Assessment to anti-epilepsy drugs in human iPSC-derived neural network

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Anti-epileptic drugs (AEDs) have different mechanisms of action depending on the generation. For example, the old generation AEDs are mainly Na+ channel inhibitory and GABA receptor agonists. On the other hand, a new generation of AEDs has a mechanism of action different from that of the previous generation, such as those that act on AMPA-type glutamate receptors and act on multiple channels and receptors. In addition, drugs with unknown mechanism of action such as Levetiracetam are also widely used as AEDs. We have developed the assay using microelectrode array (MEA) combined with hiPSC-derived neurons and have detected the responses to a lot of convulsants. In this study, it was attempted to administer AEDs to the hiPSC-derived neurons that caused seizures with convulsants, and to detect differences in the effects of old and new generation AEDs. HiPSC-derived neurons were cultured on MEA Plate, seizure-like activities (SLAs) were induced by 4-aminopyridine (4-AP), bicuculline, kainic acid, respectively, and then 6 types of AEDs were administered. Old generation AEDs were effective in case of only SLAs induced by 4-AP. On the other hand, the new generation AEDs were also effective in SLAs induced by bicucullin and kainic acid. This result suggests that MEA measurement of hiPSC-derived neurons are effective for AEDs screening.

Tumor necrosis factor alpha protects retinal ganglion cells against excitotoxicity via reduction of oxidative stress in the mice

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Excitotoxicity is thought to be involved in the neuronal cell death induced by glaucoma. We reported that tumor necrosis factor alpha (TNF α) was involved in the protective effects of a Toll-like receptor 9 agonist on the retinal ganglion cell loss induced by excitotoxicity in the mice. In the present study, we examined whether TNF α protected retinal ganglion cells against the NMDA-induced neurotoxicity via reduction of oxidative stress in the mice, *in vivo*. Male ICR mice of 8-12 weeks old were subjected to intravitreal NMDA (40 nmol/eye). TNF α (1 ng/eye) was intravitreally injected 18 hours before NMDA injection. Eyes were enucleated 24 hours and 7 days after NMDA injection, and the paraffin-embedded sections and the whole mount retinas were prepared, respectively. Immunohistochemistry using anti-8-OHdG antibody and Alexa Fluor 488-conjugated anti-NeuN antibody was carried out. TNF α significantly reduced the number of 8-OHdG-positive cells in the retinal ganglion cell layer 24 hours after NMDA injection, and the retinal ganglion cell loss 7 days after NMDA injection. These results suggest that TNF α protects retinal ganglion cells against excitotoxicity via reduction of oxidative stress in the mice.

Enhancement by a Src family inhibitor of the interaction of Pyk2 and Fyn in hypothalamic neurons

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The receptor for gonadotropin-releasing hormone (GnRH) is highly expressed in hypothalamic GnRH neurons, as well as in anterior pituitary gonadotrophs. In our previous study, we found that stimulation of the GnRH receptor activated protein kinase D1 (PKD1), and PKD1 was involved in the Fyn-mediated activation of proline-rich tyrosine kinase 2 (Pyk2) in cultured GnRH neurons (GT1-7 cells). In the present study, we examined the molecular mechanisms of Pyk2 activation and the interaction of Pyk2 and Fyn in GT1-7 cells. Experiments with site-directed mutants of Pyk2 indicated that tyrosine 402 (Tyr402) was phosphorylated both by autophosphorylation and by Fyn, whereas Tyr579 was phosphorylated exclusively by Fyn. We found that dasatinib, a Src family inhibitor, enhanced the interaction of Pyk2 and Fyn. Experiments with site-directed mutants of Pyk2 and Fyn indicated that dasatinib enhanced the binding of Pyk2 autophosphorylated at Tyr402 and the Src homology 2 domain in Fyn. Our present data may suggest that fully activated Pyk2 dissociates from Fyn after Fyn-mediated phosphorylation of Pyk2 at sites other than Tyr402 and Tyr579.

An examination whether KCC2, a K^+-Cl^- co-transporter, is efficient as a target to attenuate the neuronal dysfunction that is associated with radiation therapy for brain tumor by using oxytocin.

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Background and objective: Radiation therapy is applied to overcome brain tumors. To mitigate adverse effects including cognitive dysfunction, smaller dose of irradiation has been considered advantageous. However, it is not fully understood how the cells that survived after irradiation is damaged. Previously, it was reported that the decrease of KCC2, a K^+-Cl^- co-transporter, and consequent imbalance of responses to gamma-aminobutyric acid (GABA) are predisposition to neuronal disorders after stress (Tsukahara et al., 2015; Furukawa et al., 2017). To elucidate the association of KCC2 with the neuronal dysfunction after irradiation, we performed following experiments.

