ANNUAL RESEARCH MEETING

FOR

GRADUATE STUDENTS

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Abstracts

DETECTION OF PLATELET MITOCHONDRIAL DNA DELETIONS IN KEARNS-SAYRE SYNDROME

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To establish a noninvasive genetic diagnosing method for Kearns-Sayre syndrome, we utilized the polymerase chain reaction (PCR) technique for detecting mitochondrial DNA (mtDNA) deletions in the platelets and directly sequenced the crossover regions of the deleted mtDNA using the fluorescence-based automated sequencing system. We identified mtDNA deletions in the platelets in three of four patients. In Patient 1, a one-base repeat of G was found at the boundaries of a deleted segment spanning 8400 bp. In Patient 2, a 9-bp directly repeated sequence of 5′-ACCTCCCTC-3′ was found at the boundaries of a deleted segment spanning 7221 bp. In Patient 3, a 8-bp sequence of 5′-TCGCTGTC-3′ was found at the boundaries of a deleted segment spanning 4664 bp. Deletions were not detected neither in Patient 4 or in the mothers of the patients. The genetic diagnosis of this syndrome has required muscle biopsy specimens and the use of Southern blot analysis. On the other hand, the present method needs neither the muscle biopsy nor isotopes and is more rapid than the Southern blot method. Our results indicate that the present method is useful for noninvasive genetic diagnosis of Kearns-Sayre syndrome.

AGE-DEPENDENT INCREASE IN DELETED MITOCHONDRIAL DNA IN THE HUMAN HEART: POSSIBLE CONTRIBUTORY FACTOR TO PRESBYCARDIA

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Cardiac function deteriorates with age, and endogenous damage to mitochondrial DNA (mtDNA) is believed to be a major contributory factor to aging. Mitochondria have their own DNA, which encodes 13 subunits of the mitochondrial energy transducing system. MtDNA has been shown to have a high mutation rate, and recent advances in gene technology permit us to analyze mtNDA mutations in a small quantity of tissue. We devised rapid and accurate methods to detect mtDNA mutations, and we analyzed myocardial mtDNA in human cadavers of various ages (from 3 years old to 97 years old, mean 57 years old). A 7.4 kb deletion of mtDNA was commonly detected in elderly subjects, and the incidence of deleted mtDNA increased with age. Deleted mtDNA was observed in all subjects over 70 years old. The mutation was based on the directly repeated sequence: 5′-CATCAACAACCG-3′, which exists in both me ATPase 6 gene and the D-loop region. Some subunits of the mitochondrial energy transducing system could not be biosynthesized by the deleted mtDNA, leading to inhibition of energy transduction, which might contribute to the genesis of aging heart (presbycardia).

AGING-ASSOCIATED DELETIONS OF HUMAN DIAPHRAGMATIC MITOCHONDRIAL DNA

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It is known that respiratory function deteriorates with age. Endogeneous damage to DNA is thought to contribute to the aging process. Because mitochondria supply energy for muslce contraction and mitochondrial DNA (mtDNA) encodes 13 subunits of the mitochondrial oxidative phosphorylation system, age-related mutations in mtDNA may lead to deterioration of muscle performance. We examined the diaphragmatic mtDNA mutations obtained at autopsy from 34 cadavers. Multiple mtDNA deletions were detected particularly among the elderly and the number of deletions in those over 70 years was significantly higher than in those under 40 years. The proportion of the 3.4 kb deletion of mtDNA commonly observed in elderly to normal mtDNA increased with age. The deletion was based on the following, directly repeated sequence: 5′-TCACCCC-3′. Replication impairment occurred at that directly repeated sequence, which caused the elimination of a genome between the CO3 gene and the ND5 gene, and information of four subunits in complex I, one subunit in complex IV and five transfer RNA genes was deleted. These results indicate that the deterioration of mitochondrial electron transport function, due to aging-associated mtDNA deletions observed here, may be responsible for the decrease in respiratory muscle function associated with age.

SENSITIVE ENZYME IMMUNOASSAY FOR HUMAN 28 kDa CALBINDIN-D

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A sandwich-type enzyme immunoassay for human 28 kDa vitamin D-dependent calcium binding protein (calbindin-D) was established with a sensitivity of 1 pg/tube. Antisera were generated in rabbits injected with highly purified human kidney calbindin-D, and specific antibodies to calbindin-D were purified by the use of a column of calbindin-D-coupled Sepharose. The purified antibodies showed a single band at the position corresponding to calbindin-D on an immunoblotting test with a crude extract of human kidney. The assay system consisted of polystyrene balls with immobilized $F(ab')_2$ antibodies and the same antibodies labeled with β-D-galactosidase from *Escherichia coli.* The sassy was specific to 28 kDa calbindin-D, showing no cross-ractivity with other calcium binding proteins such as S-$100a_0$ (αα), S-100b (ββ), parvalbumin and calmodulin. The assay was also reproducible (coefficients of variation between assays were $< 10\%$). With the present method, immuno-reactive calbindin-D could be detected in various human tissues, with major concentrations in kidney and brain. The values for immunoreactive calbindin-D in various body fluids of healthy subjects varied from undectable in serum

and semen to 3.8 ± 2.0 (SD) μg/g creatinine in urine and 2.9 ± 0.8 (SD) μg/l in cerebrospinal fluid. Immunohistochemically, the calbindin-D in human kidney was localized in epithelial cells of distal tubules. (*Clin Chim Acta 201: 183–192 1991*)

EVALUATION OF RENAL DAMAGE AFTER EXTRACORPOREAL SHOCKWAVE LITHOTRIPSY BY MEASURING SERUM AND URINARY 28-kDa CALBINDIN-D

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In order to assess the renal damage following ESWL, 28-kDa calbindin-D was determined in serum and urine from the patients with renal stone and ureteral stone before and after ESWL, using a highly sensitive enzyme immunoassay method which was established recently. 28-kDa calbindin-D is a vitamin-D dependent calcium binding protein which is mainly localized in distal renal tubules and central nervous tissues. The patients were treated by Lithostar (N=59) or MPL9000 (N=22). The results of the present study indicated that: (1) MPL9000 causes stronger and longer renal damage than Lithostar; (2) Renal damage occurs during ESWL not only in the patients with renal stones but also in those with ureteral stones; (3) 28-kDa calbindin-D is a useful marker of renal damage caused by ESWL.

PURIFICATION, CHARACTERIZATION AND PARTIAL SEQUENCE ANALYSIS OF A NEWLY IDENTIFIED EF-HAND TYPE 13 kDa Ca^{2+}-BINDING PROTEIN FROM SMOOTH MUSCLE

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A novel Ca^{2+}-binding protein, tentatively designated calgizzarin, has been purified to apparent homogeneity from chicken gizzard smooth muscle by W-7 [N-(6-aminohexyl-5-chloro-1-naphthalenesulfonamide)] Sepharose affinity chromatography and ion-exchange chromatography. Application of W-7 Sepharose affinity chromatography to various tissues revealed that calgizzarin-like proteins were abundant in bovine aorta and rabbit lung. Using the same procedure, we could purify a calgizzarin-like protein from rabbit lung. Calgizzarin has a molecular weight of 13,000 as determined by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis and approximately 30,000 as determined by gel filtration on a TSK G 3000SW high performance liquid chromatography (HPLC) column, suggesting that calgizzarin seems to be a rod-like protein. The isoelectric point of calgizzarin was found to be pH 5.8. Calgizzarin can

exist as a dimer by forming a disulfide bridge. The ^{45}Ca autoradiographic technique showed that the protein binds to Ca^{2+}. On an alkaline/urea gel, calgizzarin migrated faster in the presence of [ethylenebis(oxyethylenenitrilo)] tetraacetic acid (EGTA) than in the presence of $CaCl_2$, thereby indicating a Ca^2-dependent conformational change in this protein. The partial amino acid sequence (65 amino acid residues) of calgizzarin was seen to be (SLLAVFQRYAGEGD-NLKLSKKEFRTFMNTELASFTKNQKDPAVVDRMMKRLDINSDGQLDFQEF) and two putative Ca^{2+}-binding sites (GREGDNLKLSKKE and DINSGQLDFQE) were detected. So far as the obtained 65-amino acid sequence is concerned, calgizzarin has approximately a 50% sequence homology with S-100α, 47% with S-100β and 39% with pEL-98 protein.

THE 5′-FLANKING REGION OF HUMAN DOPAMINE β-HYDROXYLASE GENE PROMOTES THE NEURON SUBTYPE-SPECIFIC GENE EXPRESSION IN THE CENTRAL NERVOUS SYSTEM OF TRANSGENIC MICE

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Dopamine β-hydroxylase (DBH, EC 1.14.17.1) catalyzes the conversion of dopamine to norepinephrine, the third step of catecholamine biosynthesis. We have previously created transgenic mice harboring a chimeric gene consisting of the 4-kb DNA fragment of the human DBH gene promoter and the human phenylethanolamine *N*-methyltransferase (PNMT, EC 2.1.1.28) cDNA, to express PNMT in norepinephrine- and epinephrine-producing cells in brain, sympathetic ganglia, and adrenal meduallary chromaffin cells [Kobayashi *et al.*, *Proc. Natl. Acad. Sci. U.S.A.*, 89 (1992) 1631–1635]. In this paper, we produced newly the antibody that can detect specifically human PNMT, but not mouse PNMT, with the synthetic oligopeptide characteristic of the human PNMT sequence, and used this antibody to investigate the cells expressing human PNMT in transgenic mice. Immunohistochemical analysis of transgenic mice showed typical expression of the human PNMT immunoreactivity in both norepinephrine and epinephrine neurons in brain, as well as in norepinephrine- and epinephrine-producing cells in adrenal gland, indicating that the 4-kb 5′-flanking region is essential for the tissue-specific expression of the DBH gene. We also detected the ectopic expression in some DBH-immunonegative cells in the olfactory bulb of transgenic mice.

