ANNUAL RESEARCH MEETING

FOR

GRADUATE STUDENTS

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Abstracts

QUANTITATIVE ANALYSIS OF HERPESVIRUS LOAD LYMPH NODES OF PATIENTS WITH HISTIOCYTIC NECROTIZING LYMPHADENITIS USING A REAL-TIME PCR ASSAY

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Histiocytic necrotizing lymphadenitis (HNL) usually affects cervical lymph nodes of young individuals and has a self-limited clinical course. Viral infection is ascribed as its cause, but its pathogenesis still remains unknown. A real-time PCR assay is useful for not only sensitive detection but also quantitation of virus DNA with a wide linear range. Using this technology, we estimated the load of herpesvirus: Epstein-Barr virus (EBV), cytomegalovirus (CMV), human herpesvirus (HHV) type 6, 7, and 8. The mean EBV-DNA copy number was $10^{2.1}$ copies/microg of DNA in lymph nodes from patients with HNL (6/20, 30%), $10^{2.4}$ in RL (12/19, 63%). By *in situ* hybridization, EBV-encoded RNA was also detected in the cases with more than $10^{1.2}$ copies/microg of EBV-DNA. HHV6-DNA was detected in 3/20 (15%) HNL cases, 1/19 (5.3%) RL. HHV7-DNA in 2/20 (10%) HNL and 4/19 (21%) RL, with no distinct difference. Neither CMV nor HHV8 was detected in any case by the real-time PCR assay. In this study we could not identify a definitely causative herpesvirus for HNL. However one HNL case had a significantly larger copy number of HHV6-DNA and positive immunostains for HHV6 early/late antigen in necrotic foci, suggesting that HHV6 infection might be associated with some cases of HNL.

FUNCTIONAL SIGNIFICANCE OF AURORA-B IN THE SEPARATION OF TYPE III INTERMEDIATE FILAMENTS DURING CYTOKINESIS

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Aurora-B is a protein kinase required for chromosome condensation/segregation and for the progression of cytokinesis during the cell cycle. We report here that Aurora-B phosphorylates type III intermediate filaments (IFs), glial fibrillary acidic protein (GFAP) and desmin, and that this phosphorylation regulates their filament organization during cytokinesis. *In vitro*, Aurora-B phosphorylates GFAP and desmin, and this phosphorylation leads to a reduction in filament forming ability. The sites phosphorylated by Aurora-B; Thr-7/Ser-13/Ser-38 of GFAP and Thr-16 of desmin are common with those related to Rho-associated kinase (Rho-kinase) which has been reported to phosphorylate GFAP and desmin at cleavage furrow during cytokinesis. We indentified Ser-11 and Ser-59 of desmin to be specific sites phosphorylated by Aurora-B. Use of antibodies that specifically recognized desmin phosphorylated at Ser-11 and Ser-59 led to the finding that these sites are also phosphorylated specifically at the cleavage furrow during cyto-

kinesis in Saos-2 cells. Desmin mutants, in which phosphorylation sites by Aurora-B and/or Rho-kinase are changed to Ala or Gly, cause dramatic defects in filament separation between daughter cells in cytokinesis. Therefore, one function of Aurora-B is the regulation of cleavage furrow-specific phosphorylation and segregation of type III IFs with Rho-kinase during cytokinesis.

WIDE DISTRIBUTION OF *PLASMODIUM OVALE* IN MYANMAR

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It has been thought that distribution of *Plasmodium ovale* in Southeast Asia is very rare. In Myanmar, the presence of *Plasmodium ovale* has never been reported. Using blood samples obtained in many villages across the country between 1996 and 2000, molecular diagnosis of *Plasmodium* species was made with nested polymerase chain reaction (PCR) with species-specific primers and confirmed with another PCR-based diagnosis, microtiterplate hybrizidation (MPH). Both methods target the same region in the A type of small ribosomal RNA gene of the four human malaria parasites. *P. ovale* DNA was amplified in samples from 65 (4.9%) of 1323 PCR-positive patients, with perfect agreement between results obtained by nested PCR and MPH. Only four *P. ovale*-infected patients had single-species infection; all others were co-infected with *P. falciparum*, *P. vivax* and/or *P. malariae*. Quadruple infections were observed in six subjects. Parasites with typical *P. ovale* morphology were found in only 19 patients by conventional microscopy of Giemsa-stained thin smears or fluorescence microscopy of acridine orange-stained thin smears. *P. ovale* infections were found in villages situated in the southern, central and western regions of Myanmar, suggesting that *P. ovale* may be widely distributing in this country. This result may suggest that the presence of this parasite in Myanmar had been overlooked by previous surveillance with Giemsa-stained thick smear microscopy. Recently, a *P. ovale* infection was detected in a Malaysian patient by using our nested PCR diagnosis. It is highly probable that *P. ovale* might distributed more widely in Southeast Asia than previously estimated.

THE CENTRAL PROJECTION OF UNMYELINATED (C) PRIMARY-AFFERENT FIBERS FROM GASTROCNEMIUS MUSCLE IN THE GUINEA PIG

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We have demonstrated the central projections of muscle C- or Group IV- afferent fibers in

the guinea pig by tracing arborizations in the spinal cord. C-afferent fibers from the gastrocnemius muscle (GCM) were electrophysiologically identified by conduction velocity (less than l m/sec). A single neuron in the lumbar 5 dorsal root ganglion (L5 DRG) was intracellularly labeled with *Phaseolus vulgaris* leucoagglutinin (PHA-L). After iontophoretic injection of PHA-L, we processed the lumbar cord and L5 DRG for PHA-L immunohistochemistry. Six muscle C-afferent fibers from 40 animals were labeled. Labeled fibers were reconstructed in serial parasagittal sections. The GCM C-afferents projected rostrocaudally for two or three segments and ran at the surface of the dorsal funiculus, giving off collaterals into laminae I, II. Based on the branching pattern, we determined that the branching of muscle C-afferent fibers showed an intermediate pattern between the terminal patterns of somatic and visceral afferents. The numbers and sizes of terminal swellings were measured on all collateral branches. The area of distribution of the terminal swellings of muscle C-afferent fibers is larger than that of somatic terminals, but the density of terminal swellings in the terminal area was lower than that of the somatic ones.

THE ROLE OF CONTRAST-ENHANCED MR MAMMOGRAPHY FOR DETERMINING CANDIDATES FOR BREAST CANCER CONSERVATION SURGERY

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PURPOSE: The aim of this study was to assess the impact of preoperative magnetic resonance Mammography (MRM) on the surgical determination of breast conservation treatment for the breast cancer patients.
METHODS: From September 1997 to March 2000, 57 consecutive breast conservation treatment candidates were prospectively evaluated with conventional imaging studies (mammography and ultrasonography) and preoperative MRM.
RESULTS: In 47 of 54 (87%) breast cancer patients, breast conservation surgery (BCS) was indicated on the basis of mammography (MMG) and ultrasonography (US). However in 40 of the 54 (74%) patients BCS was indicated on the basis of MRM. Thirty-eight of the 40 patients ultimately underwent BCS and only 1 showed a positive margin. There were 7 patients whose MRM findings suggested that more aggressive treatment that BCS was needed but for whom US/MMG suggested that BCS was appropriate. Five of the 7 patients underwent mastectomy rather than BCS based on the MRM findings, which were justified by post-surgical histological findings. Of the 2 remaining patients who underwent BCS, one had positive histological margin and one had recurrence, both of which resulted in salvage mastecromy.
CONCLUSION: One study suggests that high resolution preoperative MRM provides more accurate information compared with US and MMG for selecting candidates for BCS. Using MRM as a routine staging tool may reduce unnecessary repeated excisions. A larger study will be required to confirm these findings and to define the patients most likely to benefit from breast MR imaging.

c-JUN N-TERMINAL KINASE PATHWAY MEDIATES LACTACYSTIN-INDUCED CELL DEATH IN A NEURONAL DIFFERENTIATED NEURO2a CELL LINE

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The ubiquitin-proteasome pathway is an intracellular protein degradation pathway responsible for degradation of many regulatory proteins that must be rapidly eliminated for normal. Some recent studies reported that a proteasome dysfunction was involved in the pathogenesis of neurodegenerative diseases. Thus, there is now considerable interest in the possible role of proteasome in this regard. Here we show that inhibition of proteasomal function by Lactacystin induced cell death in a neuronal differentiated Neuro2a (nN2a) cell line but not in an undifferentiated Neuro2a (N2a) cell line. Cell death was accompanied by both the activation of c-Jun N-terminal kinase, p38 and caspase-3. A pan-caspase inhibitor, Z-VAD-FMK, or SB203580, a p38 inhibitor could not inhibit cell death induced by Lactacystin, whereas nN2a cell lines with stable expression of the dominant negative mutant of c-Jun N-terminal kinase showed a remarkable suppression of cell death. Lactacystin-induced cell death is mediated through the c-Jun N-terminal kinase pathway but not the caspase-dependent pathway in a nN2a cell line. One results shed light on the association among the proteasomal dysfunction, JKN pathway and neuronal cell death, leading to the elucidation of its possible role in the pathogenesis of neurodegenerative diseases.