Methods and Results: We performed immunofluorescence staining and found that peri-membrane KCC2 signals of the primary-cultured neuronal cells declined at 24 h after X-ray (1.5 Gy) irradiation. We further investigated GABA-induced cell death by using trypan blue exclusion test. We found that the death fraction of X-ray-irradiated cells were increased compared with non-irradiated cells. We then performed immunofluorescence staining and found that KCC2 signals were increased in the cells that were administered with oxytocin. In addition, we irradiated γ-ray (1.5 Gy) to the head region of 4-week-old mice. In the novel object recognition tests, the mice that were irradiated with γ-ray showed lower discrimination score at 1 week after irradiation compared with non-irradiated mice. On the other hand, the mice that were irradiated with γ-ray and concomitantly administered with oxytocin showed an increase in discrimination score.

Discussion: It was suggested that KCC2 expression is declined in irradiated cell. Lower KCC2 expression may lead to higher intracellular chloride concentration, which may be a cause of hyperexcitability or neurotoxicity after GABA administration to the X-ray-irradiated cells. It was also suggested that oxytocin is a possible candidate to attenuate the neuronal dysfunction after radiation therapy for brain tumor.

Effects of CNB-001, a synthetic curcumin derivative, on thrombin-induced phosphorylation of MAP kinases in primary cultured rat microglia

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We have recently found that CNB-001, a synthetic curcumin derivative, suppresses thrombin-induced nitric oxide (NO) production in cultured microglia, demonstrating that it exerts anti-neuroinflammatory effects by regulating microglial activation. In the present study, we investigated the molecular mechanisms underlying the suppressive effects of CNB-001 on thrombin-induced inflammatory responses. Western blotting analysis demonstrated that thrombin (10 U/mL) induced rapid phosphorylation of extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), and p38 mitogen-activated protein kinase (MAPK). CNB-001 significantly suppressed the thrombin-induced phosphorylation of ERK and p38 MAPK, but not JNK. In addition, the suppressive effect of CNB-001 on NO production was mimicked by blockage of the ERK and p38 MAPK signaling pathways with U0126 and SB203580. These results suggest that CNB-001 suppresses thrombin-induced microglial activation through inhibition of ERK and p38 MAPK pathways.

Prostaglandin E_2 increases expression of mRNA for cyclooxygenase-2 and microsomal prostaglandin E synthase-1 in microglia.

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Prostaglandin E_2 (PGE_2) plays an important role in modulating microglial function. In the present study, we have found that PGE_2 increases expression of mRNA for cyclooxygenase-2 (COX-2) and microsomal prostaglandin E synthase-1 (mPGES-1), which are involved in PGE_2 synthase in cultured rat microglia.

COX-2 and mPGES-1 mRNA levels were increased by PGE_2 at 10^{-6} M for 3 h in microglia. The increase of these mRNA levels was inhibited by PF-04418948 (EP_2 antagonist), but not by ONO-8713 (EP_1 antagonist), ONO-AE3 -240 (EP_3 antagonist), or ONO-AE3-208 (EP_4 antagonist) at 10^{-6} M. In addition, ONO-AE1-259-01 (EP_2 agonist), also increased COX-2 and mPGES-1 mRNA levels in a dose dependent manner, and these mRNA levels were not affected by ONO-DI-004 (EP_1 agonist), ONO-AE-248 (EP_3 agonist), or ONO-AE1-329 (EP_4 agonist) at 10^{-6} M. Moreover, PGE_2 at 10^{-6} M for 3 h decreased expression of mRNA for microsomal prostaglandin E synthase-2, and did not affect expression of mRNA levels for cyclooxygenase-1 or cytosolic prostaglandin E synthase, which are also involved in PGE_2 synthase.

Therefore, activation of EP_2 receptor results in the increase of COX-2 and mPGES-1 mRNA levels in microglia.