THE EFFECTS OF CALMODULIN INHIBITORS ON AMYLASE SECRETION FROM RAT PANCREATIC ACINI

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To investigate the role of calmodulin in stimulus-secretion coupling in pancreatic acinar cells, we studied the effects of W-7, a calmodulin inhibitor, and KN-62, a specific inhibitor of Ca^{2+}/calmodulin-dependent protein kinase II (Ca^{2+}/CaM kinase II), on amylase secretion from rat pancreatic acini. Calmodulin inhibitor (W-7, 100 μM) and Ca^{2+}/CaM kinase II inhibitor (KN-62, 10 μM) reduced amylase secretion stimulated by cholecystokinin (CCK) or carbachol. W-7 and KN-62 also inhibited amylase secretion stimulated by both calcium ionophore (A23187) and phorbol ester (12-O-tetradecanoylphorbol 13-acetate, TPA). To clarify the role of calmodulin in the interaction of intracellular mediators, pancreatic acini were permeabilized with streptolysin O. Following permeabilization, amylase secretion was stimulated by submicromolar free Ca^{2+}, and this Ca^{2+}-dependent amylase secretion was enhanced by guanosine 5′-[γ-thio]triphosphate (GTPγS), TPA or cyclic adenosine-3′,5′-monophosphate (cAMP). W-7 and KN-62 had no effects on amylase secretion stimulated by Ca^{2+} alone, but inhibited the enhancement of Ca^{2+}-dependent amylase secretion by GTPγS, TPA or cAMP. These data suggest that calmodulin plays an important role in Ca^{2+}-dependent amylase secretion from pancreatic acinar cells and in the interaction between Ca^{2+} and other intracellular messengers.

STRUCTURE OF THE MOUSE TYROSINE HYDROXYLASE GENE

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The mouse tyrosine hydroxylase (TH) gene was isolated from a genomic library by cross-hybridization with human TH cDNA probe. Nucleotide sequence analysis of two overlampping genomic clones showed that this gene is split into 13 exons distributed about 7.5-kb in length. The transcription initiation site was determined by primer extension analysis with mouse adrenal gland poly(A)$^+$RNA. The structure of the mouse TH gene was similar to that of the human TH gene, but it contained neither the alternative splice doner site around the 3′-end of the first exon nor an independent exon corresponding to the second exon of the human TH gene. There were the canonical TATA and GC boxes, cyclic AMP responsive element (CRE), and AP1 binding site in the 5′-flanking region of the mouse TH gene.

N-METHYLATION OF DOPAMINE-DERIVED 6,7-DIHYDROXY-1,2,3,4-TETRAHYDROISOQUINOLINE, (*R*)SALSOLINOL, IN RAT BRAINS: *IN VIVO* MICRODIALYSIS STUDY

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N-methylation of (*R*)1-methyl-6,7-dihydroxy-1,2,3,4-tetrahydroisoquinoline [(*R*)salsolinol], derived from dopamine was proved by an *in vivo* microdialysis study in the rat brain. The striatum was perfused with (*R*)salsolinol and N-methylated compound was identified in the dialysate using high-performance liquid chromatography and electrochemical detection with multi-channeled electrodes. N-methylation of (*R*)salsolinol was confirmed in three other regions of the brain; the substantia nigra, hypothalamus and hippocampus. In the substantia nigra, the amount of N-methylated (*R*)salsolinol was significantly larger than in the other 3 regions. These results indicate that around dopaminergic neurons, particularly in the substantia nigra, (*R*)salsolinol was methylated into N-methyl-(*R*)salsolinol, which has a chemical structure similar to that of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine, the selective dopaminergic neurotoxin. N-methylation of tetrahydroisoquinolines and β-carbolines has been proved to increase their toxicity to dopaminergic neurons and N-methylation might be an essential step for these alkaloids to increase their toxicity. On the other hand, following perfusion of (*R*)salsolinol, release of dopamine and 5-hydroxytryptamine was observed and inhibition of monoamine oxidase was indicated. (*R*)Salsolinol and its derivatives may be candidates for dopaminergic neurotoxicins.

CLINICAL EVALUATION OF SERUM TISSUE INHIBITOR OF METALLOPROTEINASES−1 (TIMP-1) LEVELS IN PATIENTS WITH LIVER DISEASES

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Serum levels of the tissue inhibitor of metalloproteinases (TIMP-1) were measured in 268 patients with liver diseases by means of a one-step sandwich enzyme immunoassay. In the cases of acute hepatitis, chronic active hepatitis (CAH), liver cirrhosis (LC) and hepatocellular carcinoma (HCC), TIMP-1 levels were higher than that of controls. There was a gradual increase in TIMP-1 levels from chronic inactive hepatitis, through CAH to LC. Serum levels of alpha fetoprotein (AFP) and TIMP-1 had a significant positive correlation in the patients with HCC. A cut-off level of TIMP-1 between LC and HCC was set at 440 ng/ml, having a low sensitivity and a high specificity. Furthermore, close relations were noted between TIMP-1 and type III

procollagen peptide (PIIIP), as well as between TIMP-1 and 7S domain of type IV collagen (7S). However, prolyl hydroxylase showed no correlation with TIMP-1, PIIIP, or 7S. These results suggest the usefulness of TIMP-1 as a tumor marker in cases of HCC where AFP levels are not elevated and also as a fibrosis marker in chronic liver diseases.

ELEVATED LEVELS OF ONCOPTERIN, N^2-(3-AMINOPROPYL)BIOPTERIN, A NEW PTERIN COMPOUND, IN URINE FROM PATIENTS WITH SOLID AND BLOOD CANCERS

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The concentrations of oncopterin, N^2-(3-aminopropyl)biopterin, a new pterin compound, were determined in urine from various cancer patients by HPLC on a reverse-phase or ion-exchange column. The concentration of oncopterin increased after acid hydrolysis, indicating that it exists as an amide in urine. The oncopterin concentrations were very low in the urine of healthy controls. Among the uring samples examined, those from cases of solid cancers, *e.g.*, hepatomas, prostatic cancer and bladder cancer, exhibited very high levels; and those from cases of blood cancers, *e.g.*, myelomas, acute myelocytic leukemia and lymphomas, showed moderate increases. Oncopterin may thus be a new biochemical marker of some types of cancer.

MEASUREMENT OF ALPHA-KETOGLUTARATE DEHYDROGENASE ACTIVITY IN HUMAN PLATELETS AND TISSUE EXTRACTS USING REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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A new method for the determination of the activity of alpha-ketoglutarate dehydrogenase complex (KGDHC) in human platelets and mouse brain and liver mitochondria is described. This method is based on the quantification of succinyl-CoA formed in the reaction catalyzed by KGDHC. Succinyl-CoA was separated using a YMC-Pack C_8 column employing isocratic elution and detected spectrophotometrically at 254 nm. The detection limit of succinyl-CoA was 0.05 nmol. Succinyl-CoA in the supernatant of the assay mixture was stable for several hours at 4°C and for a week when stored at −20°C. The enzyme showed linearity with time and added protein and all tissues demonstrated an absolute requirement for added alpha-ketoglutarate,

nicotinamide dinucleotide and coenzyme A and paratial or no requirement for thiamine pyrophosphate, $MgCl_2$, and dithiothreitol. The specific activities in liver and brain mitochondria and platelet homogenates were 19.2 ± 0.9, 18.1 ± 2.8 and 2.6 ± 0.3 nmol/min/mg protein, respectively. This method is simple, rapid, and can be readily employed for the determination of KGDHC activity in various animal tissues and human platelets.

ABNORMALITIES IN THE OLIGOSACCHARIDE MOIETIES OF IMMUNOGLOBULIN G IN PATIENTS WITH MYOTONIC DYSTROPHY

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Changes were identified in the glycosylation profiles of immunoglobulin G (IgG) from 15 patients with myotonic dystrophy (MyD). IgG oligosaccharidies released by Glycoamidase A treatment were labeled with the fluorescent compound, 2-aminopyridine, and separated by HPLC. The structural changes in the oligosaccharide moieties of IgG in these patients were characterized by marked increases in agalactosyl oligosaccharides and decreases in digalactosyl oligosaccharides. Furthermore, the ratio of the level of Galβ4GlcNAcβ2Manα6(GlcNAcβ2Manα3) Manβ4GlcNAcβ4(Fucα6)GlcNAc to the level of GlcNAcβ2Manα6(Galβ4GlcNAcβ2Manα3) Manβ4GlcNAcβ4(Fucα6)GlcNAc was 1.3 in patients with MyD, against 2.3 in controls. These data suggest that increases in agalactosyl oligosaccharides of IgG occur in patients with MyD as a result of functional disturbance in the activity of β1,4-galactosyl transferase.

AMPLIFICATION OF HLA-DQA1 GENE FROM BLOODSTAINS BY POLYMERASE CHAIN REACTION

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The DNA typing of HLA-DQA1 from bloodstains is described. Although amplification of DNA from bloodstains was sometimes difficult, the addition of BSA (40 ~ 800 μg/ml) to the PCR medium induced good amplification. When a very limited amount (2 mm^2, equivalent to 0.5 μl blood) of bloodstain was analyzed, the amplification was difficult. However, the addition of salmon sperm DNA to the DNA isolation medium, in the amount of 1 μg per tube, yielded sufficient PCR products even when this small amount of extract was analyzed. DNA from bloodstains up to one year old could be sufficiently amplified, if salmon sperm DNA was added to the DNA isolation medium, and BSA (400 μg/ml) to the PCR medium. These DNA

specimens, if sufficiently amplified, could be typed correctly. In this study, HLA-DQA1 gene from a very limited amount of bloodstains or aged bloodstains could be well amplified, and the present method seems to be useful for forensic investigations.