PROGRESSION AND PROGNOSIS IN MULTIPLE SYSTEM ATROPHY: AN ANALYSIS OF 230 JAPANESE PATIENTS

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We investigated the discase progression and survival in 230 Japanese patients with multiple system atrophy (cerebellar dysfunction (MSA-C) predominated in 155, parkinsonism (MSA-P) in 75). The median time from initial symptom to combined motor and autonomic dysfunction was 2.0 years. Median interval from onset to aid-requiring walking, confinement to a wheelchair, a bedridden state, and death were 3.0, 5.0, 8.0, and 9.0 years, respectively. Patients manifesting combined motor and autonomic involvement within 3 years of onset had a significantly increased risk of developing advanced disease stage and shorter survival ($p < 0.01$). MSA-P patients had more rapid functional deterioration than MSA-C patients but showed similar survival. Onset in older individuals showed increased risk of wheelchair, bedridden state, and death. Patients initially complaining of motor symptoms had accelerated risk of aid-requiring walking and wheelchair. The atrophy of the cerebellar vermis and pontine base showed a significant correlation with duration, but the relationship between atrophy and functional status was

highly variable among the individuals. The present study suggested that many factors are involved in the progression of MSA, but most importantly, the interval from initial symptom to combined motor and autonomic dysfunction can predict functional deterioration and survival in MSA.

A LONGITUDINAL STUDY OF ^{18}F-FDOPA AND ^{18}F-FDG POSITRON EMISSION TOMOGRAPHY IN PATIENTS WITH PARKINSON'S DISEASE

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To assess the longitudinal metabolic change in the brain of Parkinson's disease (PD), ^{18}F-FDOPA PET and ^{18}F-FDG PET scans were performed on 10 patients with PD and 20 normal subjects, both the scans were carried out again on all the 10 patients, after a mean interval of three years. Clinical assessment was made with Hoehn-Yahr rating scale, UPDRS motor score, MMSE and Raven Colored Progressive Matrix test (RCPM).

Three years apart, Hoehn-Yahr rating scale and UPDRS motor score had increased, while no intellectual ability proved to be reduced with MMSE and RCPM.

FDOPA study showed longitudinal selective decrease in dopaminergic activity in the putamen, caudate nucleus, as reported in previous studies, and midbrain. Patients with recent onset (less than 6 years) showed increased dopaminergic turnover in anterior cingulate.

FDG study showed lowered glucose metabolism in the occipital area and increase in the cerebellum at the first scan, comparing with normal subjects. Longitudinal decrease was seen in the posterior cingulate, while relative increase was seen in the fronto-parietal area.

The results suggest that decreased FDOPA uptake overtime leads to the progression of the motor dysfunction. The clinical meaning of the glucose hypometabolism in the posterior cingulated is unknown and needs further investigation.

TEL-Syk FUSION CONSTITUTIVELY ACTIVATES PI3-K/Akt SIGNAL PATHWAY AND STAT5 IN THE ABSENCE OF JAK2 ACTIVATION

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We have previously reported the fusion of *TEL* gene of *Syk* gene in myelodysplastic syndrome (MDS) with t(9;12)(q22;p12). TEL-Syk fusion transformed Interleukin-3 (IL-3) dependent murine hematopoietic cell BaF3 to growth factor independence. We investigated the intracellular signal tranduction of the stable transfectants. TEL-Syk fusion protein was associated with p85 subunit of phosphatidyl inositol 3 kinase (PI3-K) followed by the activation of Akt in the absence of IL-3. Vav, phospholipase C-γ2 (PLC-γ2) and mitogen-activated protein kinase

(MAPK) were also constitutively activated. Furthermore, TEL-Syk activated signal transducer and activator of transcription (STAT) pathway. STAT5 was tyrosine phosphorylated in the absence of Janus kinase 2 (JAK2) activation. Those constitutive kinase acitivations were not observed in the BaF3 cell transformed with TELΔPNT-Syk in which the oligomerization domain of TEL was deleted. Piceatannol, the Syk specific kinase inhibitor, inhibited the activation of STAT5 and the growth of the TEL-Syk transformed cell. Immunofluorescence technique revealed the TEL-Syk fusion products were located in cytoplasm. These data suggested that TEL-Syk fusion protein in cytoplasm led to constitutive activation of PI3-K, PLC-γ, MAPK and STAT5 signal pathway, and that was related to the pathogenesis of hematological malignancies.

TRANSCRIPTIONAL REGULATION OF FETAL *KRÜPPEL*-LIKE FACTOR 2 (FKLF-2) GENE EXPRESSED IN ERYTHROID CELLS

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FKLF-2 is a zinc finger protein cloned from mouse yolk sac cells. In human tissues FKLF-2 mRNA in highly expressed in the heart, skeletal muscles and the bone marrow, and among hematopoietic cell lines the high expression is observed in erythroid and a subset of T-cells. Chemical induction of erythroid cell lines up-regulates FKLF-2 expression, suggesting that FKLF-2 is involved in erythroid differentiation. In this study we examined molecular mechanisms of transcriptional regulation of FKLF-2 gene expression. We first determined the transcription start site by the primer extension analysis. To identify *cis*-regulatory elements, various length of truncations of the upstream region of the transcription start site were created and their promoter activities were assessed by luciferase assay. A full promoter activity existed in the proximal –0.3kb sequence to the transcription start site, in which several elements typically observed in promoters were present. In order to test the functional importance, each element was mutated by site-directed mutangenesis. Results show that a CACCC and two CCAAT motifs are key *cis*-elements of the FKLF-2 promoter. Furthermore, we found that GATA-1, an erythroid specific transcription factor, and FKLF-2 itself are capable of *trans*-activating the FKLF-2 promoter. These results suggest that GATA-1 and FKLF-2 itself is involved in transcriptional control of FKLF-2 gene in erythroid cells, which in turn may shed light on mechanisms of the increased FKLF-2 mRNA expression upon induction of erythroid differentiation.

HISTONE ACETYLATION INDUCED BY GRANULOCYTE COLONY-STIMULATING FACTOR IN A MAP KINASE-DEPENDENT MANNER

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Histone acetylation has been suggested to affect chromatin structure and gene expression. Extracellular signals are transduced into the nucleus through a variety of signal cascades to elicit changes in pattern of gene expression. The mitogen-activated protein (MAP) kinase pathway is activated by a number of cytokines and plays critical roles in hematopoietic cell survival and differentiation. A close mechanism that links extracellular signals to histone acetylation, however, remains to be elucidated. In the present study, we focused on the part of MAP kinase cascade and granulocyte colony-stimulating factor (G-CSF) in histone acetylation at the target gene. We examined the alternations of histone acetylation at the promoter associated with myeloid differentiation and the recruitment of CBP/p300 to the promoter by chromatin immunoprecipitation (ChIP) assay. In a murine myeloid cell line GM-162M, G-CSF caused rapid acetylation of histone H3 and H4 at the promoter of myeloperoxidase (MPO). In addition, two histone acetyltransferases (HATs), CBP and p300, were recruited to MPO promoter in response to G-CSF stimulation. By the use of a specific inhibitor of MAP kinase kinase (MEK), we showed that rapid histone acetylation induced by G-CSF is MAP kinase-dependent. These results illustrate how myeloid-differentiating signals via G-CSF may be coupled with histone acetylation during the process of gene expression.

ARSENITE INDUCES MEMBRANE RAFT-LINKED PRO AND ANTI-APOPTOTIC SIGNALS

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Renewed interest has recently been shown in arsenic due to its dual characteristics of being a potent carcinogen and a drug for treatment of acute promyelocytic leukemia. In this study, we found that sodium arsenite ($NaAsO_2$) simultaneously triggers both pro-apoptotic and anti-apoptotic signals in either murine T-lymphocytes or human T-cell leukemia Jurkat cells. $NaAsO_2$ induced apoptosis in these cells through activation of c-Jun amino terminal kinase (JNK), reciprocal regulation of Bel-2/Bax with the concomitant reduction of mitochondrial membrane potential, activation of caspase and fragmentation of DNA. In parallel to activation of caspase $NaAsO_2$ induced activation of Akt pathway in Jurkat cells. Inhibition of Akt pathway by a PI-3 kinase inhibitor accelerated arsenite-induced apoptosis suggesting that PI-3 kinase can be the target of manupulation of the two oppositely working arsenite-induced signals to promote pro-apoptotic signal potentially for a wider application as a drug in treating leukemia. Potentially as

the initial cell surface event for intracellular signaling, $NaAsO_2$ induced aggregation of glycosylphosphatidylinositol (GPI)-anchored protein Thy-1 and ROS production. β cyclodextrin which sequestrates cholesterol from the membrane rafts inhibited $NaAsO_2$-inducde activation of caspase, Akt and production of ROS. These results argued that membrane raft integrity-dependent cell surface event is a prerequisite for the $NaAsO_2$-mediated induction of pro- and anti-apoptotic signals.

PAEONIFLORIN INDUCES APOPTOSIS OF LYMPHOCYTES

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Paeoniflorin (PF), a glucoside isolated from paeony root, *Shakuyaku*, is the principal bioactive component of Paeoniae Radix.

Paeony root has a long history of being used as a component of traditional Japanese and Chinese drugs for treating various kinds of diseases.

