells by serum deprivation. The protective effect of IL-6 was increased by pre-incubation of PC12 with IL-6 for 20 hr before serum deprivation. The inhibition of protein synthesis by cycloheximide had no effect on the protective effect of IL-6 on the serum deprivation-induced cell death. IL-6 also inhibited the death of PC12 cells induced by addition of the calcium ionophore, A23187 to the culture medium. Specific in situ labeling of DNA cleavage was observed in PC12 cells subjected to both serum deprivation and A23187 for 24 hr. IL-6 inhibited DNA fragmentation in PC12 cells following serum deprivation. These results suggest that the death of PC12 cells induced by serum deprivation or the addition of calcium ionophore is apoptosis, and that IL-6 blocks apoptosis of PC12 cells.

CATIONIC MULTILAMELLAR LIPOSOME-MEDIATED HUMAN INTERFERON-β GENE TRANSFER INTO CERVICAL CANCER CELLS

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Interferons (IFNs) have anti-neoplastic activities, but it has been reported that the treatment with IFN alone is not effective in many cancers. To enhance the effect of growth inhibition for tumor cells by raising the concentration, we attempted the transfection of cervical cancer cells, Hela cells, with human interferon-β (HuIFN-β) cDNA contained in the expression vector pRSV delivered by cationic multilamellar liposomes, which resulted in the secretion of HuIFN-β into the medium. The concentration of HuIFN-β in the medium was 22 IU/ml by 72 h after transfection of 10 ng DNA, and provoked around 50-fold cell growth inhibitory effect compared with that with exogenously added HuIFN-β (1000 IU/ml). This strong growth inhibition was considered to be due to the action of HuIFN-β in paracrine manner, and a definite fraction of the cell death was apoptotic.

ANALYSIS OF BAX PROTEIN IN SPHINGOSINE-INDUCED APOPTOSIS IN A HUMAN LEUKEMIC CELL LINE, TF1, AND ITS BCL-2 TRANSFECTANTS

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Bax is a bcl-2 related protein that appears to counteract the death repressor activity of bcl-2, and that promotes apoptotic cell death. We examined the role of bax in apoptosis induced in a human erythroleukemic cell line, TF1, which lacks bcl-2 expression, and in a bcl-2 transfectant of TF1 (TF1-bcl2). A variety of stimuli, such as depletion of GM-CSF or exposure to methylmethane sulfonate (MMS) (100 μg/mL), ultraviolet light (UV) (15 J/m^2), X-ray irradiation (20 Gy), or sphingosine (5 μM), easily induced apoptotic cell death in TF1-mock cells. TF1-bcl2 cells, however, were resistant to most of these treatments, but remained sensitive to sphingosine. Treatment of both cell lines with fumonisin B1, which can prevent conversion of sphigosine to ceramide, did not block sphingosine-induced apoptosis, suggesting that sphingosine itself, not ceramide, possesses apoptosis-inducing activity. Western blotting, which revealed a 21 kDa bax protein in untreated TF1-mock cells, showed an additional 18 kDa protein in GM-CSF-depleted and MMS or sphingosine-treated TF1-mock cells. In TF1-bcl2 cells, this 18 kDa protein was not detected after GM-CSF depletion or MMS treatment, but was observed after sphingosine treatment. Immunoprecipitation with anti-bcl2 antibody followed by immunoblotting with anti-bax antibody showed that both the 21 kDa bax protein and the 18 kDa protein heterodimerized with bcl-2 protein. Our results suggest that sphingosine is a unique reagent for apoptosis and can overcome bcl-2, and that the induction of 18 kDa bax-related protein may play an important role in apoptosis.

ERM (EZRIN/RADIXIN/MOESIN)-BASED MOLECULAR MECHANISM OF MICROVILLAR BREAKDOWN AT AN EARLY STAGE OF APOPTOSIS

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Microvilliar breakdown is a common early event in apoptosis, but its mechanism remain unclear. ERM (ezrin/radixin/moesin) proteins are ubiquitously expressed microvillar proteins, functioning as actin filament/plasma membrane crosslinkers to form microvilli. Immunofluorescence microscopic and biochemical analyses revealed at the early phase of Fas ligand (FasL)-induced apoptosis in L cells expressing Fas, ERM proteins translocate from plasma membranes of microvilli to cytoplasm concomitant with dephosphorylation. When FasL-induced dephosphorylation of ERM proteins was suppressed by calyculin A, cytoplasmic translocation of ERM

proteins was blocked. ICE protease inhibitors suppressed the dephosphorylation as well as the cytoplasmic translocation of ERM proteins. These findings indicate that during FasL-induced apoptosis, ICE protease cascade was first activated, then ERM proteins were dephosphorylated followed by their cytoplasmic translocation, i.e. microvillar breakdown. Next I prepared DiO-labeled plasma membranes. On plasma membranes from non-treated cells, ERM proteins and actin filaments were densely detected, whereas those from FasL-treated cells were free from ERM proteins or actin filaments. I concluded that cytoplasmic translocation of ERM proteins is responsible for the microvillar breakdown at an early phase of apoptosis, and that the depletion of ERM proteins from plasma membranes results in the gross dissociation of actin-based cytoskeleton from plasma membranes.

CYTOTOXIC MOLECULE EXPRESSION IN PERIPHERAL T-CELL LYMPHOMAS

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Ten peripheral T-cell lymphomas of a cytotoxic phenotype ($CD3^+$, $CD4^-$, $CD8^+$), encountered among 98 total peripheral T-cell lymphomas (PTCLs) were studied by morphological and immunohistochemical methods. Four involved the skin, three of which were diagnosed as primary cutaneous lymphomas. Five patients died within 1 year of diagnosis. Three cases had a characteristic morphology consisting of large lymphomatous cells with massive necrosis and nuclear fragmentation. Epstein-Barr virus mRNA was detected by in situ hybridization in three cases. Apoptotic cells were detected in all cases by TdT-mediated dUTP-biotin nick end labeling. Nine tumors expressed cytotoxic molecules, namely perforin (Pf) and granzyme B (GrB) with strong positivity in the majority of the malignant cells. Expression of these molecules was also studied in 92 cases of other T/NK cell neoplasms. Most of the $CD4^+$ PTCLs (62/63) were negative for GrB, while all nasal lymphomas and natural killer cell leukemia were positive for both Pf and GrB. Variable expression was seen among the 18 anaplastic large cell lymphomas (ALCL). In conclusion, $CD8^+$ PTCLs are relatively rare, often involve extranodal sites, have an aggressive clinical course and are often associated with EBV. We believe that $CD8^+$ PTCLs are lineage-specific neoplasms of mature cytotoxic T lymphocytes.

SHO-SAIKO-TO INHIBITS THE GROWTH OF MALIGNANT MELANOMA CELLS BY UPREGULATING FAS-MEDIATED APOPTOSIS AND INDUCING G_1 ARREST THROUGH DOWN-REGULATION OF G_1 CDKS

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The rapid increase of skin melanoma incidence makes research on the prevention and treatment of malignant melanoma a pressing need. Here we investigated the mechanism of anti-tumor effect of the herbal medicine sho-saiko-to, which is most often used in Japan for hepatoma prevention, on a murine malignant melanoma cell line (Mel-ret). We found that sho-saiko-to induced the apoptotic cell death of Mel-ret cells with a definite increase of cell surface expression of Fas antigen on these cells. Fas ligand (FasL) was constitutively expressed on Mel-ret cells and was also upregulated by sho-saiko-to treatment. We also found that sho-saiko-to arrested Mel-ret cells in G_1 phase with decreasing the expression of cyclin-dependent kinase (cdk) 4 and its homologue cdk6. Kinase activities of cdk4 and cdk6 were identified to be down-regulated by sho-saiko-to in the analysis of *in vitro* kinase assay. Ingredient analysis revealed that baicalin is likely the main active constituent in the upregulation of Fas antigen and Fas ligand while glycyrrhizin is in the inhibition of cdks. In conclusion, we for the first time demonstrated that sho-saiko-to displays an anti-tumor effect on malignant melanoma cells by upregulating Fas-mediated apoptotic cell death and arresting cell cycle in G_1 phase through the inhibition of G_1 cdks.