INHOMOGENEITY OF EXCITATION IN NORMAL MYOCARDIAL TISSUE UNDER ELECTRICAL FIELD STIMULATION: POSSIBLE RELEVANCE OF DIVERSE CELL EXCITABILITY

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We investigated excitation process under electrical field stimulation (FS) using guinea pig hearts and a numerical model. Maximum upstroke velocity (V_{max}) of action potential recorded from the papillary muscles (PM) under FS was 267 ± 37 V/s, while that in the isolated ventricular cells (512 ± 68 V/s) and that near collision sites in PM (409 ± 33 V/s) were about double the value ($p < .0001$). Action potential mapping on PM under FS showed that all the upstrokes had the lower V_{max} and the phase plane trajectories with quasilinear initial ascent limbs, which were indistinguishable from those during normal propagation. The activation times appreciably varied within a PM (4.9 ± 0.8 ms). The isochrone maps showed that excitation was initiated only from a few sites, and the activation wavefronts propagated into the neighboring tissue. We hypothesized that the excitation process under FS arose from diverse cell membrane response, and tested it using a numerical model where the diverse cell membrane response was assumed. As a result, the basic experimental observations were reproduced. We conclude that inhomogeneous excitation and propagation occur in the normal myocardium under FS. The divers cell membrane response can play an important role in the tissue excitation under FS.

A STUDY OF ANTIEPILEPTIC DRUG-Ca^{2+} CHANNEL INTERACTION IN NEUROBLASTOMA CELLS

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Antiepileptic drug-Ca^{2+} channel interaction in human neuroblastoma cell line NB-I was studied with whole-cell recording mode of the patch-clamp techniques.

NB-I cells had voltage-dependent T-, N- and L-type Ca^{2+} channel currents (I_{Ca}).

5 μM phenytoin (PHT) inhibited T-type I_{Ca} by 13.0%. 100 μM carbamazepine (CBZ) had no effect on I_{Ca}. By 50 μM valploic acid (VPA), L-type I_{Ca} showed 54.8% inhibition. 50 μM zonisamide (ZNS) inhibited T and L-type I_{Ca} by 42.6% and 52.8%, respectively.

Na^+ channel blockade is probably responsible for the clinical efficacy of PHT and CBZ. Inhibition of T-type I_{Ca} by PHT may enhance the efficacy of its anticonvulsant action. The anticonvulsant activity may be related to the blockade of T-type Ca^{2+} channel in the case of ZNS. Inhibition of L-type I_{Ca} by VPA and ZNS may influence the long-term cell functions through the second messenger system.

EFFECTS OF AMMONIUM CHLORIDE ON MEMBRANE CURRENTS OF ACINAR CELLS DISPERSED FROM THE RAT PAROTID GLAND

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In various exocrine cells, it is generally accepted that activation of both basolateral K^+-selective channels and apical Cl^--selective channels by intracellular Ca^{2+} plays a very important role in stimulus-secretion coupling. In acinar cells freshly dispersed from rat parotid glands, the effects of ammonium chloride (NH_4Cl) on Ca^{2+}-dependent membrane currents were studied using the whole-cell clamp method.

When membrane currents were recorded with command pulses to 0 mV, applied at 2-s intervals from a holding potential of −70 mV, NH_4Cl (5−20 mM) transiently decreased outward currents and then slowly increased both outward and inward currents. Changes in the current/voltage relation produced by NH_4Cl (20 mM) were very similar to those produced by carbachol (10 μM). In the absence of NH_4Cl, changes in the external pH had little effect on the membrane conductance between −90 mV and −20 mV, but response of NH_4Cl was reduced by lowering the external pH. Butyrate had little effect on the resting membrane currents, but markedly inhibited the response to NH_4Cl. Carbachol-induced membrane currents were relatively resistant to Ca^{2+} removal from the external medium, but NH_4Cl-induced currents were quickly abolished in the absence of Ca^{2+}.

These results demonstrated that intracellular alkalinization increases Ca^{2+} influx and activates Ca^{2+}-dependent K^+ and Cl-channels.

INTRACELLULAR ALKALINIZATION CAUSED BY CHLORIDE REMOVAL IN THE SMOOTH MUSCLE OF GUINEA-PIG VENA CAVA

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Intracellular pH (pH_i) of smooth muscle of guinea-pig vena cava was measured with a pH-sensitive dye, 4′,5′-dimethyl-5-(and -6) carboxy-fluorescein (Me_2CF). PH_i in normal solution was 7.15. Sustained intracellular alkalinization (pH_i 7.46) was produced when Cl^- was replaced with gluconate in the presence of HCO_3^-, but not in the absence of HCO_3^-. This response was nearly completely blocked by 200 μM DIDS, a Cl^- transport inhibitor. When full alkalinization was developed in Cl^--deficient solution, removal of HCO_3^- produced only a weak recovery. pH_i at steady state (7.15) was nearly the same in the presence and absence of HCO_3^- and it was not affected by DIDS. An carbonic anhydrase inhibitor, acetazolamide (100 μM) had no clear effect on pH_i under normal conditions and also on the pH_i change in Cl^--deficient solution. Intracellular acidification caused by Na^+ removal was much less when NaCl was replaced with sucrose than with N-methyl-D-glucamine chloride. It was concluded that pH_i can be strongly altered by Cl^--HCO_3^- exchange mechanism in the guinea-pig vena cava, but that a contribution of this mechanism to pH_i regulation seems rather minor under physiological conditions.

JUVENILE DERMATOMYOSITIS: A STATISTICAL STUDY OF 114 PATIENTS WITH DERMATOMYOSITIS

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We conducted a statistical review of 114 cases of dermatomyosistis (DMS) treated primarily at the Department of Dermatology at Nagoya University Hospital over 27 years from 1965 to 1991 in order to determine the primary characteristics of juvenile DMS with the following results.

1) Juvenile DMS was found slightly more often in males than in females; the male-to-female ratio was 1.4:1. Therefore, unlike adult DMS with its preponderance of females, there was no clear gender predominance.

2) Muscular manifestations tended to follow the appearance of cutaneous manifestations, but the frequency of minor muscular manifestations was high over the entire course of the disease.

3) Laboratory findings showed increases in serum aldolase and serum creatine kinase with significant frequency when compared with adult patients ($p < 0.01$ and $p < 0.05$, respectively). Elevated serum aldolase most often occurred prior to or at the time of the appearance of muscular manifestations, suggesting its usefulness in early diagnosis. The positive rates for the

antinuclear antibody on HEp-2 cells and anti-DNA antibody were significantly lower in children than in adults ($p < 0.001$ and $p < 0.05$, respectively).
4) There were no cases of juvenile DMS complicated by malignant tumors, interstitial pneumonia, or pulmonary fibrosis. There were also no deaths, and the rate of "remission or improvement" was significantly higher than in adult DMS cases ($p < 0.05$). Adult cases which remained the same or worsened usually presented with intractable muscular manifestations. In children, however, the cutaneous manifestations were more difficult to treat.

The above conclusions from this statistical study suggest the possibility that juvenile DMS is an entity separate from adult DMS.

ANALYSIS OF VENTRICULAR ARRHYTHMIA USING RR INTERVAL PLOTTING IN CHILDREN

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Holter electrocardiographic monitoring records were analyzed using the RR interval plotting method in 83 children who had more than 1000 ventricular premature contractions (VPCs) per day. The RR interval plotting is a new Holter computer analyzing method whereby every interval of sinus QRS complex preceding a VPC is plotted on the X-axis, and every coupling interval is plotted on the Y-axis. The X-Y correlation ($Y=aX+b$) and standard deviation (SD) of coupling intervals are then calculated. We classified the patients according to the values of SD and gradient (a) into two types, fixed type: $SD < 40$ msec, $a < 0.02$; unfixed type: the others. Then we divided unfixed type into four subtypes according to the patterns of the RR interval plotting: scattered type, dependent type, biphasic type, and intermediate type. We investigated the site of origin of VPCs, organic heart disease, ventricular tachycardia (VT), and ventricular parasystole in these five subtypes.

In the right VPCs patients, the fixed type and the dependent type were more popular (31/59 vs 4/24, $p < 0.005$; 13/59 vs 1/24, $p < 0.05$) and the scattered type was less (4/59 vs 18/24, $p < 0.005$) than in the left ones. The biphasic type and the intermediate type had a tendency to be more frequent among the right VPCs patients, but not significantly. There were 13 patients who had organic heart diseases, and seven of those belonged to the fixed type. The sites of origin of these seven's VPC were right. There were 34 patients who had VT. In the scattered type, VT patients were rare (2/22, 9.1%) and in the other all, almost half of patients had VT. There were 14 exercise-induced VT patients, and six of these 14 belonged to the dependent type. All ventricular parasystole patients belonged to the scattered type and they were more frequently seen in the left VPCs patients than in the right ones (7/24 vs 4/59, $p < 0.01$).

We thought that it was useful to relate the classification of the RR interval plotting method with the site of origin of VPCs, organic heart disease, VT, and ventricular parasystole in children.

DO LIQUID CRYSTAL DISPLAYS ASSURE BETTER READABILITY THAN CATHODOE-RAY TUBES?

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Flat-panel displays, especially liquid crystal displays (LCDs) are established as important devices for information displays. Do LCDs assure better readability than cathode-ray tubes (CRTs)? An experiment was carried out to solve this question. Fifteen subjects took part in this experiment. They were five young males, five young females and five old males. Four types of displays were tested: negative and positive CRTs, and LCDs with and without backlighting. The ambient light was controled in four levels. A syllable-checking task using Japanese Kata-kana was chosen. The subjects performed this checking task for twenty seconds under each experimental condition. The number of total syllabes inspected during 20 seconds was recorded. Multiple regression analyses revealed that non-backlit LCDs significantly reduced reading performance among middle-aged subjects, but that in young subjects it was not significant. Since middle-aged workers have more difficulties than young workers, they should have more appropriate displays and even more comfortable illuminance environment than young workers.