A number of studies have recently demonstrated that PF has some important pharmacological effects: anticoagulant, antiallergic, anti-inflammatory and antihyper-glycemic actions and cognition enhancement.

However, the molecular mechanisms of these pharmacological functions of PF remain unclear. This is mainly because there have been no detailed studies on how PF affects cellular signal transduction processes.

In this study, we demonstrated for the first time that PF induces production of reactive oxygen species, reduction of mitochondrial membrane potential and activation of mitogen-activated protein family kinases and caspase, processes that lead to DNA fragmentation.

CYSTEINE RESIDUES IN THE C-TERMINAL LOBE OF Src: THEIR ROLE IN THE SUPPRESSION OF THE Src KINASE

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With a series of Src mutants in which some of cyteines were replaced to alanines, we studied the effect of SH-alkylating agents, N-[p-(2-benzimidazolyl)phenyl] maleimide (BIPM) and N-(9-acridinyl) maleimide (NAM), on their kinase activity. Of ten cysteine residuces scattered over v-Src, either a single mutation at Cys520 or multiple mutations at the four clustered cyteines. Cys483, Cys487, Cys496 and Cys498, yielded clear resistance to the treatment with 10 μM BIPM or 1 μM NAM. In contrast deletion of SH2 and SH3 did not confer the resistance to v-Src, suggesting the inactivation by the SH-alkylating agents is SH2/SH3-independent. Although Cys520-mutated v-Src was resistant to 1 μM NAM, it was inactivated by 5 μM NAM. However

combined mutation including all of Cys483, Cys487, Cys496, Cys498 and Cys520 yielded clear resistance to 5 μM NAM. Among these mutants, those with double mutations in the four clustered cysteines yielded a temperature sensitive phenotype in the transfected cells, whereas Cys520 did not, suggesting that Cys520 has, at least in part, a discrete function. In contrast to v-Src, c-Src, which lacks cysteine at position 520, was resistant to 1 μM NAM, but sensitive to 5 μM NAM. While replacement of Phe520 of c-Src to cysteine made it sensitive to 1 μM NAM, double mutation in clustered cysteines again yielded resistance to 5 μM NAM. Taken together, our results strongly suggest that the multiple cysteine residues clustered at the end of C-terminal lobe are critical for the inhibition by the SH-alkylating agents and, thereby, have an allosteric repressor effect on the catalytic activity of Src in a SH2-phosphoTyr527 independent manner.

A ROLE FOR SHPS-1/SIRPα1 IN IL-1β- AND TNFα-DEPENDENT SIGNALING

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SHPS-1/SIRPα1 is a transmembrane glycoprotein that binds SHP-1/2 upon ligand stimulation and either positively or negatively regulates Erk1/2 and Akt activation depending on the ligand and cell type. We investigated the role of SHPS1/SIRPα1 in IL-1β- and TNFα-dependent signaling that leads to the activation of Erk 1/2 and Akt. Treatment of Balb 3T3 cells with IL-1β or TNFα activated tyrosine phosphorylation of SHPS-1 and its association with SHP-2 along with the activation of Erk 1/2 and Akt, PP1, a specific inhibitor for the Src family of protein tyrosine kinases, strongly inhibited tyrosine phosphorylation of SHPS-1 and complex formation of SHPS-1 with SHP-2 by IL-1β Moreover, PP1 substantially inhibited the IL-1β- and TNFα-dependent activation of Erk 1/2 Akt. Exogenous expression of either SHPS-1 mutants that lack SHP-2 binding function or a dominant negative mutant of SHP-2 markedly inhibited the activation of Erk 1/2 and Akt by IL-1β, whereas wild type SHPS-1 did not. In addition, IL-1β stimulation induced association of SHPS-1 with IL-1R-AcP, a second sub unit of IL-1RI. Exogenous expression of ECTM mutant of SHPS-1 lacking the cytoplasmic domain inhibited this association of SHPS-1 with IL-1R-AcP along with the tyrosine phosphorylation of endogenous SHPS-1. Taken together, our results strongly suggest that activation of Erk 1/2 and Akt by proinflammatory cytokines requires tyrosine phosphorylation of SHPS-1 and subsequent association of SHPS-1 with SHP-2 and ECTM mutant has a dominant inhibitory effect on endogenous SHPS-1.

APPLICATION OF RECOMBINANT *N*-ACETYLGLUCOSAMINE-6-*O*-SULFOTRANSFERASE-1 FOR SYNTHESIS OF SULFATED OLIGOSACCHARIDES AND FOR MODIFICATION OF OLIGOSACCHARIDES IN FIBRINOGEN

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N-Acetylglucosamine-6-*O*-sulfotransferase (GlcNAc6ST) catalyzes transfer of sulfate from 3'-phosphoadenosine 5'-phosphosulfate to the C-6 position of the non-reducing GlcNAc. Human GlcNAc6ST-1 was expressed as a fusion protein with protein A in an insect cell line (Tn 5 cells) using the baculovirus system. The recombinant enzyme was purified to homogeneity by lgG Sepharose column chromatography. The substrate specificity and the kinetical properties of the enzyme were similar to those of the enzyme expressed in the mammalian system. The purified recombinant enzyme was used to synthesize 6-sulfo GlcNAcβ1-3Ga1β1-4Glc which was identified by time of flight mass spectrometry. This sulfated trisaccharide served as a better substrate of a microsomal galactosyltransferase from the mouse colon compared to 6-sulfo GlcNAc. The purified recombinant enzyme was also used to sulfate oligosaccharide chains of fibrinogen after enzymatic desialylation and degalactosylation to expose nonreducing GlcNAc residues. It is known that desialylation greatly increases the rate of clotting of fibrinogen after addition of thrombin. Subsequent sulfation of desialylated and degalactosylated fibrinogen slightly decreased the rate of clotting. The recombinant GlcNAc6ST-1 is a useful reagent to 6-sulfate exposed GlcNAc residues both in oligosaccharides and in glycoproteins.

MIDKINE BINDS TO 37 kDa LAMININ BINDING PROTEIN PRECURSOR, LEADING TO NUCLEAR TRANSPORT OF THE COMPLEX

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Midkine (MK) is a heparin binding multifunctional protein that promotes cell survival and cell migration. MK was found to bind to 37 kDa laminin binding protein precursor (LBP), a precursor of 67 kDa laminin receptor, with Kd of 1.1 nM between MK and LBP-glutathione-S-transferase fusion protein. The binding was inhibited by laminin, anti-LBP, amyloid β-peptide and heparin; the latter two are known to bind to MK. In CMT-93 mouse rectal carcinoma cells, LBP was mostly located in the cytoplasm as revealed by immunostaining with anti-LBP antibody. That a portion of LBP or 67 kDa laminin receptor was located at the surface of these cells was verified by inhibition of cell attachment to laminin-coated dishes by anti-LBP antibody. When MK was added to culture medium of these cells, a part of LBP migrated to the nucleus. The movement occurred concomitantly with nuclear transport of biotin-labeled MK.

These findings suggested that the binding of MK to LBP caused nuclear translocation of the molecular complex.

INCREASED EXPRESSION OF PLASMINOGEN ACTIVATOR INHIBITOR-1 WITH FIBRIN DEPOSITION IN A MURINE MODEL OF AGING, *"KLOTHO"* MOUSE

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Although aging accompanies specific pathological changes, including thrombosis and organ sclerosis, the underlying mechanisms of these processes remain to be elucidated. In the present study, we analyzed the gene expression of plasminogen activator inhibitor-1 (PAI-1), a key molecule in the development of thrombosis, in a murine model of aging, *klotho* mutant (*kl/kl*) mice. PAI-1 antigen in plasma and PAI-1 mRNA in several tissues were strikingly elevated in *kl/kl* mice as compared with wild-type mice. This increased PAI-1 expression was age dependent and linked to the development of ectopic calcification and glomerular fibrin deposition in the kidneys. In situ hybridization analysis of *kl/kl* mice demonstrated that strong signals for PAI-1 mRNA were localized in renal tubular epithelial cells, cardiomyocytes, adrenal medullar cells, and smooth muscle and endothelial cells in Mönckeberg's arteriosclerotic vessels. Renal glomerular fibrin deposition, as evaluated immunohistochemically, was occasionally observed only in *kl/kl* mice, and the number of fibrin-positive glomeruli increased as the *kl/kl* mice aged. These observations suggest that in the process of aging the PAI-1 gene expression is increased, contributing to the development of thrombosis.

REDUCED MYOCARDIAL SARCOPLASMIC RETICULUM CA^{2+}-ATPase mRNA EXPRESSION AND BIPHASIC FORCE-FREQUENCY RELATIONS IN PATIENTS WITH HYPERTROPHIC CARDIOMYOPATHY

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Background—The relationship between left ventricular (LV) contractile functional reserve and gene expression of Ca^{2+}-handling proteins in patients with hypertrophic cardiomyopathy (HCM) remains to be clarified.