c-Ras IS REQUIRED FOR THE SUPPRESSION OF CADHERIN-MEDIATED CELL ADHESION IN v-*fps*- AND v-*src*-TRANSFORMED CELLS

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Cell transformation by v-*src* oncogene results in strong suppression of cadherin-mediated cell-cell adhesion. To search for the signaling pathway critical for the cadherin function, we examined the effects of dominant negative *ras* (S17N ras) expression under the control of a conditionally inducible promoter in v-*src*-transformed 3Y1 (SR3Y1) and v-*fps*-transformed 3Y1 (FSV3Y1). We found that P-cadherin-dependent cell-cell adhesion was dramatically restored by S17N Ras expression in these cells. By expression of S17N Ras, the cells became flattened and tightly associated, and cadherin-catenin complexes accumulated at the cell-cell boundaries. However, the expression of P-cadherin and catenins, the complex formation between these proteins, the tyrosine phosphorylation of β-catenin and p120Cas showed no difference. In addition, cadherin-mediated cell adhesion was strongly suppressed in H-*ras* transformed 3Y1 without detectable tyrosine phosphorylation of β-catenin and p120Cas. These results strongly suggest that

endogenous Ras plays a critical role in the suppression of P- and N-cadherin mediated cell adhesion in SR3Y1 and FSV3Y1, but tyrosine phosphorylation of β-catenin and p120Cas is dispensable for the suppression.

INHIBITORY EFFECT OF α(1,2) FUCOSYLTRANSFERASE RECOMBINANT ADENO-VIRAL VECTOR ON αGAL EXPRESSION

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A major obstacle in xenotransplantation between discordant species is a hyperacute rejection (HAR) induced by the reaction of natural antibody with Gal α(1,3) Gal antigen (αGal). It has been reported that the transduction of α(1,2) fucosyltransferase (FT) gene, called "enzymatic remodeling", is efficient for preventing the xenogeneic organ from HAR. We prepared FT recombinant adeno-viral vector (AdFT) and examined the effect of the vector on downregulation of αGal expression using FACS analysis and immunohistochemistry. FACS analysis demonstrated that AdFT infected BALB/3T3 cells highly expressed H antigen, whereas αGal expression was decreased compared to AdLacZ (67% reduction in the mean fluorescence intensity value). A stronger expression of H antigen was observed in the hepatocytes of the mice which had been injected AdFT, followed by human sera. The binding of anti-human IgG antibodies and C3 deposits were decreased in the central veins and the sinusoids. The expression of αGal antigen in the liver was decreased, but not completely. It is concluded that the transduction of FT gene using adeno-viral vector is effective to inhibit αGal expression and there seemed to be an inverse relationship in the expression between αGal antigen and H antigen.

INCREASED SERUM MIDKINE LEVELS DURING HEMODIALYSIS IN CHRONIC RENAL FAILURE

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A heparin-binding growth factor, midkine (MK) has been implicated to play a role in neuron growth, angiogenesis and inflammation. In this study, to elucidate the involvement of MK in uremia, we examined serum MK levels in patients receiving hemodialysis (HD) by highly sensitive enzyme-linked immunoassay. Although there was no significant difference between control serum and serum before dialysis in HD patients, serum MK levels were increased significantly at the early stage of HD sessions using heparin and were gradually decreased after dialysis. In *in vitro* study, MK was released in time and heparin-dose dependent manner from cultured vessels, but not from peripheral leukocytes. These results indicate that MK is released immediately after

administration of heparin mainly from endothelial cells during HD. This release of MK might affect some uremic complications.

EXPRESSION OF BASIGIN, A MEMBER OF THE IMMUNOGLOBULIN SUPERFAMILY, DURING MOUSE EMBROYOGENSIS, SPERMATOGENESIS AND IN THE ADULT CENTRAL NERVOUS SYSTEM

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Basigin is a highly glycosylated transmembrane protein belonging to the immunoglobulin superfamily. We produced knockout mice lacking the basigin gene (Bsg). The Bsg (-/-) embryos show reduced efficiency in implantation. The Bsg (-/-) mice have deficits in spermatogenesis and in sensory function and/or memory. These findings prompted me to investigate the precise role of Bsg in embryogenesis, spermatogensis and construction of the central nervous system. I performed in situ hybridization histochemistry for Bsg mRNA. Around the time of implantation, Bsg mRNA was strongly expressed in the trophectoderm, embryo and uterine endometrium around the sites of implantation. In the organogenesis period, it was expressed in various epithelial tissues and brain tissue around the ventricles. In the adult central nervous system, it was expressed in the mitral cells in the olfactory bulb, the limbic system, the Purkinje cells in the cerebellum, many nuclei in midbrain and medulla, and the spinal cord. In the testis, Bsg mRNA was strongly expressed in spermatocytes and spermatid. Taking the phenotype of Bsg knockout mice into account, the present data suggest that basigin functions in a cell-autonomous manner and is involved in early embryogenesis and spermatogensis as well as learning and memory.

STRUCTURAL AND FUNCTIONAL RELATIONS IN THE MIDKINE PROTEIN, A HEPARIN-BINDING GROWTH FACTOR

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Midkine (MK) is a 13 kDa heparin-binding growth factor and is rich in basic amino acids and cysteine. It exerts a neurotrophic activity (neuronal cell attachment, survival and neurite outgrowth) and enhances plasminogen activator (PA) activity. To investigate structural and functional relation in the MK molecule, I generated and purified several mutants, and monitored

three properties of MK, i.e. heparin-binding, neurotrophic, and PA activating. Sodium chloride concentration at which the peak fraction was eluted from a heparin column was 0.78M for MK (R78KK83,84QQQ); 0.85M, MK (C-mut) (C-terminal, 106–118 residues were deleted and Ser-Leu-Ile-Asp was inserted in the position); 0.88M, MK (N-del) (N-terminal, 1–7 residues were deleted); 0.9M, MK (KK83,84QQ); and 1.0M, intact MK. In contrast, a neurotrophic activity was dramatically reduced in MK (C-mut) and MK (N-del), slightly reduced in MK (R78KK83,84QQQ) and MK (KK83,84QQ) as compared with wild type MK. Moreover, PA activation was strongly reduced at 1/100th in the cases of MK (R78KK83,84QQQ) and MK (KK83,84QQ), whereas MK (N-del) and MK (C-mut) reduced at 1/10th, indicating that lysine 83 and lysine 84 or MK are responsible for PA activity. In addition, no secreted proteins were detected in both cases of MK (K76Q) and MK (K99Q), while MK (K76R) and MK (K99R) were normally secreted, indicating that basic change of these positions are important in secretion or stabilization. Taken together, the present findings indicate that MK is a pluripotent molecule in which different sites are responsible for distinct biological properties. It is of note that neurotrophic and PA activities did not parallel in each mutation, suggesting that MK may employ distinct signaling systems for these activities.

THE SERUM LEVEL OF MIDKINE, A HEPARIN BINDING GROWTH FACTOR, AS A TUMOR MARKER

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Midkine (MK) is a heparin-binding growth factor distinct from fibroblast growth factors. Serum levels of MK were determined by enzyme-linked immunoassay using affinity-purified anti-human MK antibody. Elevated levels of MK were frequently observed in sera from patients with various carcinomas such as lung carcinoma, bile duct carcinoma, colon carcinoma and esophageal carcinoma. Most patients with lung carcinoma showed high MK serum values. In colorectal carcinoma, some correlation was observed between high MK value and tumor invasion. Surgical removal of carcinomas invariably resulted in decreases in MK level. Determination of serum MK may be useful as an aid in initial screening of certain carcinomas, such as lung carcinoma.

LACK OF ASSOCIATION OF ANGIOTENSIN-CONVERTING ENZYME GENE POLYMORPHISM OR SERUM ENZYME ACTIVITY WITH CORONARY ARTERY DISEASE IN JAPANESE

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The association of an insertion/deletion (*I/D*) polymorphism in the angiotensin-converting enzyme (ACE) gene or the serum activity of ACE with coronary artery disease (CAD) has been investigated in Japanese men and women. The ACE genotype of 947 CAD subjects who underwent coronary angiography and 893 control subjects was determined by polymerase chain reaction analysis. No association of the *DD* genotype or the *D* allele with CAD was observed in men or women. In a low-risk group defined by a body mass index below the median value and the absence of a history of hypertension, diabetes mellitus, and hypercholesterolemia, there was also no association between the ACE gene polymorphism and CAD. No significant difference in serum ACE activity was detected between CAD subjects and controls of all genotypes or of the same genotype, whereas a significant association was apparent between serum ACE activity and ACE genotype for both CAD subjects and controls in both men and women. These results indicate that the ACE *I/D* polymorphism and genotype-associated variation in serum ACE activity are not risk factors for CAD in Japanese men or women.