PRECURSOR B LYMPHOBLASTOID CELL LINES ESTABLISHED FROM A PATIENT WITH SEVERE COMBINED IMMUNODEFICIENCY SHOWED GERMLINE CONFIGURATION IN IMMUNOGLOBULIN HEAVY CHAIN GENE LOCI

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Using Epstein-Barr viruses (EBV), we established precursor B lymphoblastoid cell lines by transforming bone marrow cells of a patient with severe combined immunodeficiency (SCID), whose B and T cells were markedly decreased in members. Although these cell lines were thought to be at an early stage of B cell differentiation according to their surface phenotype, immunoglobulin heavy chain (IgH) gene loci retained the germline configuration on both chromosomes in almost all the transformants. We found that the IgH enhancer locus was not methylated and that germline Cμ transcripts were detected by Northern hybridization in these cell lines. These results suggest that the chromatin structure of IgH gene loci in these cell lines become accessible to V(D)J recombianse and is fully competent for DNA rearrangement. However, we could not detect the transcripts of RAG-1 and RAG-2 genes which were required for V(D)J recombination in Ig and T cell receptor gene loci. Thus, it seems likely that these cell lines did not initiate the V(D)J recombination process because of some deficiency in the formation of V(D)J recombinase including inability of RAG gene expression.

CORRELATION OF RESPONSE OF APLASTIC ANEMIA PATIENTS TO ANTILYMPHOCYTE GLOBULIN WITH IN VITRO LYMPHOCYTE STIMIULATORY EFFECT: PREDICTIVE VALUE OF IN VITRO TEST FOR CLINICAL RESPONSE

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Therapy with antilymphocyte globulin (ALG) has been shown to be effective in restoring hematopoiesis to some patients with aplastic anemia. The mode of immunostimulatory action of ALG is of interest in addition to its immunosuppressive action. We examined in vitro the distribution of the proliferative responses of ALG-stimulated peripheral blood mononuclear cells (PBMCs) obtained from 18 patients with aplastic anemia; eight of them responded to ALG and 10 did not. We found a significant difference in the proliferative response between PBMCs obtained from the eight responders and 10 nonresponders ($P < .01$). Two-color flow cytometry analysis of the patients' PBMCs stimulated by ALG in vitro showed that the CD4-positive subsets were activated to a greater extent than the CD8-positive subsets. Moreover, a positive correlation with the clinical response of patients to ALG with GM-CSF produced by their PBMCs stimiulated by ALG suggests that the immunostimulatory property of ALG has an important role in the treatment of aplastic anemia. Our results suggest that the clinical response to ALG therapy is correlated with its lymphocyte proliferative effect in vitro, and indicate that the assessment of the proliferative response of PBMCs in vitro would be useful in predicting the clinical response to ALG therapy.

MOLECULAR ANALYSIS OF DIFFERENTIATION-INDUCING EFFECT OF ALL-TRANS-RETINOIC ACID ON ACUTE PROMYELOCYTIC LEUKEMIA (APL)

HARUHIKO OHASHI

1st Department of Internal Medicine

Sequential molecular analyses of hematologic cells were carried out during remission induction in a patient with acute promyelocytic leukemia (APL) by all-trans-retinoic acid (ATRA). Bone marrow (BM) and periphiral blood (PB) cells sampled before and after ATRA therapy were subjected to Southern hybridization analyses. Clonality was assessed with a polymorphic X chromosome-linked probe, and rearrangement in retinoic acid receptor-α (RAR-α) gene was also analysed. BM cells sampled before ATRA therapy and BM cells and granulocytes on day 24 of therapy proved monoclonal and also showed rearrangement of RAR-α gene. In contrast, granulocytes on day 53 and BM cells on day 60 proved polyclonal, and the latter was free of

RAR-α gene alterations. It was demonstrated that remission induction of APL by ATRA proceeds in two steps: differentiation of the leukemic clone to mature granulocytes and its subsequent extinction, which is followed by restoration of polyclonal hematopoiesis.

THE PATHOGENESIS OF ANORECTAL MALFORMATION INDUCED BY ALL-TRANS RETINOIC ACID IN MICE

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The pathogenesis of high-type anorectal malformations was studied in mice using all-trans retinoic acid (RA) as a teratogen. Pregnant mice were injected intraperitoneally once with RA, suspended in corn oil, at a dose of 100 mg/kg on day 9 of pregnancy.

The RA-treated females were killed on one of days 10 to 18 of pregnancy. All fetuses observed on day 18 of pregnancy following in utero exposure to RA had anorectal malformations. In the affected male fetuses, the rectum was positioned away from the retroperitoneum toward the ventral side leading to the opening of the urethra just underneath the urinary bladder. Deficiency of the cloacal plate at the dorsal part was also observed in the affected embryos during days 10—11 of pregnancy. The cloacal plate is considered to induce the cloacal cavity to the surface, and thus the deficiency of the dorsal part may be the major cause of the high-type anorectal malformations.

A HISTOCHEMICAL STUDY ON THE TESTES FROM PATIENTS WITH IDIOPATHIC MALE INFERTILITY: IDENTIFICATION OF ACIDIC GLYCOCONJUGATES IN THE SEMINIFEROUS TUBULAR WALLS

ATSUSHI MISHIMA

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Acidic glycoconjugates in the seminiferous tubular walls in the testes from patients with idiopathic male infertility was identified light microscopically by sensitized high iron diamine method in combination with digestions with chondroitinase ABC, chondroitinase B or testicular hyaluronidase.

Tissue specimens were obtained by testicular biopsy from 37 patients with idiopathic male infertility and 9 fertile adult males. Chondroitin sulfate A, B and C, were identified in the tubular walls of oligozoospermic patients with idiopathic male infertility irrespective of the thickness of the walls. Similar results were obtained in the tubular walls of the testes from normal males. On

the other hand, chondroitin sulfate B was main acidic glycoconjugates in the tubular walls of the testes from azoospermic patients with idiopathic male infertility irrespective of the thickness of the walls. These results suggest that the etiologic factors of the impaired spermatogenesis in patients of idiopathic male infertility are not only the disturbance of nutritional transport across the seminiferous tubular walls due to peritubular thickening but also the functional alterations of the tubular walls associated with changes in components of acidic glycoconjugates in the tubular walls. The pathogenesis of oligozoospermia dose not seem to be simlar to that of azoospermia since components of acidic glycoconjugates in the peritubular tissues between both types of the disorders are quite different.

SCANNING ELECTRON MICROSCOPIC STUDY ON INNER EAR BAROTRAUMA: IN THE GUINEA PIG WITH ONE EUSTACHIAN TUBE OCCLUDED

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In order to investigate the mechanism of inner ear barotrauma, guinea pigs, with one eustachian tube occluded, were subjected to compression and decompression between 1 absolute pressure and 2 absolute pressure in a high pressure chamber. The authors separated the animals into three groups according to speed of compression and decompression. Each group contained 10 guinea pigs. Morphological changes in the cochlear sensory hairs were examined by means of a scanning electron microscope. On the occluded side, hair cells were damaged more frequently and more severely by rapid compression than by rapid decompression. There was no difference in the degree of damage produced by slow decompression and rapid decompression. On the control side, slight or moderate damage to hair cells was observed mainly as a result of rapid decompression. These findings led us to hypothesize that, in case of normal eustachian tube function, inner ear barotrauma would generally be caused by the relative positive pressure in middle ear cavity that occurs during decompression.

IMMUNOHISTOCHEMICAL STUDY OF THE ANGIOGENESIS IN EXPERIMENTAL-GASTRIC ULCER BY USING MONOCLONAL ANTIBODIES RELATED TO ADHESION MOLECULES

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1st Department of Pathology

Angiogenesis of the experimental-gastric ulcer was characterized by means of immunohistochemical staining using monoclonal antibodies (MoAbs) related to adhesion molecules. In order

to study the regulation of the antigen-expression defined by REC16-11, REC4-1 and ICAM-1, which recognize the surface antigen of the endothelial cells, we assessed their expression on the microvessels in the granulation tissues using a model of acetic acid-induced gastric ulcer in rats. Microvessels in the deep layer of granulation tissues were stained by REC16-11 within 5 days of the ulcer development. In contrast, REC4-1 and/or ICAM-1 were detected in microvessels only from 10 days, in parallel with the development of a persistent LFA-1 positive-lymphocytic infiltrate around REC4-1-positive-vessels. In cases of healed ulcer on 40 days, the distribution of the positive reaction with those MoAbs was similar to that in the normal rats. In cases of unhealed ulcers at the same period, REC16-11-positive-vessels were observed in all vascular endothelial cells of the granulation tissues. REC4-1 and/or ICAM-1-positive-vessels had the same pattern as the cases on 15 days. Functional differentiation of these microvessels in granulation tissues, which is the selective expression of the cell adhesion molecules, detected by REC4-1 and/or ICAM-1 MoAbs suggests that these antigens may play an important role in the healing process of gastric ulcer.

ATPase AND Ia-POSITIVE LANGERHANS-LIKE CELLS IN THE AVIAN EPIDERMIS

NOOR AKHTER

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Langerhans cells in the mammalian skin are migratory dendritic cells located among the epithelial cells of the skin. Although many previous studies described the presence of Langerhans cells in mammalian skin and Langerhans-like cells in the amphibian skin, until now no work was done concerning the occurrence of such cells in the avian skin. Chickens, sparrows, bunchos and quails were used. Skin fragments were incubated with collagenase or trypsin to obtain epidermal sheets. ATPase histochemistry and Ia immunohistochemistry with monoclonal antibodies were done on the epidermal sheets. Tissues were also fixed, embedded for light and electron microscopy. ATPase-positive dendritic cells were observed over the epidermal sheets of all bird species examined. With both ABC-peroxidase and FITC method MHC class II Ia antigen expressing cells were clearly observed in the quail and chicken skin. By electron microscopy cells with clear cytoplasm and processes were seen within the epithelial cells. No Birbeck granules were seen in these cells but some lysosome-like bodies were observed. These cells are ultrastructurally and histochemically similar to the mammalian Langerhans cells except the absence of Birbeck granules. These cell types or a family of cells may represent one of basic components of the immune system of birds.