Methods and Results—We calculated the maximum first derivative of LV pressure (LV dP/dt_{max}) and the LV pressure half-time ($T_{1/2}$) during pacing in 14 patients with nonobstructive HCM (LVEF > 50%) and 7 controls. Endomyocardial tissue was obtained, and mRNA levels of

sarcoplasmic reticulum Ca^{2+}-ATPase (SERCA2), ryanodine receptor-2, phospholamban, calsequestrin, and Na^{+}/Ca^{2+}-exchanger were quantified using a real-time quantitative RT-PCR. Group A consisted of 7 HCM patients who showed a progressive rise in the LV dP/dt_{max} with increased heart rate (HR). Group B consisted of 7 HCM patients in whom the HR-LV dP/dt_{max} relation was biphasic at physiologic pacing rates. Both the mean maximal wall thickness and the LV hypertrophy score in group B were greater than in group A. SERCA2 mRNA levels were significantly lower in group B compared with group A and control subjects, whereas the mRNA expression of ryanodine receptor-2, phospholamban, calsequestrin, and Na^{+}/Ca^{2+}-exchanger were similar in all groups.

Conclusion—Downregulation of SERCA2 mRNA, resulting in altered Ca^{2+} handling, may contribute to impaired LV contractile reserve in HCM patients with severe hypertrophy, even in the absence of detectable baseline systolic dysfunction.

NERVE TERMINALS EXTEND INTO THE TEMPOROMANDIBULAR JOINT OF ADJUVANT ARTHRITIC RATS

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The innervation of the temporomandibular joint (TMJ) has attracted particular interest because of the association with complex movement. Although the pathological changes of disk innervation may have a crucial role in the development of TMJ pain, the innervation of the TMJ disk by arthritis has rarely been examined.

Arthritic rats were induced by injection with Complete Freund's adjuvant. We investigated distribution of nerve fibers using immunohistochemistry for protein gene product-9.5 (PGP-9.5) and calcitonin gene-related peptide (CGRP). To clarify the possible role of nerve growth factor (NGF) and its receptor on changes in peripheral innervation of the TMJ, the expressions of trkA and p75 receptor in trigeminal ganglia were examined.

PGP-9.5 and CGRP immunoreactive (ir) fibers were seen in the peripheral part of the disk. The total length of the nerve fibers increased in arthritic rats. The innervation area of fibers proliferating in the rostro-medial part merged with that of fibers in the rostro-lateral part in the arthritic rats. The ratio of trkA- and p75-positive cells increased in trigeminal ganglia.

It is assumed that increasing innervation of the disk may be important for the pathophysiology of TMJ pain. NGF and its receptors are likely involved in pathological changes of the disk.

THROMBOSPONDIN-1 PROMOTES FIBROBLAST-MEDIATED COLLAGEN GEL CONTRACTION CAUSED BY ACTIVATION OF LATENT TRANSFORMING GROWTH FACTOR β-1

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Grafting of cultured epithelium has become a useful technique for the treatment of epithelial defects, since grafted epithelial cells secrete factors promoting wound healing. We identified one such factor produced by cultured oral epithelial cells as thrombospondin-1 (TSP-1). The role of TSP-1 in wound healing and its mechanism were investigated using human fibroblast-mediated collagen gel contraction assay as an *in vitro* model of wound healing. Human TSP-1 promoted collagen gel contraction activity, and anti-human TSP-1 and TGF-β1 antibody inhibited the activity. The diameters of the gels treated with latent TGF-β1 (LTGF) and TSP-1 were reduced to a greater extent that those of gels treated with either factor alone. Although there were no significant differences in the amounts of total TGF-β1, which include LTGF, the amount of 25 kDa TGF-β1 was 3.30-fold greater in TSP-1-treated samples than controls. These findings suggested that TSP-1 causes collagen gel contraction by activation of TGF-β1. Collagen sponges were soaked with TSP-1 and implanted subcutaneously into rats. Seven days after implantation, increased numbers of fibroblasts were observed in the sponges treated with TSP-1. TSP-1 is expected to be especially suitable for regulating wound healing.

CELL DEATH OF HUMAN ORAL SQUAMOUS CELL CARCINOMA CELL LINE INDUCED BY HERPES SIMPLEX VIRUS THYMIDINE KINASE GENE AND GANCICLOVIR

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Suicide gene therapy using herpes simplex virus thymidine kinase gene (HSVtk) and ganciclovir (GCV) is one strategy for the treatment of head and neck squamous cell carcinoma (HNSCC). The purpose of this study is to assess the mechanism of the cell death that occurs in suicide gene therapy using HSVtk and GCV.

The human oral squamous cell carcinoma cell line SAS was treated with adenovirus vector containing HSVtk gene (AdHSVtk) and GCV *in vitro*. Morphological changes including chromatin condensation, cell shrinkage, blebbing of cell membrane and ballooning formations were observed. Changes in the localization of phospholipids in the cell membrane were also observed. The results of flow cytometry showed a maximum of about 65% of cells in the early phase of apoptosis. In addition, DNA fragmentation was investigated using the TUNEL method *in vivo*. Nude mice (Balb/C, aged 4 weeks) were implanted with SAS and treated with

AdHSVtk and GCV. Tumor sections were then observed. The treatment group was confirmed to have DNA fragmentation positive cells.

These results suggest that suicide gene therapy using AdHSVtk and GCV led to apoptosis of the oral squamous cell carcinoma cell line.

RADIOIMMUNOSCINTIGRAPHY OF INTRACRANIAL GLIOMA XENOGRAFT WITH A TECHNETIUM-99M-LABELED MOUSE MONOCLONAL ANTIBODY SPECIFICALLY RECOGNIZING TYPE III MUTANT EPIDERMAL GROWTH FACTOR RECEPTOR

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Department of Neurosurgery

Object: The type III mutant Epidermal Growth Factor Receptor (EGFR) is expressed on the cell surface of a subset of glioma, but not of normal tissues. In this study, we investigated the *in vivo* kinetics of 3C10 mouse monoclonal antibody (mAb), specifically recognizing the type III mutant EGFR (EGFRvIII).

Methods: Human glioma cell line, U87MG, expressing the wild type EGFR and the transfectant, named U87MG.ΔEGFR, expressing the EGFRvIII, were transplanted subcutaneously or intracranially to nude mice. 3C10 mAb labeled with a technetium-99m (^{99m}Tc) was intravenously injected into these nude mice and then the mice were sacrificed at 24 hours later, and the ^{99m}Tc-uptake by xenografts and major normal organs were measured to determine the biodistribution of mAb. Furthermore, at 3, 6 and 24 hours after injection of ^{99m}Tc-labeled 3C10 mAb, whole-body scintigraphy was performed.

Results: 3C10 mAb significantly accumulated to U87MG.ΔEGFR xenografts transplanted subcutaneously or intracranially in nude mice, showing tumor-to-blood ratio of 10.23 and 4.01, respectively. In scintigrams, intracranially transplanted U87MG.ΔEGFR xenografts were detectable at 3 hours after injection of ^{99m}Tc-labeled mAb.

Conclusion: 3C10 mAb is a potential diagnostic and therapeutic agent for patients with gliomas expressing the EGFRvIII.

RATE OF AXONAL REGENERATION IN CAT RETINAL GANGLION CELLS—DIFFERENCES BETWEEN CELL TYPES

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Transected axons of retinal ganglion cells (RGCs) regenerate into a transplanted peripheral

nerve (PN) segment, but the numbers of regenerated RGCs are at most 4% of the cat retina. Enhancing axonal regrowth is promising methods to obtain more regenerated RGCs. I estimated the rate of regrowth of RGC axons regenerating in a PN graft as the control value. After 4, 6, and 8 week survival, regenerated RGCs were double-labeled with two fluorescent dyes injected separately at 10 mm and 20 mm from the connected site. From a scatter diagram of double-labeling ratios, I estimated that axons reached 20 mm by 3.2 weeks. Immunostaining suggested that axon sprouts first entered into a PN segment on day 4. These values enabled me to estimate average rates of axonal regeneration as 1.1 mm/d for all the RGCs. Proportions of cell types of regenerated RGCs were obtained with Lucifer yellow injections. From analysis on scatter diagrams I estimated axonal growth rate of alpha, beta, and non alpha/beta cells as 1.4, 1.1, 1.0 mm/d, respectively. Higher regeneration rate of alpha cells may reflect their greater regenerative ability than other cell types. The present system provides control values when methods to promote axonal regeneration are developed.

FUNCTION AND MORPHOLOGY OF MACULA BEFORE AND AFTER REMOVAL OF IDIOPATHIC EPIRETINAL MEMBRANE

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PURPOSE: To study the function and morphology of the macula of eyes before and after the removal of unilateral idiopathic epiretinal membrane (ERM).
METHODS: Focal macular electroretinograms (FMERGs) elicited by a 15-degree stimulus were recorded from 37 eyes of 37 patients with a unilateral ERM. The amplitudes of the a- and b-waves, and the oscillatory potentials (OPs) were compared to the corresponding waves in the normal fellow eyes before and after removal of ERM. In 29 eyes followed for more than 6 months postoperatively, the FMERGs and foveal and parafoveal thickness, measured by optical coherence tomography (OCT), were evaluated.
RESULTS: Preoperatively, the mean amplitudes of all components of the FMERGs were significantly smaller than that in the fellow eyes with the decrease largest for the OPs, followed by the b-waves, and then the a-waves. The eyes with less severely reduced a-wave amplitude (>70% of the fellow eyes) had significantly lower b-wave to a-wave (b/a) ratios. Postoperatively, the amplitudes of the b-wave and OPs were still significantly smaller in the affected eyes. The mean foveal and parafoveal thickness was significantly thinner postoperatively, however, the thickness was still thicker in the affected eyes. The decrease of the OPs remained after surgery and was correlated with the increased parafoveal thickness ($r = –0.460$, $P = 0.011$).
CONCLUSIONS: The decreased FMERGs indicate that macular function is impaired in eyes with an ERM. The decrease of the b-wave and OPs in the 29 eyes examined after vitrectomy may be due to the still thickened macular retina.