IMMUNOHISTOCHEMICAL CO-LOCALIZATION OF GLYCOXIDATION PRODUCTS AND LIPID PEROXIDATION PRODUCTS IN DIABETIC RENAL GLOMERULAR LESIONS: IMPLICATION FOR GLYCOXIDATIVE STRESS IN THE PATHOGENESIS OF DIABETIC NEPHROPATHY

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Accumulation of advanced glycation end products (AGEs) including a variety of protein adducts alters the structure and function of tissue proteins, and stimulates cellular responses. They have been implicated in tissue damage associated with diabetic complications. In order to amalyze the relation between AGE accumulation and the development of diabetic nephropathy (DN), we examined the immunohistochemical localization of various AGE structures postulated so far, i.e., pentosidine, carboxymethyllysine (CML), and pyrraline, in diabetic and control kidneys. CML and pentosidine accumulated in expanded mesangial matrix and thickened glomerular capillary walls of early DN and in nodular lesions and arterial walls of advanced DN, but were absent in control kidneys. By contrast, pyrraline was not found within diabetic glomeruli but was detected in the interstitial connective tissue of both normal and diabetic kidneys.

Although the distribution of pyrraline was topographically identical to type III collagen, those of pentosidine and CML were not specific for collagen type, suggesting that the difference of matrix could not explain the AGE localization. Since oxidation is closely linked to the formation of pentosidine and CML, we also immunostained malondialdehyde (MDA), a lipid peroxidation product whose formation is accelerated by oxidative stress, assuming that local oxidative stress may serve as a mechanism of pentosidine and CML accumulation. Consistent with our assumption, diabetic nodular lesions were positive for MDA. These findings show that AGE localization in DN varies according to AGE structure, and suggest that the co-localization of markers of glycoxidation (pentosidine and CML) with that of lipid peroxidation reflect a local oxidative stress in association with the pathogenesis of diabetic glomerular lesions. Thus, glycoxidation markers may serve as useful biomarkers of oxidative damage in DN.

GENETIC MAPPING OF THE PROTEINURIA SUSCEPTIBLE LOCUS, *pur-1*, IN BUF/Mna RATS TO A REGION ON CHROMOSOME 13

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The BUF/Mna (BUF) strain is a high proteinuria line of rats, and virtually all rats develop overt proteinuria by the age of 20 weeks. Genetic analysis revealed that proteinuria susceptibility was determined principally by two autosomal recessive genes *pur-1* and *pur-2* (proteinuria susceptible genes of the rat). These findings prompted us to perform genetic mapping of the genes. (BUF/Mna × WKY/NCrj) F1 × BUF/Mna backcross rats were raised and maintained for 40–60 weeks to detect proteinuria. DNA were extracted from ears of these rats and were examined by linkage study using polymerase chain reaction (PCR) with 120 microsatellite markers. 54 out of 167 rats developed proteinuria. DNA of 51 out of these 54 rats showed homozygote BUF/BUF genotype in the D13Mgh4 and D13N1 markers located on chromosome 13. The D13Rat1, D13Mgh2, D13Rat13, D13Mgh3, SYT2, REN, D13Rat25, D13Mit2, D13Mgh5, and D13N2 markers located on the chromosome also showed statistically significant linkage to the proteinuria development, whereas the other 108 markers showed no linkage. The *pur-1*, which regulate the proteinuria development, resides on a region less than 2.0 cM distal from the D13Mgh4 and D13N1.

UP-REGULATION OF VASCULAR ENDOTHELIAL GROWTH FACTOR IN RESPONSE TO GLUCOSE DEPRIVATION

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Vascular endothelial growth factor (VEGF), an endothelial specific mitogen, is a potent inducer of angiogenesis. It has been reported that hypoxia induces VEGF mRNA expression. Since glucose is an important energy source along with oxygen in mammalian cells, we hypothesized that there would be a parallel effect of glucose deprivation on VEGF induction. In the present study we examined the effect of glucose deprivation on VEGF mRNA expression using U-937 cell, human monocytic cell line. Both VEGF mRNA in U-937 cells and VEGF protein production increased after exposure to low glucose condition. Addition of L-glucose, the L-stereoisomer of D-glucose, did not prevent the upregulation of VEGF expression. Correction of glucose concentration of the conditioned medium into control level did not enhance VEGF mRNA expression. D-glucose supplementation recovered the observed enhanced VEGF mRNA expression in glucose-deprived cells. These results suggest that local hypoglycemia may act as an essential trigger for angiogenesis through the up-regulation of VEGF gene expression.

INVOLVEMENT OF OXIDATIVE STRESS IN THE ACCELERATED FORMATION OF PENTOSIDINE IN PATIENTS WITH END-STAGE RENAL FAILURE

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Advanced glycation end products (AGEs) have been found to accumulate in hemodialysis patients (HD), implicating the piossible involvement of AGEs-modified protein in the pathogenesis in dialysis-related amyloidosis. Pentosidine, an AGE, is a specific marker for AGEs. In the present study, the levels of plasma pentosidine, fructoselysine, advanced oxidation protein products (AOPP) and glutathione peroxidase (GSHPx) were measured to elucidate the role of oxidative stress in pentosidine formation in HD patients. The plasma pentosidine level did not correlate with the fructoselysine level; plasma AOPP levels were significantly higher than those in normal subjects and positively correlated with plasma pentosidine in HD patients; the levels of plasma GSHPx were significantly lower than those in normal subjects and negatively correlated with plasma pentosidine in HD patients. These findings implicated the involvement of oxidative stress in the accelerated formation of pentosidine in uremia and suggested that pentosidine could be considered as an oxidative stress biomarker to estimate the degree of oxidative stress-mediated protein damage.

AGE-ASSOCIATED DECREASE IN RESPONSE OF RAT AQUAPORIN-2 GENE EXPRESSION TO DEHYDRATION

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It is well known that urine-concentrating ability decreases with aging and that this decreasing ability results from a reduced sensitivity of renal collecting ducts to arginine vasopressin (AVP). AVP regulates aquaporin-2 (AQP2) through V2 receptors and increases the water permeability of collecting ducts. To elucidate the mechanism of change with aging in urine-concentrating ability, we investigated the change of V2 receptor (V2R) and AQP2 mRNA expression in young (8-week-old) and older (7-month-old) rats after dehydration for 2 days. After dehydration, the plasma AVP levels in older rats were higher than young rats, and the urinary osmolality in older rats was lower than young rats. By Northern bloting, there was no significant difference between young and older rats in both V2R and AQP2 mRNA expression before dehydration. After dehydration, V2R mRNA expression in young and older rats decreased in the same degree, suggesting the down-regulation of V2Rs occurs in the mRNA level. Northern blotting and *in situ* hybridization histochemistry showed that AQP2 mRNA expression increased and the increased expression in older rats was less than in young rats. These results suggest the decreasing urine-concentrating ability with aging might result at least partially from the reduced response of AQP2 mRNA expression to elevated plasma AVP.

RESPIRATORY DEFECT ALTERS PEROXIDE PRODUCTION, MITOCHONDRIAL MEMBRANE POTENTIAL AND PROTEIN IMPORT INTO MITOCHONDRIA OF CULTURED CELLS CARRYING DISEASE-ASSOCIATED mtDNA MUTATIONS

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The functional analyses in cybrids by cytoplast transfer of mitochondria from the patients with mitochondrial diseases into human mtDNA-lacking cell lines (ρ^0 cells) have demonstrated a clear correlation between mtDNA mutations and respiratory defect. However, it still remains unanswered how such a respiratory defect affects the intracellular physiological states which are essential for maintenance of the cellular activity in patients with mitochondrial diseases. We investigated alterations in intracellular states in cybrids (syn^-) carring mutations in mtDNA, as compared with two cell lines carrying a normal mtDNA (ρ^+) or lacking it (ρ^0). The peroxide production monitored using 2′,7′-dichlorodihydrofluorescein-diacetate (H2DCFDA) increased in the syn^- cell lines. The mitochondrial membrane potential ($\Delta\Psi m$) monitored using 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolocarbocyanine iodine (JC-1) decreased in the syn^-

cell lines. Morphological, fluorometrical, and immunochemical analyses demonstrated that import of a green fluorescent protein (GFP) with a mitochondrial signal peptide was impaired in the syn^- cells. These results imply that the respiratory defect due to mtDNA mutations leads to changes in intracellular states including reduction in the $\Delta\Psi m$, enhanced releases of peroxides from mitochondria, and impaired import of mitochondrial proteins. These results suggest that these mitochondrial alterations contribute to the pathophysiology of mitochondrial diseases.