THREE DISTINCT REGIONS INVOLVED IN 3P DELETION IN HUMAN LUNG CANCER

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There is now good evidence that a series of genetic lesions in both dominant oncogenes and tumor suppressor genes are involved in the pathogenesis of human lung cancer. The 3p deletion was first noted by cytogenetic analysis and was later confirmed by several independent studies using restriction fragment length polymorphism (RFLP) probes. As an initial step towards positional cloning (reverse genetics) of the tumor suppressor gene(s) on 3p, a detailed analysis of the minimum deleted region(s) on 3p was performed with 16 RFLP probes and 102 paired human lung cancer samples. All 10 SCLC cases (100%) and 52 of 92 NSCLC cases (57%) showed allelic loss at one or more loci mapped on 3p. We show here that three distinct regions on 3p appear to be frequently deleted in lung cancer. These regions include 3p25, 3p21.3 and 3p14-cen. The present study should warrant future work focusing on these chromosomal regions on 3p, and may ultimately lead to the isolation of tumor suppressor genes involved in the pathogenesis of lung cancer.

EXPRESSION OF PROTO-ONCOGENES AND TUMOR SUPPRESSOR GENES IN *IN VITRO* CELL LINES DERIVED FROM A THYMUS, THYMOMA, AND MALIGNANT THYMOMA OF RATS

YOSHIHISA SAKAI

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To analyze the processes of the development of thymoma and malignant thymoma from normal thymic epithelial cells, we have examined the expression of 15 proto-oncogenes and tumor suppressor genes in 7 *in vitro* epithelial cell lines established from a normal thymus (TuD1-1, TuD1-3, and TuD1-5), thymoma (TaD1-3 and TaD1-8), and malignant thymoma (MTHC-1 and MTHC-3) of rats. Northern blot analysis indicated that most of these genes examined were transcribed at similar levels. However, higher levels of transcription of epidermal growth factor receptor (EGFR) were observed in TuD1-1, TuD1-3, TuD1-5, TaD1-3, and TaD1-8 cells than in MTHC-1 and MTHC-3 cells. Conversely, 4 of the former 5 cell lines showed no TGF-β transcription while the latter 2 cell lines did high levels of its expression. In addition, c-*fos* proto-oncogene was highly expressed in TuD1-5 cells which showed the fastest growth rate among the 7 cell lines. These results denote that some molecular changes in the regulation of gene expression occurred in the processes of malignant transformation of thymic epithelial cells.

GROWTH INHIBITORY EFFECT OF BESTATIN ON CHORIOCARCINOMA CELL LINES *IN VITRO*

KAZUHIKO INO

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Bestatin, one of the biological response modifiers (BRMs), is an inhibitor of aminopeptidase M(N) (AP-M)/CD13, aminopeptidase B(AP-B) and leucine aminopeptidase (LAP). We investigated the direct effect of bestatin on the growth of cancer cells *in vitro* using established four choriocarcinoma cell lines. *In vitro* chemosensitivity was evaluated by the succinate dehydrogenase inhibition (SDI) test. Bestatin showed the growth inhibitory effect on all the choriocarcinoma cell lines dose-dependently, especially on NaUCC-4 cells. Both an isomer of bestatin with no inhibitory activity against aminopeptidases, (2R,3S)-AHPA-(R)-Leu, and another isomer with stronger inhibitory activity against AP-B than bestatin, (2S,3S)-AHPA-(R)-Leu, did not show growth inhibition on NaUCC-4 cells. So it is suggested that one of the possible mechanisms responsible for the direct action of bestatin on the choriocarcinoma cells may be related to the inhibition of activity of LAP or AP-M rather than that of AP-B. Furthermore, cytotoxicity of actinomycin D on the choriocarcinoma cells was significantly enhanced by combination with bestatin. These results suggest that bestatin shows not only an indirect host-mediated anti-tumor activity, but also a direct growth inhibitory effect on some kinds of cancer cells, probably by inhibiting aminopeptidase activity existing on them.

ESTABLISHMENT AND BIOCHEMICAL ANALYSIS OF A MALIGNANT MELANOCYTIC TUMOR CELL LINE EXPRESSING THE *RET* ONCOGENE

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2nd Department of Pathology

We established a cell line (designated Mel-ret) from a melanocytic tumor developed in a metallothionein/*ret* transgenic mouse. Unlike primary melanocytic tumors, which did not show malignant features, when the Mel-ret cells were transplanted into nude mice, they invaded into surrounding tissues and had metastatic ability. Although the Ret proteins were expressed at similar levels in the cell line and the primary tumors, the level of tyrosine phosphorylation in the Mel-ret cells was much higher than that in the primary tumors. In paraticular, an 85kDa tyrosine-phosphorylated band was specifically detected in the Mel-ret cells. These results suggested that the increase in tyrosine phosphorylation may be responsible for malignant transformation of the Mel-ret cells. Immunofluorescence and cell fractionation studies showed that the Ret proteins and most of tyrosine-phosphorylated proteins in the Mel-ret cells localized in the membrane fraction. No activation of phosphatidylinositol-3 kinase (PI-3 kinase), a target protein for several tyrosine kinases, was detected in the Mel-ret cells.

ESTABLISHMENT OF MOUSE OLIGODENDROCYTE/TYPE-2 ASTROCYTE LINEAGE CELL LINE BY TRANSFECTION WITH ORIGIN-DEFECTIVE SIMIAN VIRUS 40 DNA

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A permanent glial cell line has been established from the neonatal mouse primary mixed glial cell cultures by transfection with replication origin-defective simian virus 40 DNA. This cell line, designated OS3, has morphological similarity to type-2 astrocyte and expresses an astrocyte-specific marker, glial fibrillary acidic protein (GFAP) when cultured in the presence of 10% calf serum (CS). OS3 cells do not express the O4 antigen, galactocerebroside (GalC) and A2B5 under this culture condition. When cultured in the medium containing 2% CS or chemically defined medium, these cells undergo morphological transformation. Some of these cells express O4 antigen and/or GalC, and the percentage of GFAP positive cells decreases under these conditions. Thus depending on the culture conditions, the OS3 cells display either type-2 astrocyte properties or immature oligodendrocyte characteristics. Furthermore, the OS3 cells show similar responses to the various growth factors as do oligodendrocyte/type-2 astrocyte (O-2A) progenitors. Therefore, the OS3 cell line is an unique mouse bipotential permanent O-2A lineage cell line which may be useful to analyze the developmental properties of these glial cells.

GEOMETRIC RESPONSE TO NERVE GROWTH FACTOR IS PRESERVED IN AGED RAT SENSORY NEURONS; A SINGLE-NEURON CULTURE STUDY

TAKAYUKI ITOH

Department of Neurology

To analyze the primary effects of nerve growth factor (NGF) on dorsal root ganglion (DRG) neurons, we established a single-neuron culture system which was exclusively free of non-neuronal cells producing several neurotrophic factors and free of serum-containing trophic molecules. By applying this technique, we previously reported that NGF promoted neurite arborization in young adult rat (4 to 60-month-old) DRG neurons. In this study, we demonstrated that the effects of NGF on neurite regeneration in DRG neurons was well preserved in aged rats (20 to 24-month-old and 33-month-old). NGF did not increase the percent process-bearing neurons in aged rats, which indicated that neuronal survival was not promoted by NGF, but it significantly enhanced the number of branching points, total neurite length and soma size in aged neurons. These effects of NGF on neurite geometry tended to be reduced to some extent in aged neurons and the initiation of neurite-outgrowth in aged neurons was also delayed as compared with young adult neurons. NGF-responsive subpopulation of neurons, found in the entire range of

neuronal size, was preserved in aged rats. These findings indicate that NGF could play an important role in regeneration of injured DRG neurons of aged animals.

AGE-RELATED CHANGE OF T CELL SUBSETS IN INTESTINAL INTRAEPITHELIAL LYMPHOCYTES AND LIVER LYMPHOCYTES OF MICE

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Age-related changes in T cell subsets were examined in intestinal intraepithelial lymphocytes (i-IEL) and liver lymphocytes, which contain unique T cells differentiating extrathymically. In 2 months-old mice bred under conventional condition, i-IEL consisted of a large number of $CD4^{-}CD8\alpha/\alpha^{+}$ cells bearing either $Tc R\alpha/\beta$ or $Tc R\gamma/\delta$ and only a few $CD4^{+}CD8\alpha^{-}$ cells, while unique $CD4^{+}CD8\alpha/\alpha^{+}$ i-IEL bearing $Tc R\alpha/\beta$ increased in number and conversely the proportion of $Tc R\gamma/\delta^{+}$ i-IEL decreased in 12 months-old and 24-months-old mice. Such an increase in number of $CD4^{+}CD8\alpha/\alpha^{+}$ i-IEL was not evident in 14 months-old germfree mice, while a significant number of $CD4^{+}CD8\alpha/\alpha^{+}$ cells were detected in i-IEL both in 14 months-old nude mice and 14 months-old neonatally thymectomized mice, albeit a smaller number as compared with those in aged-matched euthymic mice, suggesting that $Tc R\alpha\beta^{+}$ $CD4^{+}CD8\alpha/\alpha^{+}$ i-IEL may differentiate extrathymically and expand in response to stimulation with intestinal microflora with age. In liver lymphocytes, instead of $CD4^{+}CD8\alpha^{+}$ T cells, $CD4^{-}CD8\alpha^{+}$ T cells expressing $CD8\alpha/\alpha$ homodimer, which are thought to be a thymus-independent population, significantly increased in proportion with age under conventional conditions. These results suggest that a significant fraction of $Tc R\alpha/\beta$ T cells in i-iEL and liver lymphocytes can develop along an extrathymic pathway under the influence of intestinal microflora.