MORPHOLOGICAL ASSESMENT OF IDIOPATHIC MACULAR HOLES AFTER VITRECTOMY WITH AND WITHOUT INTERNAL LIMITING MEMBRANE REMOVAL

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Purpose: To evaluate the effect of internal limiting membrane (ILM) removal on foveal configuration after idiopathic macular hole (IMH) surgery.
Methods: Optical coherence tomography (OCT) was performed on 47 eyes of 46 consecutive patients with IMH 6 to 12 months (average 7.5 months) after successful macular hole surgery. OCT images were classified into the U type, nearly normal foveal shape, or the pseudohole type, steep edge and flat fovea. The ILM was randomly removed in 27 eyes (ILM-off) and not removed in 20 eyes (ILM-on).
Results: All TMHs were closed after the first surgery in both qroups. At the first postoperative evaluation (1 month), 2 eyes (7%) of the ILM-off eyes and 2 eyes (9%) of the ILM-on eyes had the P-type shape. These differences were not significant between two groups.

At the last evaluation, there were 9 P-type eyes. Two were ILM-off eyes (2/25; 7%) and 7 were ILM-on eyes (7/13; 35%). The P-type macula was found in significantly higher numbers in the ILM-on group than ILM-off group. (P = 0.026; Fisher's probability test). The difference was not significant in the postoperative best corrected visual acuity between two groups.
Conclusion: ILM removal may be effective in maintaining good foveal shape after successful macular hole surgery.

ROLE OF D AND E DOMAINS IN THE MIGRATION OF VASCULAR SMOOTH MUSCLE CELLS INTO FIBRIN GELS

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We examined the mechanisms of the migration of vascular smooth muscle cells (SMCs) into fibrin gels, using an in vitro assay system. Cultured SMCs from bovine fetal aortic media migrated into fibrin gels prepared with thrombin, which cleaves both fibrinopeptides A and B from fibrinogen, without other chemotactic stimuli. Both desA fibrin gels prepared with batroxobin, which cleaves only fibrinopeptide A, and desB fibrin gels prepared with Agkistrodon contortrix thrombin-like enzyme (ACTE), which cleaves only fibrinopeptide B, similarly induced the migration of SMCs compared to fibrin gels prepared with thrombin. The addition of GRGDS, a synthetic RGD-containing peptide, but not that of GRGES, a control peptide, partially inhibited the migration of SMCs into fibrin gels, suggesting that the migration of SMCs into fibrin gels is at least in part dependent on the RGD-containing region of the α chain. The migration of SMCs into fibrin gels was also inhibited by a monoclonal antibody for integrin

αvβ3 and α5β1, indicating that migration is dependent on these integrins. Furthermore, both fibrin(ogen) fragments D and E inhibited the migration of SMCs into fibrin gels, suggesting that these fragments, generated during fibrino(geno)lysis, may be relevant in the regulation of SMC migration into fibrin gels.

CHARACTERIZATION OF A FUNCTIONALLY EXPRESSED STRETCH-ACTIVATED BKca CHANNEL FROM CHICK VENTRICULAR MYOCYTES IN CHINESE HAMSTER OVARY CELLS

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We have cloned a stretch-activated Kca channel (SAKca) gene from chick ventricular myocytes (CCVM) and expressed it in Chinese hamster ovary cells (CHO). Electrophysiological and pharmacological properties of the expressed channel were studied by using the patch clamp single channel recording technique. Our results indicated that the cloned SAKca keeps most of the key properties of the native SAKca in CCVM, such as conductance, ion selectivity, voltage- and Ca^{2+}-dependencies. However, there was a slight difference between these channels in the effects of channel blockers, such as TEA, CTX and gadolinium (Gd^{3+}). As the involvement of some auxiliary components was suspected, we cloned the most probable candidate component, a β-subunit from CCVM, and coexpressed it with the cloned SAKca in CHO cells to test its effect on the SAKca. Although the pharmacological properties of the SAKca became to be very similar to those of the native SAKca by the coexpressed β-subunit, the key characteristics of SAKca such as voltage- and Ca^{2+}-dependencies were greatly altered. Therefore we concluded that native SAKca in CCVM does not interact with the endogenous β-subunit. The difference in pharmacological properties between the expressed SAKca in CHO and the native one in CCVM suggests that some properties of cloned one in CHO or (and) the native one in CCVM are modulated by unknown auxiliary components in CHO or (and) in CCVM.

PHOSPHORYLATION OF NEUROGLYCAN C, A BRAIN-SPECIFIC TRANSMEMBRANE CHONDROITIN SULFATE PROTEOGLYCAN, AND ITS LOCALIZATION IN THE LIPID RAFTS

SHINOBU YAMAUCHI

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Neuroglycan C (NGC) is a brain-specific transmembrane chondroitin sulfate proteoglycan. In the present study, we examined whether NGC could be phosphorylated in the brain. On meta-

bolic labeling of cultured cerebral cortical cells from the rat fetus with [^{32}P] orthophosphate, serine residues in NGC were radio-labeled. Some NGC became detectable in the raft fraction from the rat cerebrum, a signaling microdomain of the plasma membrane, with cerebral development. NGC from the non-raft fraction, not the raft fraction, could be phosphorylated by an *in vitro* kinase reaction. The phosphorylation of NGC was inhibited by adding to the reaction mixture a recombinant peptide representing the ectodomain of NGC but not its cytoplasmic domain. NGC could be labeled by an *in vitro* kinase reaction using [γ-^{32}P]GTP as well as [γ-^{32}P]ATP, and this kinase activity was partially inhibited by 5,6-dichloro-1-β-$_D$-ribofuranosylbenzimidazole, a selective inhibitor for casein kinase II (CKII). In addition to the intracellular phosphorylation, NGC was also phosphorylated at the cell surface by an ectoprotein kinase. This is the first report to demonstrate that NGC can be phosphorylated both intracellularly and pericellularly, and our findings suggest that a kinase with a specificity similar to that of CKII is responsible for the NGC ectodomain phosphorylation.

PHOSPHORYLATION OF COLLAPSIN RESPONSE MEDIATOR PROTEIN-2 BY RHO-KINASE: EVIDENCE FOR TWO SEPARATE SIGNALING PATHWAYS FOR GROWTH CONE COLLAPSE

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We previously identified Rho-associated protein kinase (Rho-kinase) as a specific effector of Rho. In this study, we identified collapsing response mediator protein-2 (CRMP-2), as a novel Rho-kinase substrate in brain. CRMP-2 is a neuronal protein whose expression is upregulated during development. Rho-kinase phosphorylated CRMP-2 at Thr-555 *in vitro*. I produced an antibody that specifically recognizes CRMP-2 phosphorylated at Thr-555. Using this antibody, I found Rho-kinase phosphorylated CRMP-2 downstream of Rho in COS7 cells. Phosphorylation of CRMP-2 was observed in chick dorsal root ganglion (DRG) neurons during lysophosphatidic acid (LPA)-induced growth cone collapse, whereas the phosphorylation was not detected during Semaphorin-3A (Sema3A)-induced growth cone collapse. Both LPA-induced CRMP-2 phosphorylation and LPA-induced growth cone collapse were inhibited by Rho-kinase inhibitor, HA1077 or Y-32885. LPA-induced growth cone collapse was also blocked by a dominant negative form of Rho-kinase. On the other hand, Sema3A-induced growth cone collapse was not inhibited by a dominant negative form of Rho-kinase. Furthermore, overexpression of a mutant CRMP-2 in which Thr-555 was replaced by Ala significantly inhibited LPA-induced growth cone collapse. These results demonstrate the existence of Rho-kinase-dependent and -independent pathways for growth cone collapse and suggest that CRMP-2 phosphorylation by Rho-kinase is involved in the former pathway.

TRANSLOCATION OF Na^+, K^+-ATPase IS INDUCED BY RHO SMALL GTPase IN RENAL EPITHELIAL CELLS

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The distribution of transmembrane proteins is considered to be crucial for their activities because these proteins mediate the information coming from outside of cells. A small GTPase Rho participates in many cellular functions through its downstream effectors. In this study, we examined the effects of RhoA on the distribution of Na^+, K^+-ATPase, one of the transmembrane proteins. In polarized renal epithelium, Na^+, K^+-ATPase is known to be localized at the basolateral membrane. By microinjection of the constitutively active mutant of RhoA ($RhoA^{Val14}$) into cultured renal epithelial cells, NA^+, K^+-ATPase was translocated to the spike-like protrusions over the apical surfaces. Microinjection of the constitutively active mutant of other Rho family GTPases, Rac1 or Cdcd42, did not induce the translocation. The translocation induced by $RhoA^{Val14}$ was inhibited by treatment with Y-27632, a Rho-kinase specific inhibitor, or by coinjection of the dominant negative mutant of Rho-kinase. These results indicate that Rho and Rho-kinase are involved in the regulation of the localization of Na^+, K^+-ATPase. We also found that Na^+, K^+-ATPase seemed to be colocalized with ERM proteins phosphorylated at T567 (ezrin), T564 (radixin) and T558 (moesin) in cells microinjected with $RhoA^{Val14}$.