Roles for microglial N-type Ca^{2+} channel in aging-related enhanced neuroinflammation

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Voltage-dependent calcium channel (VDCC) is generally known to be functional only in excitable cells. However, we have reported recently that N-type VDCC (Cav2.2) could become functional in non-excitable cells under pathological conditions. In the present study, we have demonstrated that Cav2.2 channels are also functional in physiological microglial activation process. MG6 cells, a mouse microglial cell line, showed an enhanced neuroprotective M2 transition in the presence of a Cav2.2 blocker but no changes in the efficacy of the neuroinflammatory M1 transition. Treatment with a specific blocker of hypoxia inducible factor 2 (HIF-2) completely abolished this enhancement, suggesting an involvement of HIF-2 in this process. It is known that enhanced neuroinflammation occurs in aging brains. And we found that the efficacy of microglial M1 transition was enhanced but that M2 transition was reduced by aging in primary culture experiments. Interestingly, blockade of microglial Cav2.2 expression restored this aging-dependent reduction of microglial M2 transition and reduced the aging-induced exaggerated cytokine response, as revealed by a fast recovery from depressive-like behaviors in microglia-specific Cav2.2 deficient mice. These results suggest a critical role for microglial Cav2.2 channel in the aging-related neuroinflammation.

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Dysfunction of microglia in hyperglycemia

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Microglia play important roles in maintaining brain homeostasis. Dysfunction of microglia is implicated in various neurological disorders. Recent studies have shown that microglia are also involved in the metabolic diseases. In hyperlipidemia, long chain fatty acids induced microglial activation, resulting in exacerbation of the disease. However, the involvement of hyperglycemia in microglial functions has not been clarified.

First, we investigated the effect of high glucose on primary mouse microglia. Chronic high glucose treatment (2-3 weeks) increased inflammatory cytokine levels such as IL-1β and TNFα, and decreased microglial phagocytosis and migration, indicating that chronic exposure of high glucose led to dysregulation of microglial function. Then, we examined the impact of chronic hyperglycemia on microglia using streptozotocin-induced diabetic model mice. IL-1β and TNFα expressions in microglia from diabetic mice were increased compared with control mice. Minocycline, a microglial inhibitor, decreased the expressions of these cytokines. Minocycline intervened in diabetic mice also decreased food intake and blood glucose level with the enhanced expression of proopiomelanocortin which inhibited appetite.

Our study might demonstrate that chronic hyperglycemia activated microglia and induced hypothalamic inflammation, leading to the stimulation of appetite and the exacerbation of hyperglycemia. Abnormally activated microglia in diabetic conditions might be one of the therapeutic targets for hyperglycemia.

TLR4-activated p38 and NF-κB and GM-CSF receptor-mediated JAK2/STAT5 pathways are important for microglial long-term survival

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We have previously reported that the activation of Toll-like receptor 4 (TLR4) by lipopolysaccharide (LPS) induced rapid death of primary cultured rat microglia. However, a subpopulation of microglia survived much longer than two days, in which time all control cells had died. These surviving microglia may have neuroprotective functions because the neurons remained viable in co-cultures with these microglia. Moreover, the LPS-stimulated microglia may produce GM-CSF to promote survival. However, signaling mechanism of TLR4-mediated microglial long-term survival remains unknown. Therefore, in this study, we investigated TLR4 signaling pathways that control microglial survival, focusing on p38 MAP kinase and NF-κB, which are known to be important for innate immune response and control of apoptosis. Furthermore, we examined the involvement of GM-CSF receptor downstream signaling intermediates, JAK2 and STAT5, which are known to regulate the transcription of survival genes. LPS stimulation resulted in the phosphorylation of p38 MAP kinase, NF-κB and STAT5 in primary rat microglia. Moreover, a p38 MAP kinase inhibitor, SB202190, and a NF-κB inhibitor, BAY11-7082, suppressed LPS-stimulated microglial survival. Inhibition of JAK2 by NVP-BSK805 also inhibited the survival of these microglia. These results suggest that p38 and/or NF-κB pathways may play important roles in TLR4-mediated microglial survival. Furthermore, microglia-producing GM-CSF may activate cytoprotective JAK2/STAT5 signals to support their survival.