HYPOTONICALLY INDUCED WHOLE CELL CURRENTS IN A6 CELLS: RELATIONSHIP WITH CELL VOLUME AND CYTOPLASMA CA^{2+}

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We investigated changes in whole cell currents, cell volume, and intracellular calcium concentration ($[Ca^{2+}]_i$) during hypotonic stimulation in amphibian renal cells (A6 cells). When exposed to hypotonic solution, A6 cells swelled. This peaked in 5 minutes, followed by a progressive decrease in cell volume termed regulatory volume decrease (RVD). Following the cell swelling there were large increases in both outward- and inward-currents, which seemed to be carried by K^+ and Cl^- efflux, respectively. A K^+ channel blocker (TEA or quinine) or a Cl^- channel blocker (NPPB or SITS) significantly inhibited both currents and RVD, suggesting that the inward- and outward-currents are highly correlated and essential to RVD. Hypotonic stimulation also induced a transient $[Ca^{2+}]_i$ increase the time course of which was essentially similar to that of the currents. When internal and external Ca^{2+} were deprived to eliminate the $[Ca^{2+}]_i$ increase, whole cell currents and RVD were strongly inhibited. On the other hand channel blockers, which inhibited currents and RVD, did not inhibit the $[Ca^{2+}]_i$ increase. It is concluded that hypotonic stimulation first induces cell swelling, which is followed by a $[Ca^{2+}]_i$ increase leading to coactivation of K^+ and Cl^- channels. This coactivation may accelerate K^+ and Cl^- effluxes resulting in RVD.

DYNAMIC PROPERTIES OF INDIVIDUAL WATER MOLECULES IN A HYDROPHOBIC PORE LINED WITH ACYL CHAINS: A MOLECULAR DYNAMICS STUDY

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It is generally believed that the pore of an ion channel should be lined with charged or polar residues to alternatively replace the solvation structure of permeant ions. However, this belief has been challenged by recent findings that the amino acid residues of the pore linings of some

natural channel proteins such as nicotinic acetylcholine receptor (nAChR) and shaker K^+ channel are surprisingly hydrophobic. As an initial step toward understanding ion permeation mechanisms across such a hydrophobic pore, systematic molecular dynamics simulations were performed on intrapore water molecules of a hydrophobic channel constructed from a dimer of alanine-N'-acylated cyclic octa-peptide. Dynamic energy profile for water molecules indicated that the energy barrier is approximately 2—3 kcal/mol. Energetics analyses demonstrated that the mutual interactions among intrapore water molecules are the major factor to give favorable interaction (negative energy contribution) for themselves. The pore, despite being lined with acyl chains, has a favorable van der Waals interaction with intrapore water molecules. These results may help to explain why water-filled channels can be formed by the hydrophobic helices in natural channels.

FOCAL MACULAR ELECTRORETINOGRAM IN IDIOPATHIC EPIMACULAR MEMBRANE

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Idiopathic epimacular membrane (EMM) can be treated by vitrectomy, when patients have significant symptoms and substantially reduced visual acuity. Morphological and pathological studies have been performed by many authors, but the mechanism of reduction in visual acuity is still unknown. In this study, we recorded focal macular electroretinogram (MERG) to investigate functional impairment of the retina underlying the membrane. Focal macular electroretinograms were recorded with rectangular stimuli of 10° in diameter from 30 patients with unilateral EMM. The a-, b-waves, and oscillatory potentials (OP) of each responses were compared in both eyes. The amplitudes of the a- and b-wave and OP were significantly reduced, inducing a smaller b/a ratio. When expressed the mean ($\pm$ SE) amplitudes of affected eyes as percentages of those of the fellow eyes, the reduction of the b-wave (60.2 ± 3.9) and OP (40.1 ± 7.2) was significantly greater than that of the a-wave (74.8 ± 3.9). The peak time of a- and b-wave and OP were delayed significantly. There was significant but small correlation between the relative b-wave amplitude and visual acuity ($P < 0.01$, $r = 0.50$). These findings were quite similar to those found in macular edema (Miyake, et al: Am J Ophthalmol 1993). The reduced b/a ratio probably suggests vulnerability of ON-bipolar cells to pathology caused by the epiretinal membrane.

LEVELS OF HEPATOCYTE GROWTH FACTOR AND ITS MESSENGER RIBONUCLEIC ACID IN UNCOMPLICATED PREGNANCIES AND THOSE COMPLICATED BY PREECLAMPSIA

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The purpose of this study was to elucidate the possible relationship between hepatocyte growth factor (HGF) expression and the pathogenesis of preeclampsia. The concentration of immunoreactive HGF (mRNA) assessed in human placentas obtained from two groups: uncomplicated and preeclamptic pregnancies at various gestational weeks. In addition, the localization of HGF mRNA and c-met protein was analyzed using in situ hybridization and immunohistochemical staining, respectively. The epxression of HGF mRNA and the concentration of immunoreactive HGF were highest in the second trimester and were significantly decreased in preeclamptic placentas compared with the uncomplicated cases in the third trimester. HGF mRNA was localized to placental mesenchymal cells, whereas c-met protein was demonstrated on cytotrophoblast. These results provide evidence of an abnormality of HGF expression in the preeclamptic placentas. Preeclampsia is known to be often accompanied by abnormally shallow trophoblast invasion of the uterus. The reduced expression of HGF could well account for this morphometric change.

NUMEROUS VESSELS IN VILLOUS STROMA OF COMPLETE HYDATIDIFORM MOLE DETECTED BY CD34

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Department of Laboratory Medicine

It has been considered that the villous stroma of complete hydatidiform mole (CHM) is usually avascular. Vessels would normally form if embryogenesis had occurred. But judging by the structural maturity of molar villi, we suspected there must be a considerable number of blood vessels in the stroma, so we tried to demonstrate the blood vessels. The putative vascular endothelial cell markers, such as factor VIII related antigen (FVIII-RAg), Ulex europaeus 1 agglutinin (UEA-1) and CD31, were of no use in demonstrating the vessels in molar villi. A monoclonal antibody, QBEND/10, raised against the CD34 antigen in human endothelial cell membranes and hemopoietic progenitor cells, was detected for its usefulness as a marker of villous vascular endothelial cells in CHM. Immunohistochemically, numerous blood vessels were found using CD34 antibody in the stroma of CHM. Their numbers corresponded to those found in normal villi of gestational age 8–12 weeks. Moreover, electron microscopy was also used to confirm the presence of blood vessels. In this study, we concluded that numerous vessels in villous stroma of CHM, and CD34 antibody was found to be the best of all the markers investigated for endothelial cells in villous stroma on formalin-fixed, paraffin-embedded tissue sections.

DIFFERENTIAL TEMPORAL EXPRESSION OF mRNAs FOR CILIARY NEUROTROPHIC FACTOR (CNTF), LEUKEMIA INHIBITORY FACTOR (LIF), INTERLEUKIN-6 (IL-6), AND THEIR RECEPTORS (CNTFRα, LIFRβ, IL-6Rα AND gp130) IN INJURED PERIPHERAL NERVES

YASUHIRO ITO

Department of Neurology

The mRNA expression of the neuropoietic cytokines, ciliary neurotrophic factor (CNTF), leukemia inhibitory factor (LIF), interleukin-6 (IL-6), and their receptor components (CNTFRα, LIFRβ, IL-6Rα and gp130) was examined in peripheral nerves after two different types of injury, crush and transection. The CNTF mRNA expression levels decreased after injury and remained low in the transected model, but recovered in 4 weeks in the crushed. The LIF mRNA rapidly increased after damage and returned gradually to control levels. The IL-6 mRNA expression increased rapidly within 1 day after injury but dramatically decreased soon after. The CNTFRα mRNA levels gradually increased after nerve injury. LIFRβ was expressed in the intact nerve and decreased slightly after injury. The IL-6Rα expression was observed faintly in the intact nerve and increased significantly soon after injury. There was also an increase in the expression of gp130. Although the temporal expression of these neuropoietic cytokines and receptors was extremely different, its pattern was similar between the crushed and transected models except for CNTF. These results suggest that the expression of the ligands and receptors are differentially regulated after peripheral nerve injury, implying that each cytokine and signal transduction system has entirely distinctive functions in neuronal regeneration and repair.