THE Gly952 RESIDUE IN *SACCHAROMYCES CEREVISIAE* DNA POLYMERASE α IS IMPORTANT IN DISCRIMINATING CORRECT dNTPs FROM INCORRECT ONES

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The conserved residue Gly952 of *Saccharomyces cerevisiae* DNA polymerase α (pol α) is strictly required for genetic complementation and polymerase catalytic activity. To analyze the role of Gly952, biochemical propertics of mutant were characterized by determining nucleotide incorporation specificity. A mutant pot α, G952A, incorporated nucleotides with extraordinarily low fidelity. In a steady state kinetic assay, the G952A mutant polymerase incorporated incorrect nucleotides more efficiently than correct nucleotides opposite to C, G and T. The fidelity of the G952A mutant polymerase was highest at template A, where the ratio of incorporation efficiency of dCMP to dTMP was as high as 0.37. Correct nucleotide insertion was 500- to 3600- fold lower than wild type pol α, with up to 22- fold increase in pyrimidine misincorporation in G952A. The K_m values for G952A pol α bound to mismatched termini T:T, T:C, C:A and A:C were 70- to 460- fold lower than to matched termini. Furthermore, pol α G952A preferentially incorporated pyrimidine instead of dAMP opposite an abasic site, *cis-syn*

cyclobutane di-thymine, and (6-4) di-thymine photoproduct. These data demonstrate that the Gly952 is a critical residue for catalytic efficiency and error prevention in *S. cerevisiae* pol α.

NOVEL PHENALENONE DERIVATIVES FROM A MARINE-DERIVED FUNGUS EXHIBIT DISTINCT INHIBITION SPECTRA AGAINST EUKARYOTIC DNA POLYMERASES

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A number of compounds used for cancer chemotherapy exert their effects by inhibiting DNA replication. This study tested effects of two phenalenone-skeleton based compounds, isolated from a marine-derived fungus *Penicillium sp*., sculezonone-B and sculezonone-A, upon DNA polymerase activity. Both compounds inhibited bovine polymerase α and γ, moderately affected activity of polymerase ε and had almost no effect on HIV-reverse transcriptase and *E. coli* polymerase I Klenow fragment. Most notably, whereas SCUL-A inhibited polymerase β (IC_{50} = 17 μM), SCUL-B has only a weak influence upon this polymerase (IC_{50} = 90 μM). Kinetics studies showed that polymerase α and β inhibition by either SCUL-A or SCUL-B was competitive with dTTP and noncompetitive with the template-primer. Whereas pol α inhibition by SCUL-B is competitive with dATP, the inhibition by SCUL-A was mixed type with dATP. SCUL-B K_i values were 1.8 and 6.8 μM for polymerases α and γ, respectively. Polymerase β K_i for SCUL-A was 12 μM and for polymerase α, 16 μM. Therefore, deletion of OH-group at C12 enhanced inhibition of polymerase β. Since computational analyses of these two inhibitors revealed a difference in distribution of negative electrostatic charge on the molecule surface, we infer that different electrostatic charges might elicit different inhibition spectra from these two compounds.

PRE- AND POST-GANGLIONIC PARASYMPATHETIC CONTROL IN RAT WITH RIGHT VENTRICULAR HEART FAILURE

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Congestive heart failure (CHF) is associated with parasympathetic withdrawal as well as with an activation of sympathetic nervous systems, and such autonomic imbalance is supposed to increase the risk of sudden cardiac death. We investigated the effects of pre- and post-ganglionic vagal nerve stimulation (VS) and ACh application on the heart rate of rats with CHF. Right-sided CHF secondary to pulmonary hypertension was produced in rats (n=6) by a single

injection of monocrotaline. Heart rate reduction in response to pre-ganglionic VS in rats in-vivo expressed in % increment of cycle length (dCL) was significantly attenuated in CHF rats than that of control rats. The suppression of spontaneous beating of isolated right atria including the whole sinoatrial node (SAN) in response to post-ganglionic VS was significantly attenuated in CHF rats. In contrast, ACh application to the atrial preparations resulted in a significantly greater suppression of spontaneous beating in CHF rats than controls. IC_{50} were 0.09 μM in CHF whereas 0.13 μM in control. In conclusion, these results suggest that the M2 receptor-mediated inhibitory response of pacemaker cells in the SAN of CHF animals is functionally upregulated despite of an obvious dysfunction of pre- or post-ganglionic vagal input.

CHANGES IN DISTINET SPECIES OF 1,2-DIACYLGLYCEROL IN CARDIAC HYPERTROPHY DUE TO ENERGY METABOLIC DISORDER

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The juvenile visceral steatosis mouse (JVS), a genetic model of systemic carnitine deficiency resulting from carnitine transport mutation, develops cardiac hypertrophy. We determined a putative lipid messenger 1,2-diacylglycerol (DAG), an activator of protein kinase C, in JVS, carnitine palmitoyltransferase-I (CPT-I) inhibitor etomoxir-treated mice (ET) and carnitine-treated JVS mice (CT), a model of inhibited cardiac hypertrophy. DAG level and their fatty acid composition of all groups were measured at 8 wk. DAG markedly increased in both JVS and ET compared with that in controls (1677±84, 1258±49, and 585±58 ng/dry wt, respectively; $P<0.01$), whereas it was decreased significantly in CT compared with that in JVS (1066±54 ng/dry wt, $P<0.01$). Furthermore, the fatty acid composition of DAG was similar between JVS and ET mice: in particular, 18:1 and 18:2 were significantly elevated in the myocardium ($P<0.01$ versus controls). On the other hand, that of DAG in CT was similar to that of the control group. Pharmacological intervention of etomoxir mimics changes in the lipid second messenger characteristic of genetic JVS mice. The results suggest that the increases in distinct DAG species might be involved in the pathogenesis of cardiac hypertrophy as a result of disorder of fatty acid transport.

ELUSION FROM CTL EPITOPE PROCESSING AND PRESENTATION IS A MAJOR ESCAPE MECHANISM FROM CTL RECOGNITION IN HIV-1 INFECTION

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Investigating the mechanisms of HIV-1 mutants escaping from specific cytotoxic T lymphocytes (CTLs) is essential to understand the pathogenesis of HIV-1 infection and to develop effective vaccines. We hypothesized that field isolates often escape from the CTL immune pressure via the escape from the processing and presentation of CTL epitope in HIV-1 infected cells. To address this issue, we have developed new HIV-1 vector to make target cells which endogenously express Gag of field isolate. Three of four field variants in an A*0201-restricted p17 epitope were escaped from the wild-type epitope specific CTL lines, while exogenously loaded synthetic peptides of two escaping variants were well-recognized. Similarly, four of five field variants in an A24-restricted p17 epitope were escaped from A24-restricted CTL lines although synthetic peptides of three escape variants were recognized. Further experiments have demonstrated that mutations within the epitope regions were largely responsible for these discordant results. Our results indicate that the escape from epitope processing and presentation frequently occurs in HIV-1. Detail analysis of epitope processing and presentation among HIV-1 field isolates including non-B subtypes is essential to understand further how the viruses elude the host immune pressure and will escape from the immunity elicited by future vaccines.

MOLECULAR IDENTIFICATION OF A RENAL URATE-ANION EXCHANGER THAT REGULATES BLOOD URATE LEVELS

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Urate, a naturally occurring product of purine metabolism, is a scavenger of biological oxidants implicated in numerous disease processes, as demonstrated by its capacity of neuroprotection. It is present at higher levels in human blood (200–500 μM) than in other mammals, because humans have an effective renal urate reabsorption system, despite their evolutionary loss of hepatic uricase by mutational silencing. The molecular basis for urate handling in the human kidney remains unclear, because of difficulties in understanding diverse urate transport and species differences. Here we describe the identification of the long-hypothesized urate transporter in the human kidney (URAT1, encoded by *SLC22A12*), a urate-anion exchanger regulating blood urate levels and targeted by uricosuric and antiuricosuric agents (which affect excretion of uric acid). Moreover, we provide evidence that patients with idiopathic renal hypouricaemia (lack of blood uric acid) have defects in *SLC22A12*. Identification of URAT1 should provide insights into the nature of urate homeostasis, as well as lead to the development

of better agents against hyperuricaemia, a disadvantage that is concomitant with the evolution for humans.