LAMININ A, B1 AND B2 CHAIN GENE EXPRESSION IN TRANSECTED AND REGENERATING NERVES: REGULATION BY AXONAL SIGNALS

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Department of Neurology

Laminin A, B1 and B2 chain mRNA levels in degenerating and regenerating mouse sciatic nerves were examined using Northern blot analysis. When the nerves were crushed, the steady-state levels of B1 and B2 mRNA per mg wet tissue weight of the distal segments of the nerves increased six fold over that of control as the total RNA and β-actin mRNA increased,

suggesting that these increases were the consequence of Schwann cell (Sch) proliferation after axotomy. The normalized levels of B1 and B2 mRNA as the ratio to the total RNA or β-actin mRNA levels were high in intact nerves, but they drastically decreased to 10—20% of the normal levels one day after injury. In the crushed nerves, B1 and B2 levels gradually increased as the regenerating axons reestablished axon-Sch contact, and returned to normal levels on day 21. In the transected nerves, where Sch continued being disconnected from axons, both levels remained low. B1 and B2 mRNA levels in cultured Sch were significantly increased when Sch were cocultured with sensory neurons. Laminin A mRNA was not detectable in the normal, axotomized nerves or in cultured Sch. These data indicate that Sch expressed laminin B1 and B2 chain mRNA which were up-regulated by axonal or neuronal contact, but they did not express A chain mRNA.

IMMUNOHISTOCHEMICAL EXAMINATION OF THE INFLAMMATORY RESPONSE INDUCED BY LIPOPOLYSACCHARIDE (LPS)

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Until now, little is known about the kinetics of B-lymphocytes in the primary inflammatory response as a non-specific self-defense mechanism. When exogenous antigens such as bacteria invade *in vivo*, neutrophils and macrophages play an important role in the primary inflammatory response to exclude these antigens. Meanwhile, B-lymphocytes are known to be activated polyclonally by LPS and appear to relate to the primary inflammatory response. By using immunohistochemical methods, we studied the inflammatory response which was induced in the regional skins and lymph nodes of SMA mice after the subcutaneous injection of LPS, extracted from *Klebsiella pneumonia* strain LEN-111(O3:K1-). The results were consistent with the view that immature B-lymphocytes were generated in the early stage of the inflammatory response and they differentiated into plasma cells directly in the regional lesions preceding the construction of germinal centers. Moreover, some of these B-lymphocytes expressed Ly-1 antigens (CD5). These results indicated that these B-lymphocytes related to both the primary inflammatory response and the autoimmunity. Based on the view, we classified these B-lymphocytes functionally as non-follicular B-lymphocytes. On the other hand, the morphological characters of these B-lymphocytes resembled monocytoid B-lymphocytes in immature sinus histiocytosis (ISH) of human lymphadenitis. This finding suggested that the non-follicular B-lymphocytes were closely related to the monocytoid B-lymphocytes.

FURTHER EVALUATION OF THE PREGNANCY-LINKED DOWN REGULATION OF THE PATERNAL ANTIGEN-SPECIFIC SPLENIC CYTOTOXIC T LYMPHOCYTE ACTIVITY IN ALLOGENEICALLY PREGNANT MICE

MAHBUBA PARVIN

Department of Obstetrics and Gynecology

Cytotoxic T lymphocyte (CTL) activity directed against paternal alloantigens was examined in allogeneically pregnant mice using various allogeneic combinations. The spleen cells from pregnant C57BL/6 (H-2^b) mice mated with BALB/c (H-2^d) male mice generated less anti H-2^d CTL after in vitro sensitization than those from unpregnant or syngeneically mated C57BL/6 mice. Different allogeneic combinations including the incompatibility at only D region of H-2 or minor histocompatibility loci were effective for downregulating the anti-paternal CTL activity in pregnancy. The downregulation of anti-paternal CTL activity induced by allogeneic pregnancy occurred at day 10 to day 18 of pregnancy, most extensively at day 14. The allogeneic pregnancy also downregulated the allogeneic CTL activities that had been amplified by injecting alloantigens before mating. The present study examined the level of the CTL immunity against the paternal alloantigen in allogeneically pregnant mice under new conditions, in order to determine whether or not such a systemic regulatory mechanism exists and to further characterize the mode of the regulation.

DETECTION OF VIRAL DNA IN NEONATAL HERPES SIMPLEX VIRUS INFECTIONS: FREQUENT AND PROLONGED PRESENCE IN SERUM AND CEREBROSPINAL FLUID

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Polymerase chain reaction (PCR) assay was used to detect herpes simplex virus (HSV) in mouth, skin, serum or cerebrospinal fluid (CSF) from seven neonates with HSV infections. In a culture-negative patient, the diagnosis was confirmed by detection of HSV DNA. Serial examinations revealed that HSV DNA remained in the serum and/or CSF of several patients for 1 − 2 weeks after the beginning of treatment. Next, the results of PCR assay in neonatal HSV infections were compared with those in older children with herpes simplex encephalitis (HSE). HSV DNA was detected in CSF from four neonates with central nervous system involvement and in CSF from all nine children with HSE. Sera were positive for HSV DNA in five of seven neonates, including two cases of localized infections, but in none of the children with HSE. These results suggest that HSV may spread principally via viremia in neonates. PCR assay could be

useful for the confirmative diagnosis of neonatal HSV infections, especially in culture-negative cases.

DOWN-REGULATION OF THE SURFACE EXPRESSION OF CLASS I MHC ANTIGENS BY HUMAN CYTOMEGALOVIRUS

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Class I major histocompatibility complex (MHC) antigens of higher eukaryotes play a crucial role in the recognition of human cytomegalovirus (HCMV)-infected cells by cytotoxic T lymphocytes. In the present study, we have demonstrated that HCMV infection resulted in a marked reduction in the surface expression of class I MHC antigens on human embryonic lung fibroblasts (HEL). Even when HCMV-infected HEL was cultured in the presence of 9-(1,3-dihydroxy-2-propoxymethyl)-guanine (DHPG), the reduction was observed to the same extent, indicating that the HCMV DNA synthesis was not required for this phenomenon. However, immunoprecipitation studies have shown that there is no significant reduction in the synthesis of either the heavy chain or the light chain of class I MHC antigens after HCMV infection. In addition, western blotting studies have revealed that the total amount of the antigens was almost unaltered after HCMV infection. These results suggest that the reduction in the cell surface expression of class I MHC antigens is due to some defect occurring posttranslationally, most likely at the level of the correct compelx formation and/or the intracellular transport of class I MHC antigens.

MUSCLE SYMPATHETIC OUTFLOW IN BUERGER'S DISEASE

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1st Department of Surgery

One of the pathophysiological features in Buerger's disease i.e. thromboangiitis obliterans (TAO) contains sympathetic contribution which relates vasospastic phenomenon. The purpose of this study is to clarify sympathetic mechanisms in TAO. Muscle sympathetic nerve activity (MSA) was recorded using a microneurographic technique in patients suffering from TAO. The cold pressor test by immersing the hand of the subject into the ice water was used to examine the sympathetic responsiveness to local cold stimulus. The basal level of the MSA in TAO group was lower than that in control group (control group vs TAO group: 39.8 ± 12.8 bursts/min vs 26.1 ± 12.1 bursts/min in burst rate, $P < 0.05$; 61.8 ± 8.3 bursts/100 heart beats vs 39.5 ± 22.3 bursts/100 heartbeats in burst incidence, $P < 0.05$). The responsiveness of MSA to the cold pressor test in TAO group was higher than that in control group (TAO group vs

control group: 385 ± 191% total MSA vs 198 ± 156% total MSA, $P < 0.05$). We directly demonstrated higher responsiveness of MSA to the local cold stimulus in patients with TAO. This hyperresponsive MSA might contribute to pathophysiologic features in TAO.

GALANIN AS A PHYSIOLOGICAL NEUROTRANSMITTER IN HEMODYNAMIC CONTROL OF ARGININE VASOPRESSIN RELEASE IN RATS

KUNIKAZU KONDO

1st Department of Internal Medicine

The present study investigates the contribution of endogenous galanin (GAL) to the plasma volume-mediated control of arginine vasopressin (AVP) release in conscious rats. Intracisternal injection of a synthetic rat GAL dose dependently suppressed plasma AVP increased by polyethylene glycol-induced hypovolemia. In contrast, when plasma AVP was suppressed by isotonic plasma volume expansion, immunoneutralization of endogenous GAL by intracisternal injection of antiGAL-IgG significantly reversed the suppression. On the other hand, intracisternal injection of GAL failed to suppress plasma AVP increased by hypertonic saline-induced hyperosmolality. These results suggest that GAL is physiologically involved in the plasma volume-mediated control of AVP release through an inhibitory action, and that GAL injected around the brain stem acts on the site specific to the plasma volume-mediated, but not the osmolality-mediated, pathway. Since primary afferent nerves from volume receptor terminate at the nucleus of the solitary tract in dorsal medulla oblongata, and since GAL and its binding sites are densely localized there, it is concluded that endogenous GAL acts as a physiological neurotransmitter in the plasma volume-mediated inhibition of AVP release, presumably at the nucleus of the solitary tract.