MITOCHONDRIAL GENOTYPE ASSOCIATED WITH LONGEVITY

JIAN-SHENG GONG

2nd Department of Biochemistry

To identify an mtDNA genotype associated with longevity, we analyzed the entire coding regions of mtDNA from 11 centenarians. Two nucleotide substitutions causing amino acid replacements as well as a transition within the 16S rRNA gene were more frequently observed in the centenarians than in the controls. Among these variations, we focused on Mt5178A, which showed the highest statistical significance, and screened 37 centenarians and 252 healthy blood donors. The frequency of Mt5178A was significantly higher in the centenarians (62%) than in the blood donors (45%). To evaluate the effect of mtDNA variations on the occurrence of diseases, we analysed the frequencies of Mt5178A and Mt5178C in 338 randomly selected patients. The age distribution of the patients indicated that the frequency of Mt5178C was almost the same as that of Mt5178A among the young patients, whereas the frequency of Mt5178C increased more markedly than that of Mt5178A among old patients. The ratio of

Mt5178 A/C was significantly lower in the old patients than in both the centenarians and the healthy controls. These results suggest that to carry an mtDNA genotype predisposing resistance to adult-onset diseases is one of the genetic factors for longevity.

NEWLY DEVELOPED DIABETIC RETINOPATHY AND ITS PRECEDING CHANGES IN BIOLOGICAL MARKERS

MASAYO KOJIMA

Department of Preventive Medicine

To disclose the chronological changes prior to the manifestation of diabetic retinopathy (DR), we analyzed the time course of biological markers among apparently healthy diabetic subjects in a case-control study out of 21,579 adults who had undergone comprehensive health examinations for ≥ 10 years, and identified 54 cases who had newly developed DR. As a referent group, we selected 108 adults without fundus abnormalities, matching them for sex, age, and fasting plasma glucose (FPG) at the onset of the patients group's retinopathy from the same population. In a multivariate analysis, a high average FPG (>175 mg/dl) and a final-year FPG reduction ($<-3\%$) were significantly associated by a 5.4 (95%CI, 1.8–15.7)- and 5.0 (95%CI, 1.0–24.7)-fold increased risk of DR, respectively. Thus, sustained hyperglycemia and a subsequent drop in FPG might promote retinopathy in non-insulin dependent diabetes mellitus.

REGIONAL DIFFERENCE IN LIPOLYSIS CAUSED BY A β-ADRENERGIC AGONIST AS DETERMINED BY THE MICRODIALYSIS TECHNIQUE

NOBUKO IWAO

1st Division of Health Promotion Science

We have investigated the difference in lipolysis caused by a β-adrenergic agent between visceral and abdominal subcutaneous adipose tissues *in vivo*. Glycerol levels were continuously monitored in mesenteric and abdominal subcutaneous adipose tissues of anesthetized Wistar rats using the microdialysis technique. During microdialysis, increasing concentrations of the lipolytic agent, isoproterenol (10^{-8}, 10^{-7}, 10^{-6}, 10^{-5} mil L^{-1}), were added to the perfusion. Glycerol concentrations in dialysate at each isoproterenol concentration, blood glucose concentrations during the experiment, and plasma insulin concentrations before and immediately after the experiment were measured. Effect of isoproterenol on local blood flow was investigated using ethanol technique. The clearance rate of ethanol from the perfusion medium was used as an index of local blood flow. There was no significant change in blood glucose or plasma insulin concentrations during the study. Glycerol levels in dialysate were significantly higher in mesenteric than in abdominal subcutaneous adipose tissues at all isoproterenol concentrations. Percent change of

baseline ethanol ratio was not changed by increasing isoproterenol concentrations both mesenteric and subcutaneous adipose tissues. There was also no significant difference in % change of baseline ethanol ratio between mesenteric and abdominal subcutaneous adipose tissues. These results suggest that mesenteric adipose tissue is characterized by an even higher β-adrenergic agonist-induced lipolysis than abdominal subcutaneous adipose tissue.

VENTILATORY CONTROL DURING EXERCISE IN NORMAL CHILDREN

YOSHIKO NAGANO

Department of Pediatrics

We investigated the relation between age and ventilatory control during exercise in 80 children aged 6.4—17.6 years. At rest and at peak exercise, the ratio of minute ventilation to carbon dioxide production ($\dot{V}E/\dot{V}CO_2$), the ratio of effective alveolar ventilation to carbon dioxide production ($\dot{V}A/\dot{V}CO_2$), the ratio of pulmonary dead space to tidal volume (Vd/Vt), and arterial partial pressure of carbon dioxide ($PaCO_2$) were not correlated with age. At the exercise intensity of ventilatory anaerobic threshold, the $PaCO_2$ increased, and the $\dot{V}A/\dot{V}CO_2$ decreased, significantly with age, whereas the Vd/Vt was independent of age. Linear regression analysis demonstrated that the slope of the relationship between $\dot{V}E$ and $\dot{V}CO_2$ ($\Delta\dot{V}E/\Delta\dot{V}CO_2$) ($r = -0.43$, $p < 0.01$) and the slope of the relationship between $\dot{V}A$ and $\dot{V}CO_2$ ($\Delta\dot{V}A/\Delta\dot{V}CO_2$) ($r = -0.44$, $p < 0.01$) decreased with age. There were no gender differences. Results show that younger children breathe more during exercise to eliminate a given amount of CO_2 to keep $PaCO_2$ set-point slightly but significantly lower than older children. This age dependence must be considered in investigating ventilatory control during exercise in children.

DNA EXTRACTION WITH CTAB PRECIPITATION FROM HAIR SHAFTS AND HLA-DQA1 TYPING USING A SEMI-NESTED PCR

HIDEKI NOZAWA

Department of Legal Medicine

It is well known that DNA typing from human hair shafts is hardly possible. We tried to determine HLA-DQA1 genotype from hair shaft portions with CTAB (cetyl-trimethyl ammonium bromide) precipitation followed by a semi-nested PCR. We also compared a commercially available kit, "GENECLEAN Kit for Ancient DNA", with CTAB precipitation. Several pieces of hair shafts equivalent to 40cm in length were washed in distilled water and 100% ethanol, cut into pieces and digested in a solution containing TEN buffer, SDS, DTT and proteinase K. The mixture was incubated overnight at 37°C and DNA was extracted with phenol and

chloroform. The DNA extract was then concentrated in a Centricon 30 device and purified by CTAB precipitation. The specific region of HLA-DQA1 gene was amplified by a semi-nested PCR using the extracted DNA as template, and the PCR product was cloned using a TA Cloning Kit. The HLA-DQA1 genotype was determined using the ABI Model 373A automated DNA sequencer. HLA-DQA1 genotype was correctly determined from all 40cm hair shaft samples with CTAB precipitation followed by a semi-nested PCR. CTAB precipitation could yield more PCR product from hair shafts than GENECLEAN kit.

STUDY OF ORGAN-LOCALIZED AUTOIMMUNE DISEASE WITH MODEL MICE ANALYSIS OF AUTOANTIGENS IN SJÖGREN'S SYNDROME

KATSUHIRO OHNO

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Sjögren's syndrome (SS) in human is characterized by focal lymphocytic infiltration into the salivary and lacrimal glands, resulting in symptoms with dry mouth and eye. Hypofunction and destruction of exocrine glands with massive lymphocytic infiltration is believed to be an autoimmune phenomenon; however, the immunopathogenesis is unclear. BALB/c nude mice that have been grafted with embryonic rat thymus (TG nude mice) are useful experimental model animals with multiple localized autoimmune diseases including SS. Transfer of $CD4^+$ cells from TG nude mice leads to such lesions in the recipient C.B-17-scid (SCID) mice. In TG nude mice, appearance of autoantibody(ies) against cytoplasm of salivary and lacrimal glands was closely correlated with the onset of inflammation in the target organs. Protein immunoblotting was performed using homogenates of the target organs and sera from TG nude mice with various histological grades. The density and the number of bands detected increased with the advance of histological grade. Each protein observed in the initial histological stage may be the trigger protein (submandibular gland: 42kD, sublingual gland: 270kD and lacrimal gland: 42kD). The 270kD protein in SLG, which had a unique NH_2-terminal sequence of "PRAPLNVAF", was novel protein.