DECREASE IN RENAL MEDULLARY ENDOTHELIAL NITRIC OXIDE SYNTHASE OF FRUCTOSE-FED, SALT-SENSITIVE HYPERTENSIVE RATS

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We investigated the expression of endothelial NO synthase (eNOS) in the kidneys of fructose-fed insulin-resistant rats (FFR) with a low- or high-sodium diet. Male Sprague-Dawley rats were fed a control (C) or high-fructose (40% fructose; F) diet, with cach coming in low-sodium (0.024% NaCl; LS-C or LS-F) or high-sodium (3% NaCl; HS-C or HS-F) varieties, for two weeks. Half of the FFR were orally administered pioglitazone ($10mg \cdot kg^{-1} \cdot day^{-1}$), an insulin-sensitizing agent (LS-FP or HS-FP). The systolic blood pressure was significantly higher in the HS-F rats than in the LS-F rats or the HS-C rats, which indicated the salt dependence of hypertension in FFR. The protein expression of eNOS in the renal medulla of FFR was significantly lower than that in control rats during a high sodium load. The administration of pioglitazone prevented the hypertension and the reduction of medullary eNOS protein expression in HS-F rats. There was no significant difference in cNOS expression in the renal cortex or aorta between FFR and control rats, regardless of sodium load. These results suggest that the decrease in renal medullary NO production by eNOS during a high sodium load may play a role in fructose-fed, salt-sensitive hypertension.

ABERRANT DNA METHYLATION OF $P57^{PIP2}$ GENE IN THE PROMOTER REGION IN LYMPHOID MALIGNANCIES OF B-CELL PHENOTYPE

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The cyclin-dependent kinase (CDK) inhibitor $p57^{PIP2}$, is thought to be a potential tumor suppressor gene (TSG). The present study examines this possibility. We found that expression of $p57^{PIP2}$ gene is absent in various hematological cell lines. Exposing cell lines to the DNA demethylating agent, 5-aza-2'-deoxycytidine, restored $p57^{PIP2}$ gene expression. Bisulfite sequencing analysis of its promoter region showed that $p57^{PIP2}$ DNA was completely methylated in cell lines that did not express the $p57^{PIP2}$ gene. Thus, DNA methylation of its promoter might lead to inactivation of the $p57^{PIP2}$ gene. DNA methylation of this region is thought to be an aberrant alteration, since DNA was not methylated in normal peripheral blood mononuclear cells and in reactive lymphadenitis. Methylation-specific PCR analysis found frequent DNA methylated of

the $p57^{PIP2}$ gene in primary diffuse large B cell lymphoma (54.9%) and in follicular lymphoma (44.0%), but methylation was infrequent in myelodysplastic syndrome and adult T cell leukemia (3.0% and 2.0% respectively). These findings directly indicate that the $p57^{PIP2}$ profile of the gene corresponds that of a TSG.

RESPONSES OF PRIMARY AFFERENT C-FIBERS TO COLD WERE FACILITATED IN ADJUVANT-MONOARTHRITIC RATS HYPERSENSITIVE TO COLD

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Cold allodynia is an annoying symptom in conditions of chronic inflammation such as rheumatoid arthritis. To examine whether primary afferent nerve activities are associated with hypersensitivity to cold, we recorded single nerve activity in the rat *in vivo*. Persistent inflammation was induced by an injection of complete Freund's adjuvant solution into the tibio-tarsal joint. Behavioral experiments using cold immersion revealed that inflamed rats became hypersensitive to innocuous temperature of 25°C 2 to 3 weeks after the injection, showing an increase in paw shaking behavior. Recordings from the sural nerve were carried out during this period, focused on C-fibers. Thermal (2°C cold) stimuli were applied to the receptive fields of cutaneous receptors, using a feedback-controlled Peltier thermode.

In inflamed rats, we found that the suprathreshold response of C-low threshold mechanoreceptors to cold increased 1.8 fold, without significant change in the cold threshold (ca. 27°C). Besides, the proportion of cold-sensitive units in C-nociceptors was significantly greater in the inflamed group (9 out of 18 units, 50.0%; mean threshold: 10.0 ± 2.6°C) than in the intact group (1 out of 14 units, 7.1%; threshold: 4.0°C). These results suggest possible involvement of peripheral mechanisms in cold hypersensitivity in persistent inflammation conditions.

STEROID RECEPTOR COACTIVATOR-1 DEFICIENCY CAUSES VARIABLE ALTERATIONS IN THE MODULATION OF T_3-REGULATED TRANSCRIPTION OF GENES *IN VIVO*

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Department of Teratology and Genetics

[Aim] SRC-1 belongs to a family of coactivators to mediate hormone-dependent transcriptional activation. Recently we have shown that inactivation of the SRC-1 gene in the mouse (SRC-$1^{-/-}$) causes similar thyroid functions to those found in a patient with resistance to thyroid hor-

mone. In this study we determined whether SRC-1 deficiency affects T_3-dependent transcriptional regulation globally or the effect is restricted to specific genes.
[Methods] SRC-$1^{-/-}$ and SRC-$1^{+/+}$ mice were rendered hypothyroid by 5-propyl-2-thiouracil (PTU). Both SRC-$1^{-/-}$ and SRC-$1^{+/+}$ hypothyroid mice were treated with either L-T_3 (0.2 μg/mouse/day) or vehicle only. The mRNA levels of T_3-responsive genes were determined by Northern blots.
[Results] Hypothyroidism produced a comparable increase in pituitary TSH β mRNA in both genotypes, but its suppression in SRC-$1^{-/-}$ mice by L-T_3 was attenuated. In SRC-$1^{-/-}$ mice, hypothyroidism failed to reduce liver spot 14 mRNA levels that were significantly suppressed in SRC-$1^{-/-}$ mice, resulting in the lack of T_3-responsiveness of this gene in these mice. SRC-1 deficiency had no effect on the expression of the rest of the T_3-responsive genes examined.
[Conclusion/discussion] SRC-1 deficiency caused alterations in the transcriptional regulation of certain T_3-responsive genes, but the effect was not global. In addition, the results of the present study have suggested that SRC-1 is involved not only in transcriptional activation by liganded TRs, but also in T_3-dependent of T_3-independent transcriptional suppression of T_3-responsive genes.

LOCALIZATION OF A CALCINEURIN INHIBITORY PROTEIN, ZAKI-4, IN MOUSE BRAIN

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Department of Teratology and Genetics

[Purpose] ZAKI-4 was identified as a thyroid hormone response gene from human skin fibroblasts. From this gene, two isoforms, ZAKI-4α and β, are generated by alternative splicing. Recent *in vitro* studies have demonstrated that both ZAKI-4α and β belong to a family proteins that inhibit the activity of calcineurin, calcium/calmodulin dependent phosphatase. Since calcineurin has been reported to be involved in development and functions of brain, it is speculated that ZAKI-4α and β play an important role by regulating calcineurin activity. We thus examined whether ZAKI-4 isoforms localize in the position where they exert the inhibitory action of calcineurin activity, by investigating the spatial distribution of ZAKI-4α and β isoforms and comparing these with distribution of calcineurin. [Method] *In situ* hybridization (ISH) was employed to examine the spatial expression of ZAKI-4α and β mRNA, and distributions of ZAKI-4α and β protein were studied by immunohistochemistry using confocal and electron microscopies. [Result] ISH showed that both ZAKI-4α and β mRNAs were widely expressed in the entire brain. It was noted that strong signals were detected in the cerebral cortex, olfactory bulb, hippocampus and cerebellum. In these regions, calcineurin was also highly expressed. ZAKI-4α and β proteins showed similar distribution patterns as these observed at mRNA levels. Though both isoforms were expressed mainly in neurons of these areas, ZAKI-4α and β showed relatively distinct intracellular localization in neurons; ZAKI-4α was localized mainly in the soma whereas ZAKI-4β located mainly in the trunks of dendrites. On the other hands, calcineruin localized mainly in the periphery of dendrites. [Conclusion] Since both ZAKI-4α and β were abundantly expressed where calcineurin was also strongly expressed in mouse brain, it is suggested that ZAKI-4 proteins localized where they are able to exert the regulator action on calcineurin activity *in vivo*. More close intracellular localization of ZAKI-4β to that of

calcineurin as compared with ZAKI-4α may indicate that ZAKI-4β interact with calcineuron more than ZAKI-4α.

CASPASE-1 AND -3 mRNAs ARE DIFFERENTIALLY UPREGULATED IN MOTOR NEURONS AND GLIAL CELLS IN MUTANT SOD1 TRANSGENIC MOUSE SPINAL CORD — A STUDY USING LASER MICRODISSECTION AND REAL-TIME RT-PCR

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Amyotrophic lateral sclerosis is characterized by selective motor neuron degeneration. An apoptotic pathway is thought to be involved. It is difficult, however, to analyze the molecular pathogenic mechanism in single motor neurons because of complexity in the neural tissue which consists of multiple lineages of cells neighboring motor neurons. We quantified the caspase-1 and -3 mRNA in single motor neurons and neighboring glial cells isolated from the spinal ventral horn of mutant SOD1 transgenic (Tg) mice and littermates. Motor neurons and neighboring glial cells were isolated from spinal sections by laser microdissection, and the mRNAs were quantified by real-time RT-PCR. In the Tg mice, caspase-1 mRNA was first upregulated in motor neurons and secondly in glial cells. The caspase-3 mRNA was increased in motor neurons following the caspase-1 mRNA. These results indicated that caspase-1 and -3 mRNAs are differentially upregulated in motor neurons and glial cells of the Tg mice, and that mRNAs in isolated cells can be accurately assessed using our procedures.