CLONING AND SEQUENCING OF A LARGE CHONDROITIN SULFATE PROTEOGLYCAN (PG-M) IN CHICK LIMB BUDS: EVIDENCE FOR THE CHICKEN EQUIVALENT OF HUMAN VERSICAN

YOSHIHIRO NISHIDA

Department of Orthopedic Surgery

The cDNA sequence coding PG-M, the large chondroitin sulfate proteoglycan in limb buds has been partially determined. The deduced amino acid sequence revealed a complement regulatory protein-like domain, a lectin-like domain, two EGF-like domains from the carboxy terminal with an extremely high homology to respective domains of versican, the human fibroblast

large proteoglycan. The fusion protein expressed by constructed plasmid for the Ver-27b clone which is cDNA clone coding the N-terminal portion of versican core protein, has been stained with anti-PG-M antibodies. Together with the immunological evidence, it is likely that PG-M is the chicken equivalent to human versican. The occurrence of such evolutionarily conserved domain structures suggests important roles in cellular interactions.

COLLAGEN AND ALKALINE PHOSPHATASE GENE EXPRESSION DURING CARTILAGE AND BONE DIFFERENTIATION BY BONE MORPHOGENETIC PROTEIN

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Endochondral bone formation induced by bone morphogenetic protein (BMP) involves expression of collagen Types I and II and alkaline phosphatase (ALP) genes. Expression of these genes was studied in mice after implantation of BMP. The amount of Type I collagen mRNA increased from Day 3 to Day 7, when mesenchymal cell aggregation was observed. On Day 17, Type I collagen mRNA expression was correlated with an increased number of osteoblasts. Type II collagen mRNA increased from Day 7 coincided with chondroblast appearance. This increase was suppressed by Day 17, although hypertrophic and degenerative chondrocytes were present. ALP mRNA increased markedly from Day 7 with the appearance of chondroblasts. The high level of ALP mRNA continued until Day 11, during chondrogenesis. Mineral deposition was first observed roentgenographically on Day 11. Thus, BMP-induced bone formation occurs with the expression of collagen Types I and II and ALP genes.

HYPERTHERMIC AND HYPERGLYCEMIC EFFECTS OF CENTRAL $PGF_2\alpha$

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A relationship between hyperthermia and hyperglycemia induced by intraventricular prostaglandin (PG) $F_2\alpha$ was examined in anesthetized rats. Iv curare completely prevented the $PGF_2\alpha$-induced hyperthermia, but enhanced the hyperglycemic effect of $PGF_2\alpha$. Adrenal demedullation completely prevented the hyperglycemia, but did not affect the hyperthermic effect of $PGF_2\alpha$ into the preoptic area produced hyperthermia, but not hyperglycemia. These results suggest that $PGF_2\alpha$ affects the central nervous system to produce hyperthermia via shivering, stimulated somatic motor system, and to produce hyperglycemia by stimulating central

sympathetic outflow to the adrenal medulla, but these operate independently under the different neural regulation, and these sensitive sites are organically dissociated in the CNS. Further examinations were performed for the effects of the histamine H_1 receptor antagonists diphenhydramine and pyrilamine, the H_2 receptor antagonist ranitidine and the muscarinic receptor antagonist atropine injected into the third cerebral ventricle on the $PGF_2\alpha$-induced hyperglycemia. The concomitant injection of diphenhydramine or pyrilamine or atropine with $PGF_2\alpha$ suppressed the increase in plasma glucose and epinephrine concentration, but ranitidine had no effect. These findings demonstrae that $PGF_2\alpha$-induced hyperglycemia is mediated by the muscarinic receptors of cholinoceptive neurons and in part by H_1 receptors in the central nervous system.

CNS STIMULATION DOES NOT AFFECT HEPATIC VENOUS GLUCOSE CONCENTRATION IN SEVERELY DIABETIC RATS

HISAYUKI MIURA

3rd Department of Internal Medicine

To assess the role of the central nervous system (CNS) in carbohydrate metabolism in insulin-dependent diabetes, neostigmine was injected into the third cerebral ventricle in fed rats with streptozotocin (STZ; 80 mg/kg)-induced diabetes under pentobarbital sodium anesthesia. Changes in hepatic venous plasma glucose concentrations were monitored. Neostigmiine injection caused no significant changes in the hepatic glucose concentration in untreated diabetic rats, whereas the glucose level increased significantly in insulin-treated diabetic rats similarly to the changes in normal control rats. In diabetic rats, the plasma levels of glucagon, epinephrine, and norepinephrine were increased significantly by neostigmine. Systemic infusions of epinephrine and glucagon induced hepatic venous plasma hyperglycemia in normal rats but not in severely diabetic rats. After various doses (35—80 mg/kg) were given to rats, it was found that the higher the STZ doses, the lower was the hepatic glycogen content and the smaller was the glycemic response to neostigmine. Our results indicate that, in severe diabetes, CNS stimulation with neostigmine fails to increase hepatic glucose output, because glycogen stores are nearly exhausted and gluconeogenesis is already maximal.

EFFECTS OF GLIMEPIRIDE ON IN VIVO INSULIN SENSITIVITY AND RESPONSIVENESS IN NORMAL AND DIABETIC RATS

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To evaluate the effects of glimepiride on insulin action in peripheral tissues, insulin-induced glucose uptake was investigated in normal and diabetic rats using euglycermic clamp

procedures (insulin infusion rates: 6 and 30 mU/kg/min).

Normal rats: Glimepiride-administrated rats were gavaged with glimepiride (0.1 mg/kg/day). Control rats were gavaged with saline. After administration for 2 weeks, euglycemic clamp procedures were performed. During submaximal hyperinsulinemia (500—700 pmol/L), metabolic clearance rates of glucose (MCR) in glimepiride-administrated rats were significantly higher than those in the control rats (25.1 ± 2.3 vs. 17.7 ± 1.5 ml/kg/min, $p < 0.05$). During maximal hyperinsulinemia (> 4500 pmol/L), MCR in glimepiride-administrated rats tended to be higher than in controls (43.1 ± 3.0 and 38.8 ± 3.1).

Diabetic rats: Streptozotocin-induced diabetic rats were divided into four groups: GI (glimepiride, 0.1 mg/kg/day po and insulin, 5 U/day sc treatment), I (insulin treatment), G (glimepiride treatment), and C (saline administration). After 2 weeks of treatment, MCR in the four groups were similar during 6 mU/kg/min clmaps. During 30 mU/kg/min clamps, MCR in GI were significantly higher than those in G and C (23.4 ± 3.0 vs. 9.0 ± 0.9 and 12.2 ± 2.0 ml/kg/min, $p < 0.01$), and MCR in I (17.4 ± 1.7) tended to be higher than in C but lower than in GI.

These results suggest that glimepiride enhances insulin action in peripheral tissues, and in the diabetic state glimepiride coexistent with insulin improves insulin resistance effectively.

CORRELATION BETWEEN ALDOSE REDUCTASE ACTIVITY AND SORBITOL LEVELS IN ERYTHROCYTES FROM DIABETIC PATIENTS

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The accumulation of sorbitol in tissues through the polyol pathway has been implicated in the pathogenesis of diabetic complications. It is unknown whether there is a variety in the activity of aldose reductase (AR), the first enzyme of the pathway, among diabetic patients and whether the enzyme activity can be correlated with tissue sorbitol levels.

To clarify these issues, we measured the activity of erythrocyte AR in diabetic and nondiabetic subjects and compared it with the simultaneously measured erythrocyte sorbitol levels.

The results showed that both erythrocyte sorbitol levels and AR activity were significantly increased in diabetic patients compared with nondiabetic subjects but there were three and six fold differences in the sorbitol levels and AR activity among diabetic patients, respectively. There was a significant correlation between erythrocyte sorbitol levels and AR activity or plasma glucose levels. The sorbitol levels were higher in patients who had high enzyme activity than in those having low enzyme activity who had similar glucose levels. The product of AR activity and plasma glucose levels or a calculated sorbitol production rate correlated the sorbitol levels with higher significance. We conclude that both AR activity and plasma glucose levels are important determinants of tissue sorbitol levels.

PROTECTIVE EFFECTS OF A PROSTAGLANDIN E_1 OLIGOMER ON TAUROCHOLATE-INDUCED RAT PANCREATITIS

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Effects of prostaglandin E (PGE) on acute pancreatitis have been controversial. Effects of PGE_1 oligomer, MR-356, on trypsin-taurocholate-induced acute pancreatitis were studied in rats. Divided doses of intraperitoneal administration of 0.6 mg/rat increased 24-h survival rates when the oligomer was given both at 1 h before and after (A group) and immediately and 3 h after (B group) induction of pancreatitis. In A group MR-356 significantly improved the survival rates at 18 h (94% vs 61%, $p<0.05$) and 24 h (68% vs 33%, $p<0.05$) when compared with controls. MR-356 improved the survival rates dose-dependently up to 0.6 mg/rat when given by the same protocol of A group. In B group MR-356 also improved the survival rate (72% vs 39%, $p<0.05$) only at 24 h , while other parameters (amylase in serum, ascites, and pancreatic tissue, trypsin-like enzyme activity in ascites and pancreatic tissue, immunoreactive cationic trypsin in serum and ascites, histological examination of pancreas) failed to improve. The present results suggest that the PGE_1 oligomer may play a beneficial role in bile-induced pancreatitis probably through its proposed effects of stabilization of lysosomal membranes, maintenance of microcirculation, and inhibition of protease in the pancreas.

RITODRINE ENHANCES PROLIFERATION OF FETAL HEPATOCYTES IN PRIMARY CULTURE

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Ritodrine is a β_2-sympathomimetic agent which is widely used in obstetrics to inhibit premature labor. Although ritodrine crosses the placenta freely, its direct effect on fetal cell proliferation has not been reported. Liver is an essential organ for the fetal growth. It is known that β_2-adrenergic receptors are highly expressed only in the fetal, neonatal, and regenerating livers. Taken together, we hypothesized that β_2-adrenergic receptor stimulation could promote the fetal liver growth.

Ritodrine was added to the serum- and hormone-free primary cultures of fetal, neonatal, or adult rat hepatocytes, or fetal human hepatocytes. We measured [^{3}H]thymidine incorporation into DNA, and nucleus number. The effect of ritodrine on cell cycle was also analyzed with flow cytometry.