OCCURRENCE OF PG-Lb, A LEUCINE-RICH SMALL CHONDROITIN/DERMATAN SULPHATE PROTEOGLYCAN IN MAMMALIAN EPIPHYSEAL CARTILAGE: MOLECULAR CLONING AND SEQUENCE ANALYSIS OF THE MOUSE cDNA

KAZUHIRO KURITA

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PG-Lb is a chondroitin/dermatan sulphate proteoglycan first isolated from chick embryo limb cartilage. It had been assumed that osteoglycin represents its mammalian homologue. However, partial amino acid sequences of a novel proteoglycan from bovine epiphyseal cartilage showed high identity with those of chick PG-Lb (personal communication). Reverse transcriptase PCR using degenerate oligonucleotide primers gave a cDNA fragment that might correspond to mouse PG-Lb. We isolated a clone from a cDNA library of newborn mouse epiphyseal cartilage using the cDNA fragment as a probe. The cloned cDNA was 1430 bp long and contained a 966 bp open reading frame which encoded the core protein consisting of 322 amino acid residues. The deduced amino acid sequence showed a high overall identity with chick PG-Lb. In addition, the amino acid sequence contained a signal peptide, six cysteine residues at the invariant relative position to chick PG-Lb, six leucine-rich repeats at the carboxyl two-thirds, three possible glycosaminoglycan-attachment sites and two possible Asn-glycosylation sites near the C-terminus. Northern blot analysis demonstrated the specific expression of a 1.5 kb message in cartilage and testis. These structural features and the characteristic expression suggest that the cloned molecule is mouse PG-Lb.

EFFECT OF DIBUTYRYL CYCLIC ADENOSINE MONOPHOSPHATE ON SKELETAL MUSCLE REPERFUSION INJURY IN RATS

TAKESI OHSHIMA

Department of Orthopaedic Surgery

We investigated the effect of dibutyryl cyclic adenosine monophosphate (DBcAMP) on reperfused ischemic skeletal muscle using the hindlimbs of 18 male Lewis rats. Total ischemia was produced by clamping the femoral artery and vein with a microvascular clamp for 4 hours, followed by 1 hour of reperfusion. DBcAMP or saline (control) was infused continuously from 1 hour before ischemia to 1 hour after ischemia. At 15 and 30 min. after reperfusion, increase in the blood flow change was significantly higher in the DBcAMP group than in the control group. Tissue ATP and Pcr was significantly higher in the DBcAMP group than in the control group. Skeletal muscle LPO did not differ between the DBcAMP group and the control group, but

serum LPO level was significantly lower in the DBcAMP group than in the control group. These data confirm that in this reperfusion model the administration of DBcAMP enhances the viability of skeletal muscle cells. Moreover, mediated by an effect on vascular endothelial cells, this agent is thought to be of help in mitigating the vascular endothelial cell injury occurring in acute ischemic injury. DBcAMP may be a useful agent in mitigating skeletal muscle ischemia-reperfusion injury.

DIFFERENTIAL DISPLAY ANALYSIS OF MURINE COLLAGEN-INDUCED ARTHRITIS: CLONING OF THE cDNA ENCODING MURINE ATPASE INHIBITOR

EIJI YAMADA

Department of Orthopaedic Surgery

We used the differential display technique in order to detect a new gene involved in the murine type II collagen-induced arthritis (CIA). In this study we identified a novel gene, IF1, whose expression level is increased during the natural course of CIA. Northern blot analyses suggest that IF1 is involved in the natural course of CIA except as a trigger of CIA. IF1 is considered to be the murine ATPase inhibitor gene by the following reasons. First, IF1 shows an extremely high homology to the rat ATPase inhibitor; the region of amino acid residues 22–45, which is the minimum sequence showing ATPase inhibitory activities and highly conserved between rat and bovine, is also highly conserved in IF1. Second, IF1 possesses a histidine-rich region in the corresponding area, which is thought to be important for the regulation of mammalian inhibitors. Third, the tissue distribution of IF1 is very suggestive. Expression of IF1 is very strong in energetic organs such as the heart, brain and kidney. It is known that development of arthritis requires great amounts of ATP. As the arthritis develops rapidly, the cellular ATP pool may be decreased. Before the ATP pool is exhausted, the ATPase inhibitor may serve as a brake for ATP hydrolysis. If the supply of free energy can be reduced, the inflammation may in turn be restored. Our hypothesis is that the ATPase inhibitor is involved in regulating the inflammatory responses.

HISTOLOGICAL STUDY OF THE EFFECT OF HYPERBARIC OXYGEN THERAPY ON AUTOGENOUS FREE BONE GRAFT

TOSHIHIRO SAWAI

Department of Oral Surgery

This study was undertaken to evaluate the effect of hyperbaric oxygen (HBO) therapy on autogenous free bone graft transplanted from iliac crest to mandibles of rabbits.

A piece of bone harvested from iliac crest was grafted to a same-size defect of mandibles in 16 Japanese white rabbits. In 8 rabbits, 20 and 10 sessions of HBO treatments were carried out twice per day (2.4 ATA 60 min.) before and after operation, respectively. The other 8 rabbits served as the controls. The grafted and surrounding bone were sampled at 1, 2, 4, or 8 weeks after transplantation, and the effects of HBO were evaluated by light micrography and contacto-microradiography.

At 1 week after the grafting, osteoid formation in the experimental group was much greater than that in the control group. Union between the grafted bone and host bone was observed in the experimental group at 2 weeks after grafting, but not in the control group until 4 weeks. At 4 weeks after, grafted bone and host bone turned into undistinguishable in the HBO treated group, but they could be differentiated each other in the control group.

These results indicated that HBO accelerated the union of autogenous free bone graft.

ROLE OF BRADYKININ B_2 RECEPTORS AND MAST CELLS IN THE BRADYKININ-INDUCED SKIN RESPONSE IN THE RAT

MASASHI MORISHITA

Department of Pediatrics

We investigated the role of activation of bradykinin receptors and mast cells in the microvascular leakage of the vessels of the skin induced by the intracutaneous (i.c.) injection of bradykinin in the rat. We evaluated the effects of HOE140, a bradykinin B_2 receptor antagonist, and ketotifen, a histamine H_1 receptor antagonist with mast cell stabilizing properties, on the skin response. Evans blue dye extravasation served as an index of the increase in vascular permeability. Bradykinin (2 to 100 nmol/site i.c.) induced the extravasation of Evans blue dye in a dose-dependent manner. Ketotifen (20 mg/kg i.p.) significantly inhibited the leakage of dye induced by bradykinin (10 nmol/site i.c.) by 66.2%, while HOE140 (1 mg/kg i.v.) had no effect. The concomitant injection of HOE140 (0.2, 2 nmol/site) and bradykinin (10 nmol/site i.c.), also did not significantly reduce the extravasation of dye. We conclude that the extravasation of plasma induced by the i.c. injection of bradykinin is mediated mainly by stimulation of the skin mast cells, but not by bradykinin B_2 receptors.