SKIN SYMPATHETIC NERVE FUNCTION DURING SLEEP: A STUDY WITH EFFECTOR RESPONSES

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We investigated the effector-organ activities corresponding to skin sympathetic nerve activity (SSNA) during sleep in eight healthy adult volunteers. The following parameters of SSNA were recorded during night sleep: the spontaneous skin vasoconstriction and skin blood flow volume, by laser Doppler flowmetry; sweating, by the ventilated capsule method; and the galvanic skin response (GSR). Fluctuations of sweating and GSR were mainly observed on the dorsal side of the hand during night sleep. The frequency of GSR and sweat rate on the dorsal side of the hand were significantly lower during REM sleep than during NREM sleep. The frequcney of spontaneous skin vasoconstriction was higher and blood flow was lower during REM sleep than

during NREM sleep. These results indicate that sweating and blood flow in the skin are differentially regulated depending on the sleep stage. Our results also suggest that the sleep-regulating system is closely linked to thermoregulation, which is controlled by the sympathetic nervous system.

SPINAL CORD MAGNETIC RESONANCE IMAGING DEMONSTRATES SENSORY NEURONAL INVOLVEMENT AND CLINICAL SEVERITY IN NEURONOPATHY ASSOCIATED WITH SJÖGREN'S SYNDROME

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Objectives—To determine spinal cord magnetic resonance imaging (MRI) findings in Sjögren's syndrome-associated neuronopathy and their correlation with severity of sensory impairment. *Methods*—Clinical and electrophysiologic features, pathologic findings in the sural nerve, and hyperintensity on T2*-weighted MRI in the spinal dorsal columns were evaluated in 14 patients with Sjögren's syndrome-associated neuronopathy. *Results*—Of 14 patients, 12 showed high intensity by T2*-weighted MRI in the posterior columns of the cervical cord. High-intensity areas were seen in both the fasciculus cuneatus and gracilis in 9 patients, who showed severe and widespread sensory deficits in the limbs and trunk; these patients also had a high frequency of autonomic symptoms. Somatosensory evoked potentials often could not be elicited. Hyperintensity restricted to the fasciculus gracilis was seen in 3 patients, who showed sensory deficits restricted to lower limbs without trunk involvement, or with only partial limb involvement; no autonomic symptoms were noted. The 2 patients who did not show high-intensity areas in the dorsal columns showed restricted sensory involvement in the limbs. All patients showed axonal loss predominantly affecting large fibres, without axonal sprouting. *Conclusions*—High intensity areas on T2*-weighted MRI in the spinal dorsal columns reflect the degree of sensory neuronal involvement in Sjögren's syndrome-associated neuronopathy; this finding also could be a helpful marker for estimating severity of this neuronopathy.

HYPERBARIC OXYGEN THERAPY FOR THE TREATMENT OF LARGE VASTIBULAR AQUEDUCT SYNDROME

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Eight patients with large vestibular aqueduct (LVA) syndrome and acute sensorineural hearing loss that had not responded to intravenous treatment were treated with hyperbaric oxygen

(HBO_2) therapy. This was performed daily, with 15 treatments per course. Pure tone and speech audiometry were performed on all patients. The mean hearing recovery following HBO_2 therapy (20.8 ± 12.1 dB) was significantly higher than following intravenous treatment (2.3 ± 7.2dB) ($P < 0.01$), although treatment duration was not different between the two therapies. Of the eight patients, five showed complete or partial recovery after one course of HBO_2 therapy, and their hearing levels have not since changed. The other three patients showed hearing recovery during therapy, but treatment had to be repeated for two or more courses because of progressive or fluctuating hearing loss. We consider HBO_2 therapy should be used to treat acute hearing deterioration associated with LVA syndrome if patients do not recover their hearing ability following conventional treatment.

DETECTION OF MITOCHONDRIAL DNA ALTERATIONS IN THE SERUM OF HEPATOCELLULAR CARCINOMA PATIENTS

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Purpose: Somatic mutations in mitochondrial DNA (mtDNA) have recently been detected in various cancers. These mutations could possibly be detected in serum because mtDNA has a higher copy number than nuclear DNA. Thus, we examined genetic alterations in the D-loop region of mtDNA in hepatocellular carcinoma (HCC) patients.
Experimental Design: Fifty patients with HCC were investigated in this study. Somatic mutations in the D-loop region of tumor mtDNA were screened by direct sequencing, and then the paired serum samples were investigated using mutation-specific mismatch ligation assay.
Results: Fifteen of 100 sequence variants that were detected in tumor mtDNA have not been recorded previously. True somatic mutations in the D-loop region were detected in 17 of 50 patients (34%). Subsequent screening for paired serum by mismatch ligation assay revealed that 5 of 15 paired serum samples (33%) contained the same mutations as primary tumors.
Conclusions: mtDNA mutation may be a novel tumor maker of HCC and prove effective for detection of tumor DNA in the serum.

Cys611Ser MUTATION IN *RET* PROTO-ONCOGENE IN A KINDRED WITH MEDULLARY THYROID CARCINOMA AND HIRSCHSPRUNG'S DISEASE

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Germline mutations in the *RET* proto-oncogene are responsible for the development of human hereditary diseases, including multiple endocrine neoplasia (MEN) type 2A and 2B,

familial medullary thyroid carcinoma (FMTC), and Hirschsprung's disease (HSCR). It has been reported that some families developed both MEN 2A/FMTC and HSCR, in which a mutation in a cysteine residue at codon 609, 618, or 620 in the *RET* gene was present. Here we report a novel *RET* mutation detected in a Japanese family with medullary thyroid carcinoma (MTC) and HSCR. A germline mutation in cysteine 611 of the *RET* gene was identified in this family, which introduced an amino acid change from cysteine to serine. Using NIH3T3 transfection assay, we previously predicted the potentiality that amino acid substitution for cysteine 611 as well as cysteines 609, 618, and 620 would promote the development of both MEN 2A/FMTC and HSCR. This clinical case substantiates our suggestion for the mechanism of the development of both MEN 2A/FMTC and HSCR.

PGP9.5 AS A MARKER FOR INVASIVE COLORECTAL CANCER

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We recently proved that PGP9.5-negative pancreatic cancer had significantly better survival rates compared to those who were PGP9.5 positive, and PGP9.5 may be a novel marker for indicating the prognosis of pancreatic cancer patients. In this study, we examined the expression of PGP9.5 in primary colorectal cancers using immunohistochemistry. Of 74 colorectal cancer specimens examined, 33 cases (46%) showed positive staining with PGP9.5 in most tumor cells, while no PGP9.5 expression was detected in adjacent normal epithelium. Subsequently, we correlated PGP9.5 expression in tumors with the clinicopathological features of affected patients and found two significant differences in maximal tumor size and the extent of tumor (p = 0.035 and 0.019, respectively). This result suggests that PGP9.5 expression is related to tumor progression and may be useful as a marker for invasive colorectal cancer.

MOLECULAR DETECTION OF *p16* PROMOTER METHYLATION IN THE SERUM OF COLORECTAL CANCER PATIENTS

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Assays based on the molecular detection of genetic changes in serum have been shown as potential diagnostic tools for colorectal cancer. We examined the methylation status of *p16* in colorectal cancers using methylation-specific PCR (MSP). Forty-four of 94 (47%) cancer DNA exhibited abnormal promoter methylation of *p16* gene while no corresponding normal DNA exhibited such methylation. Subsequently, we examined whether aberrant methylation could be detected in corresponding serum DNA, and found that 13 of 44 (30%) patients with *p16* promoter methylation in tumor DNA demonstrated abnormal methylation in their serum DNA.

Moreover, abnormal methylation was found in the serum of patients in all clinical stages, suggesting that early colorectal cancer could be detected using the MSP method.

DIPEPTIDYL PEPTIDASE IV EXPRESSION IN ENDOMETRIAL ENDOMETRIOID ADENOCARCINOMA AND ITS INVERSE CORRELATION WITH TUMOR GRADE

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Objective: Dipeptidyl peptidase IV (DPPIV)/CD26 is a cell surface aminopeptidase. The present study investigated the expression and localization of DPPIV in endomentrial endometrioid adenocarcinomas of different grades.

Study Design: Immunohistochemical analysis was performed using DPPIV and RANTES (regulated on activation, normal T cell expressed and secreted) specific monoclonal antibodies. Cell proliferation was evaluated by BrdU uptake assay.

Results: Immunohistochemical analyses showed that DPPIV was strongly or moderately stained in glandular cell of the normal secretory phase. In endometrial adenocarcinoma, the DPPIV expression decreased with advancing grade ($p < 0.01$). Furthermore, RANTES, one of the possible DPPIV substrates, was highly expressed in all grades of endometrial adenocarcinoma cells. Addition of RANTES to endometrial adenocarcinoma cells increased proliferation in a concentration-dependent manner.

Conclusion: DPPIV is expressed in normal endometrial glandular cells, but its expression in endometrial adenocarcinoma is down-regulated with increasing grade. Our data also suggest a regulatory role of this ectoenzyme in neoplastic transformation and progression of endometrial adenocarcinomas possibly by degrading certain bioactive peptides such as RANTES.