Ritodrine enhanced the proliferation of fetal rat hepatocytes. Ritodrine remarkably stimulated DNA synthesis of fetal and neonatal but not adult hepatocytes. The effect was dose-dependent and was antagonized by propranolol. Similar results were shown in the experiments of fetal human hepatocytes. Analysis of the nuclear DNA content derived from flow cytometry

revealed that cells stimulated by ritodrine entered S phase.

These results indicate that ritodrine may promote the proliferation of fetal hepatocytes through the stimulation of β_2-adrenergic receptors followed by induction of DNA synthesis.

Part of this study was presented at the First Internation Congress of Perinatal Medicine, Tokyo, Japan, 1991.

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POLY(ADP-RIBOSE) POLYMERASE STIMULATES DNA POLYMERASE α BY PHYSICAL ASSOCIATION

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The direct effect of the eukaryotic nuclear DNA-binding protein poly(ADP-ribose) polymerase (PARP) on the activity of DNA polymerase α was investigated. Homogenously purified PARP (5 to 10 μg/ml) stimulated the activity of immunoaffinity-purified calf thymus or human DNA polymerase α by about 6 to 60 fold in a dose-dependent manner. It had no effect on the activities of DNA polymerase β, DNA polymerase γ, and primase, indicating that its effect is specific for DNA plymerase α. Apparently poly(ADP-ribosyl)ation of DNA polymerase α was not necessary for the stimulation. The stimulatory activity is due to PARP itself since it was immunoprecipitated with a monoclonal antibody directed against PARP. Kinetic analysis showed that, in the presence of PARP, the saturation curve for DNA temple-primer became sigmoida; at very low concentrations of DNA, it rather inhibited the reaction in competition with template DNA, while, at higher DNA doses, it greatly stimulated the reaction by increasing the V_{max} of the reaction. By the automodification of PARP, however, both inhibition at low DNA and stimulation at high DNA doses were largely lost. Furthermore, stimulation by PARP could not be attributed to its DNA-binding function alone since its fragment, containing only the DNA-binding domain, could not exert full stimulatory effect, as of the intact enzyme. PARP is co-immunoprecipitated with DNA polymerase α, using anti-DNA polymerase α antibody, clearly showing that PARP may be physically associated with DNA polymerase α. In a crude extract of calf thymus, a part of PARP activity existed in a 400 kDa, as well as a larger 700 kDa complex containing DNA polymerase α, suggesting the existence *in vivo* of a complex of these two enzymes.

DNA HELICASES ASSOCIATED WITH DNA POLYMERASES

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To elucidate mechanism of DNA replication of mammalian cells, we are purifying and characterizing DNA replication factors. One of these factors is DNA helicase, which displaces double-stranded DNA to single-stranded form. Because there are more than ten kinds of DNA helicases present in the cells, I focused onto the DNA helicases co-purified with DNA polymerases, which are necessary for DNA replication. DNA polymerase α, δ and ε from HeLa cells were separated from each other by a hydroxylapatite column. DNA helicase activities were detected in DNA polymerase α and δ fractions but not in ε fraction. By a repeated gel-filtration of Superose 6 (SMART system), activities of DNA polymerase α and δ were eluted at approx. 600 kDa and 400 kDa respectively and the activities of DNA helicase were well associated with those of corresponding DNA polymerases. These data strongly suggest that DNA helicases may be physically associated with DNA polymerase α and δ to make large complexes.

Anti-oncogene product p53 regulates cell growth as a negative regulator and is reported to be co-localized with DNA replication machineries. I showed that p53 bound to DNA helicase in vitro and in vivo. These data suggest that p53 interacts directly to the DNA replication machinery and exerts its function in the negative regulation of cell growth through a protein-protein interaction.

HEART ALLOGRAFT REJECTION IS MAINLY MEDIATED BY T CELLS STIMULATED WITH MHC CLASS I ALLOANTIGEN

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We have developed a new technique for heterotropic heart transplantation in mice using a cuff technique. To investigate the difference in the nature of rejections between heart allograft and skin allograft, heart and skin transplantations have carried out in combination with C57BL/6 (B6) and MHC class I-disparate B6-C-H-2^{bm1} (bm1) or MHC class II-disparate B6-C-H-2^{bm12} (bm12) mice. In accordance with previous reports, both skin allografts from bm1 and bm12 mice were rejected in B6 recidient mice within 23 days. In case of heart transplantation, B6 recipient mice rejected MHC class I-disparate but class II-matched bm1 heart grafts within 27 days, while heart allografts from MHC class I-matched but MHC class II-disparate bm12 mice survived in B6 recipient mice beyond 100 days. The bm12 skin grafts were rejected within 10 days in B6 recipient mice which had carried bm12 heart grafts for over 100 days. The LN cells of these B6 mice carrying bm12 heart grafts showed a significant level of proliferative response to bm12 spleen cells, suggesting that the anti-bm1 T cells in these mice are not rendered to tolerance. Our results suggest that T cells specific for MHC class I alloantigens may be important for rejection of vasculized allogeneic heart graft.

EXPERIMENTAL RAT PANCREAS TRANSPLANT: SURGICAL TECHNIQUE AND IMMUNOLOGICAL CONSIDERATION

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Vascularized whole pancreas transplantation was performed using diabetes-induced rats. Two different exocrine drainage procedures, intestinal or ureter drainage were used with a cuff technique for vascular anastomoses. In the isograft transplant models, hyperglycemia was ameliorated immediately after transplantation. In the allograft transplant models without immunosuppression, graft rejection as defined by recurrence of hyperglycemia (blood glucose > 200 mg/dl) occurred 6 to 9 days posttransplant. However, urine amylase levels started to decline 2 to 3 days posttransplant. This finding correlated with the histological evidence of acute cellular rejection which was observed as early as on day 3 posttransplant. Allograft rejection could be delayed approximately one month after transplant with a short term use of FK506. We conclude that whole pancreas transplant with ureter exocrine drainage is an ideal model for experimental studies in the allograft pancreas transplantation.

IDENTIFICATION AND DNA SEQUENCE OF PHOSPHOMANNOMUTASE AND GDP-MANNOSE PYROPHOSPHORYLASE GENES IN *Escherichia coli* O9 *rfb* CLUSTER

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We identified phosphomannomutase (PMM) and GDP-mannose pyrophosphorylase (GMP) genes, *rfbK* and *rfbM*, of *Escherichia coli* O9 and determined their nucleotide sequences. *rfbK* and *rfbM* of *E. coli* O9 encode proteins of 460 amino acids (molecular weight of 50,809), and 471 amino acids (molecular weight of 52,789), respectively. The amino acid sequences of GMP are highly conserved in the bacterial mannose pathway genes, *cpsB* of *S. typhimurium, algA* of *Pseudomonas aeruginosa, xanB* of *Xanthomonas campestris*, and *rfbM* genes of *Salmonella* group B and C2. From the data of amino acid identities of PMM genes, they are classified into three groups of *E. coli* O9 type (*rfbK* of *E. coli, cpsG*, and *xanA*), *algC* type, and *Salmonella* type (*rfbK* of *Salmonella* group B and C2). There are no striking similarities among these groups of PMM. the phylogenetic trees of PMM and GMP suggest that these genes are evolved independently in bacteria though both participate in GDP-mannose pathway.

ENHANCEMENT OF SYNTHESIS OF EXTRACAPSULAR POLYSACCHARIDIE IN *KLEBSIELLA PNEUMONIAE* BY RmpA2, CARRYING SIMILAR DOMAIN TO NtrC AND UhpA

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Klebsiella pneumoniae is one of the important opportunistic pathogens that usually cause serious diseases in immunocompromised hosts. Among at least 77 distinguished serotypes, *K. pneumoniae* strains belonging to serotype 2 (K2) were usually isolated from patients with bacteremia. Clinically isolated encapsulated strains of *K. pneumoniae* serotype O1:K2 are divided into two groups by colony phenotype and virulence. It has been known that capsular polysaccharide is one of important virulence factors of *K. pneumoniae.* We previously cloned a *cps* structural gene cluster for K2 capsule production from chromosome of *K. pneumoniae* strain Chedid (O1:K2) which is one of the muco-viscous and virulent strains. We later found that a 2.0 kb *Hin*dIII-*Eco*RI fragment containing *rmpA2* region cloned from a resident large plasmid of Chedid was needed in association with *cps* cluster for the expression of K2 capsule in *Escherichia coli* HB101. RmpA2, a 212 amino acid peptide, is similar in its central part to the central domains of NtrC and UhpA, transcriptional regulators belonging to a two-component regulatory system. Another open reading frame, ORF2, was idientified upstream of RmpA2 and was found to be highly homologous to insertion sequence IS3 of many *Enterobacteriaceae.* Southern hybridization analysis suggested that all of the muco-viscous and virulent K2 tested strains of *K. pheumoniae* have *rmpA2* region on their resident large plasmids, however, avirulent and slightly muco-viscous K2 strains have not. Freeze substitution electron microscopy and FITC staining showed that all muco-viscous, virulent K2 strains including Chedid had dense and thick capsule (180 nm) with dense reticulated extracapsular polysaccharide, whereas K2-215, one of slightly muco-viscous, avirulent strains, had a capsule which is looser and thinner (120 nm) than that of Chedid but nearly without extracapsular substance. Introduction of a plasmid pROJ31 carrying *rmpA2* region into a slightly muco-viscous and avirulent *K. pneumoniae* strain K2-215 as well as *K. pneumoniae* K9, and K72 reference strains resulted in changing of the colony phenotype to highly muco-viscous through abundant production of extracapsular substance which were positive to anti-K2, K9 or K72 serum, respectively, as their parental strains. From these results it is suggested that RmpA2 confers highly muco-viscous phenotype on cells of various serotypes of *K. pneumoniae* through enhancement of synthesis of extracapsular polysaccharide.