DIRECT PCR DETECTION OF THE METALLO-β-LACTAMASE GENE (bla_{IMP}) FROM URINE SAMPLES FOR EARLY RECOGNITION OF COLONIZING CARBAPENEM-RESISTANT BACTERIA

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Since gram-negative bacterial strains producing IMP-1-type metallo-β-lactamase usually demonstrate a wide range of resistance to all β-lactams including cephamycins, carbapenems and related combination drugs consisting of β-lactam and β-lactamase inhibitor, early recognition of IMP-1 producing strains is essential for rigorous infection control and prevention. In our previous studies, IMP-1 producing strains, such as *Pseudomonas aeruginosa* and *Serratia marcescens*, tended to be isolated from urine samples with high frequency. Therefore, polymerase chain reaction (PCR) was applied to a rapid and direct detection of the metallo-β-lactamase gene (bla_{IMP}) from urine samples. DNA templates were prepared directly from urine samples and specific amplification of the bla_{IMP} gene fragments was done using two PCR-primers (5′CTACCGCAGCAGAGTCTTTG3′, 5′AACCAGTTTTGCCTTACCAT3′). 587 bp fragments of the bla_{IMP} gene were successfully amplified from all urine samples, from which IMP-1 producing strains were isolated later by the ordinally culture method. According to the result of control studies, it was possible to detect 1×10^2 cfu/ml of bla_{IMP}-bearing bacteria in urine sample with this method. Hence, we conclude that the PCR-aided rapid detection of the bla_{IMP} gene from urine samples is helpful for early recognition of IMP-1 producing strains, and this enable us to prevent nosocomial transmission of multiple β-lactam-resistant gram-negative rods.

MORPHOLOGICAL STUDY OF PACEMAKER CELLS IN THE GUINEA-PIG COLON

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The smooth muscle tissue at the mesenteric border of the flexure region in the guinea-pig colon produces spontaneous regular contractions. Several experiments have suggested that in the colon spontaneous rhythmical contractions originated from the pacemaker cells expressing c-Kit which are located along the submucosal surface of the circular muscle layer. In this study, we examined the submucosal surfaces at the mesenteric border of the flexure region in the guinea-pig colon by electronmicroscopical, immunocytochemistrical, immunohistochemistrical and physiological methods, and compared to proximal and distal colons. Bi-polar and multi-polar type special smooth muscle cells were distributed in all regions, of which features were identified as the c-Kit immunopositive cells. Their slender cell processes constituted a cellular network. Caveolae, filament structures expressing smooth muscle actin, vimentin and little desmin, and

basal lamina were characteristic features of these cells. Their population was highest in the flexure region, and the circular muscle layer at the flexure region was thicker than in other regions. Spontaneous contractions in the flexure region showed the highest frequency and regularity. Thus it is suggested that bi-polar and multi-polar special smooth muscle cells correspond to the intestinal pacemaker cells. Their high population and the thicker muscle layer in the flexure region may produce quite regular rhythmical contractions, which may produce peristaltic movement.

PORTAL VEIN BRANCH LIGATION INDUCES SPHINGOMYELIN BREAKDOWN IN RAT HEPATOCYTE NUCLEI PRIOR TO DNA FRAGMENTATION

KYOJI TSUGANE

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Progression of cell death and its association with sphingomyelin pathway were investigated after ligation of a portal vein branch supplying 70% of the liver in rats, as a model of percutaneous transhepatic portal vein branch embolization, which is now an essential prophylactic procedure for postoperative liver failure following major hepatectomy. DNA fragmentation was detected in hepatocytes with the use of both terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling and agarose gel electrophoresis. 90 minutes after portal vein ligation, apoptotic DNA fragmentation was detectable in the tissue of the ligated hepatic lobes. In nuclei of hepatocytes in the ligated lobes, Mg^{2+} dependent neutral sphingomyelinase was activated at 15 minutes after ligation or later in parallel with its reaction product, ceramide. Nuclear ceramidase in the ligated lobes was also activated at 30 minutes after ligation and thereafter with simultaneous increase of its reaction product, sphingosine. In contrast, no significant change was observed in the nonligated lobes. These results suggest that portal vein branch ligation induces apoptosis within 90 minutes via activating nuclear sphingomyelin breakdown.

SERUM LEVELS OF TISSUE INHIBITOR OF METALLOPROTEINASES-2 AND OF PRECURSOR FORM OF MATRIX METALLOPROTEINASE-2 IN PATIENTS WITH LIVER DISEASE

MIEKO EBATA

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Serum levels of tissue inhibitor of metalloproteinases-2 (TIMP2) and of precursor form of matrix metalloproteinase-2 (proMMP2) were determined in patients with chronic hepatitis and

hepatocellular carcinoma by a one-step sandwich enzyme immunoassay. Serum levels of TIMP2 and proMMP2 were significantly higher in patients with chronic liver disease than in normal controls. Serum levels of TIMP2 showed a weak negative correlation with the serum albumin level and prothrombin time (PT). Serum levels of proMMP2 in patients with chronic hepatitis were strongly correlated with those of type IV collagen and were negatively correlated with PT and serum albumin levels. Serum proMMP2 levels were also significantly correlated with histological stages. These data indicate that serum levels of proMMP2 might be useful in the follow-up of patients with chronic hepatitis.

SERUM DNA POLYMERASE β AS AN INDICATOR FOR FATAL LIVER INJURY OF RAT INDUCED BY D-GALACTOSAMINE HYDROCHLORIDE AND LIPOPOLYSACCHARIDE

OSAMU KATO

2nd Department of Internal Medicine

DNA polymerase β (pol β) is a nuclear enzyme that is tightly bound to chromatin. Release of the pol β activity into serum, therefore, may indicate the occurrence of massive destruction of cell nuclei in organs or tissues. In the present study, we made a liver injury model rat by the intraperitoneal injection of D-galactosamine hydrochloride (GalN, 500 mg/kg) and lipopolysaccharide (LPS, 100 μg/kg). Serum from the GalN/LPS-treated rats showed a high level of pol β activity up to 118 pmol/0.5 μl serum (4,700 cpm) at 12 h after the treatment, while the control rat serum showed the background level (3.8 pmol/0.5 μl, 150 ± 70 cpm). The serum pol β activity was sensitive to inhibition by 2′,3′-dideoxyTTP and by an anti-rat pol β antibody. Among 30 rats treated with GalN/LPS, 10 rats died within 120 h (dead group). Serum pol β activity in the dead group was as high as 23.0 ± 19.5 p mol/0.5 μl (925 ± 778 cpm) at 10 h after the treatment, while in alive group (n=20), it was 3.7 ± 3.2 pmol. Levels of the serum pol β activity correlated well with the prognosis of GalN/LPS-treated rats based on an analysis of the receiver-operator characteristic curves.

CELLULAR SOURCE AND BIOLOGICAL ACTIONS OF GUANYLIN IN THE GASTROINTESTINAL TRACT

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Guanylin isolated from the rat small intestine is a peptide structurally related to the heat-stable enterotoxin (STa) produced by pathogenic Escherichia coli. In order to identify

proguanylin-producing cells, we raised an antiserum against proguanylin (1—15) fragment and have examined proguanylin-positive cells in the human and rat gastrointestinal tract by immunohistochemical methods. Proguanylin (1—15) immuno-postive cells were abundant in the stomach and duodenum but few in the ileum and colon. They were present in both crypts and villi and their morphological features were similar to those of endocrine cells in the gut. Double staining with anti-serotonin or -somatostatin monoclonal antibodies revealed that proguanylin (1—15) immuno-positive cells were also positive for somatostatin but not for serotonin, indicating that they are D cells. Luminal guanylin (10^{-8}—10^{-6}M), like STa, inhibited net water and NaCl absorption from the closed jejunal loop in anesthetized rats. We conclude that proguanylin (1—15) producing cells are intestinal D cells and guanylin released into the intestinal lumen may regulate intestinal fluid transport.

DIETARY MALTITOL DECREASES THE INCIDENCE OF 1,2-DIMETHYLHYDRAZINE-INDUCED CECUM AND PROXIMAL COLON TUMORS IN RATS

MIDORIKO TSUKAMURA

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Maltitol is fermented in the colon, due to only partial hydrolysis in the small intestine. In the present study, we examined effects of dietary maltitol on dimethylhydrazine-induced intestinal tumors in rats. In Experiment 1, rats were fed on a fiber-free diet or diets supplemented with 1 or 5g/100g maltitol for 27 weeks. Each group of rats was injected with dimethylhydrazine or vehicle alone for the first 14 weeks of the experimental period. Maltitol supplementation at 1% of the diet significantly reduced tumor incidence in the cecum and the 5% supplement reduced tumor incidence in both the cecum and proximal colon in dimethylhydrazine-treated rats. In Experiment 2, we investigated the effect of the 1% maltitol diet on the short chain fatty acid concentrations in cecal contents of placebo and dimethylhydrazine-treated rats. Intake of the 1% maltitol diet doubled ($P < 0.05$) the concentration of butyrate, but did not affect acetate or propionate, in the cecal contents. These results suggest that dietary maltitol has a protective effect against dimethylhydrazine-induced tumors in the rat cecum and proximal colon, and that butyrate produced by bacterial fermentation of maltitol in the cecum may be involved in the protection.

FIBRONECTIN SECRETION FROM HUMAN PERITONEAL TISSUE INDUCES Mr92,000 TYPE IV COLLAGENASE EXPRESSION AND INVASION IN OVARIAN CANCER CELL LINES

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Ovarian cancer cells disseminate by attachment to the peritoneal mesothelial cell surface of the abdominal cavity. We found that conditioned medium (CM) from human peritoneal tissue increased the secretion of matrix metalloproteinases (MMPs) by ovarian cancer cells. In an effort to identify this MMP-9 stimulating factor, we examined the effects of extra cellular matrix (ECM) components. We found that fibronectin increased the MMP-9 activity of ovarian cancer cells (NOM1) CM in a concentration-dependent manner and the peritoneal CM contained high level of fibronectin. An increase of MMP-9 activity in NOM1 cells CM by the peritoneal CM was almost completely blocked by 20 mg/ml of anti-integrin a5/FnR antibody and RGD polypeptides. Fibronectin and the peritoneal CM also increased MMP-9 activity and expression in NOM1 cell lysate and these effects were blocked by anti-integrin a5/FnR antibody. Invasiveness of NOM1 cells was enhanced by fibronectin and the peritoneal CM in a concentration-dependent manner, and anti-integrin a5/FnR the antibody blocked these effects. These results suggest that fibronectin secreted from peritoneum increased MMP-9 activity and expression, in turn invasiveness of ovarian cancer cells.

TSH-DEPENDENT INDUCTION OF TRANSCRIPTIONALLY ACTIVE NF-κB BY TNF-α IN RAT THYROID FRTL-5 CELLS

TOYONE KIKUMORI

2nd Department of Surgery

In autoimmune thyroid diseases, it has been suggested that TNF-α plays a key role in etiology of the diseases by stimulating the production of cytokines from the thyroid cells. Recently, TNF-α has been shown to activate NF-κB. We thus investigated TNF-α-mediated activation of NF-κB in FRTL-5 cells. Furthermore, it was studied whether TSH modifies the activation of NF-κB by TNF-α. In the absence of TSH, TNF-α induced the activation of p50 homodimer NF-κB, while in the presence of TSH, TNF-α induced p65-p50 heterodimer as well as p50 homodimer. Reporter gene experiment revealed that p65-p50 heterodimer is the transcriptionally active NF-κB. When the expression of IL-6, one of the target genes for NF-κB, was examined, the mRNA level was increased by TNF-α only in the presence of TSH. The treatment of the FRTL-5 cells with high-titer TSAb instead of TSH also induced the activation of p65-p50 heterodimer NF-κB by TNF-α. It is thus suggested that high levels of TSH or TSAb may aggravate autoimmune thyroid disorder by exerting their effects on cytokine-induced activation of transcriptionally active NF-κB.

INTERACTION OF BOTH THE C2A AND C2B DOMAINS OF RABPHILIN3 WITH Ca^{2+} AND PHOSPHOLIPID

HIDETO OISHI

3rd Department of Internal Medicine

Available evidence suggests that rabphilin3 is involved in Ca^{2+}-dependent neurotransmitter release as both a downstream target molecule of the Rab3 subfamily members and a Ca^{2+} sensor. Rabphilin3 has two C2 domains: the C2A and C2B domains. The C2 domain was originally found in protein kinase C, which is activated by Ca^{2+} and phospholipid. We have recently shown that overexpression of the C2B domain of rabphilin3, but not that of the C2A domain, inhibits Ca^{2+}-dependent exocytosis from PC12 cells, suggesting that the functions of these two domains are different. We have found here that both the C2A and C2B domains of rabphilin3 interact with Ca^{2+} only in a phospholipid-dependent manner with the similar kinetics. We have moreover found here that the C2A and C2B domains interact with phospholipid with slightly different kinetics: the C2A domain interacts with phospholipid in a Ca^{2+}-independent manner, whereas the C2B domain interacts with it in a Ca^{2+}-dependent manner. Physiological significance of the interaction of the C2 domains of rabphilin3 with phospholipid remains unknown. However, one fascinating function could be proposed that it may be related to the docking and/or fusion of synaptic vesicles with the presynaptic plasma membrane.

ISOFORM-SPECIFIC ACTIVATION AND STRUCTURAL DIVERSITY OF CAM KINASE I

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Department of Pharmacology

We earlier confirmed that there are isoforms of Ca^{2+}/calmodulin-dependent protein kinase I (CaM kinase I) (CaM kinase Iβ1, and Iγ) beside CaM kinase Iα by cDNA cloning. Here, we demonstrate the existence of an isoform-specific activation mechanism of CaM kinase I and alternative splicing specifically regulating CaM kinase I (CaM kinase Iβ2) in the central nervous system. To cast light on isoform structure-enzyme activity relationships, CaM kinase Iβ1, Iβ2, and Iα were expressed separately using a baculovirus/Sf9 cell expression system. The novel CaM kinase Iβ2 isoform demonstrated similar catalytic activity to those of CaM kinase Iβ1 and Iα. Interestingly, CaM kinase Iβ1 and Iβ2 both can activate CaM kinase Iα activity via phosphorylation at Thr177. RT-PCR analysis showed that CaM kinase Iβ2 is dominant in cerebrum and cerebellum, whereas CaM kinase Iβ1 is present in peripheral tissues such as liver, heart, lung, kidney, spleen and testis. CaM kinase Iβ2 was also detected with an anti-CaM kinase Iβ2 antibody in PC12 cells. The results indicate that alternative splicing is a means for tissue-specific expression of CaM kinase Iβ. Thus the Thr177 residue of CaM kinase Iα is phosphorylated by not only CaM kinase kinase but also CaM kinase Iβ for activation of the enzyme.

PHOSPHATIDYLINOSITOL 3 KINASE-DEPENDENT TYROSINE PHOSPHORYLATION OF FOCAL ADHESION KINASE, PAXILLIN AND p130Cas BY GDNF

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Ret is a receptor tyrosine kinase which plays a crucial role in the development of the kidney and the enteric nerve system. Recently, Glial cell line-derived neurotorophic factor (GDNF) which has been shown to be a potent survival factor for dopaminergic neurons was identified as a ligand for Ret. In order to characterize the signal-transduction pathway mediated by Ret tyrosine kinase, we investigated whether adaptor proteins such as Shc, Crk and Nck are bound to Ret activated by GDNF. The result demonstrated that Shc and Crk are bound to activated Ret and that Crk is also bound to tyrosine phosphorylated Paxillin and p130Crk associated substrate (p130Cas). GDNF induced tyrosine phosphorylation of Paxillin and p130Cas as well as Focal adhesion kinase (FAK) transiently and increased polymerization of actins. Pretreatment of cells with wortmannin, a specific inhibitor of PI 3-kinase, resulted in an inhibition of tyrosine phosphorylation of these focal adhesion proteins induced by GDNF. These results thus suggest that GDNF stimulates Paxillin, FAK and p130Cas tyrosine phosphorylation in a PI 3-kinase-dependent manner and that these focal adhesion proteins could be novel signaling components in GDNF-mediated signal transduction.

IDENTIFICATION OF A NOVEL NUCLEAR SPECKLE-TYPE PROTEIN, SPOP

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A novel antigen recognized by serum from a scleroderma patient was identified by expression cloning from the HeLa cell cDNA library. The cloned cDNA encoded a 374-amino acid protein with a relative molecular mass of 47,000 (Mr 47K) and a predicted amino acid sequence 62.7% identical to the hypothetical protein of *C. elegans*, T16H12.5. The deduced amino acid sequence had a typical POZ domain and an unidentified region conserved during evolution. No zinc finger or RNA recognition motifs were found in this clone. The 2 kb mRNA encoding the novel clone SPOP (speckle-type POZ protein) was found to be expressed in all human tissues examined. HA-tagged SPOP, transfected and overexpressed in COS7 cells, exhibited a discrete speckled pattern in the nuclei and was co-localized with the splicing factor, snRNP B′/B. Deletion analysis revealed that both the POZ domain and the evolutionally conserved region at the amino-terminus were required for the nuclear speckled accumulation of SPOP.