Involvement of EPAC and TPL2 in IL-1β production in microglial cells following activation of β-adrenergic receptors

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Endogenous noradrenaline (NA) has multiple bioactive functions and, in the central nervous system (CNS), has been implicated in modulating neuroinflammation via β-adrenergic receptors (β-ARs). Microglia, resident macrophages in the CNS, have a central role in the brain immune system and have been reported to be activated by NA. However, intracellular signaling mechanisms of the AR-mediated proinflammatory responses of microglia are not fully understood. Using a rapid and stable *in vitro* reporter assay system to evaluate IL-1β production in microglial BV2 cells, we found that NA and the β-AR agonist isoproterenol upregulated the IL-1β reporter activity. This effect was suppressed by β-AR antagonists. We further examined the involvement of EPAC (exchange protein directly activated by cAMP) and TPL2 (tumor progression locus 2, MAP3K8) and found that inhibitors for EPAC and TPL2 reduced AR agonist-induced-IL-1β reporter activity. These inhibitors also suppressed NA-induced endogenous *Il1b* mRNA and IL-1β protein. Our results suggest that EPAC and TPL2 are involved in β-AR-mediated IL-1β production in microglial cells, and extend our understanding of its intracellular signaling mechanism.

Activation of toll-like receptor 4 induces downregulation of sigma-1 receptor in microglia

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Background: Inflammatory responses could be involved in induction of neurodegenerative diseases. Microglia are known to act as the main immune cell in the central nervous system, and contribute to regulate inflammatory reactions in the brain. The activation of sigma 1 receptor (Sig1R) in microglia is neuroprotective, while microglial Sig1R expression is reduced in the brains of patients with neurodegenerative diseases such as Alzheimer's disease and Parkinson's disease. The current study has investigated mechanisms underlying downregulation of microglial Sig1R expression by activation of toll-like receptor 4 (TLR4), which plays an important role in inflammatory responses.
Method: Primary cultured microglia were prepared from the cortex of neonatal Wistar rats. Expression levels of mRNA or protein were measured by the real-time PCR or Western blot, respectively.
Results: TLR4 activation by ☒lipopolysaccharide(LPS), an agonist of TLR4, significantly reduced the expression level of microglial Sig1R in dose and time dependent manner. Inhibition of p38 mitogen-activated protein kinases (p38 MAPK) and histone deacetylase (HDAC) restored decrease of Sig1R mRNA levels. Among HDACs, HDAC6 was specifically involved in the LPS-induced downregulation of Sig1R.
Conclusions: The current study indicates that the expression level of Sig1R in microglia is regulated via p38 MAPK and HDAC6 under inflammatory conditions.

$P2Y_2$ receptor is involved in upregulation of phagocytic receptor AXL tyrosine kinase in TLR4-activated microglia

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Microglia are professional phagocytes which play an important role in homeostasis maintenance in the central nervous system. These cells rapidly detect and remove apoptotic cells which expose phosphatidylserine (PtdSer) as the key "eat-me" signal on the cell surface. This process is largely mediated through TAM receptor tyrosine kinases, MER and AXL, and TAM ligands which are soluble bridging proteins binding to PtdSer. We have previously reported that LPS-stimulated microglia promoted dying cell phagocytosis via purinergic $P2Y_2$ receptor signaling. However, the mechanism underlying $P2Y_2$ receptor-mediated phagocytosis remains unknown. In this study, we examined the involvement of $P2Y_2$ receptor in the regulation of TAM receptor MER and AXL expression in LPS-stimulated microglia. In primary rat microglia, LPS stimulation decreased MER and increased AXL mRNA expression, indicating that MER and AXL play distinct roles in microglial phagocytosis depending on physiological and inflammatory conditions. Furthermore, AR-C118925, a selective $P2Y_2$ receptor antagonist, significantly suppressed LPS-induced AXL mRNA expression. These results suggest that $P2Y_2$ receptor may be implicated in dying cell phagocytosis at least through mediating up-regulation of phagocytic receptor AXL tyrosine kinase in TLR4-activated microglia.

Expression of nucleoside transporters and hydrogen peroxide-induced thymidine incorporation in astrocytes

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We have found that cultured differentiated astrocytes pretreated with *N*^{6}, *2'-O*-dibutyryladenosine 3',5'-cyclic monophosphate (DBcAMP), a permeable analogue of cAMP, incorporate thymidine, but not uridine, via nucleoside transporters into TCA insoluble fraction for repair on DNA injury in the presence of hydrogen peroxide (H_2O_2) at an early time, and these phenomena are specific in differentiated astrocytes, but not undifferentiated astrocytes and neurons.

We studied expression of nucleoside transporters in cultured astrocytes by RT-PCR, western blot analysis and immunocytochemistry. We could confirm CNT2, that is pyrimidine selective nucleoside transporter, CNT3, that is non-selective nucleoside transporter, ENT1, that is hypersensitive nucleoside transporter, and ENT2, that is low-sensitive nucleoside transporter, but not CNT1, that is purine selective nucleoside transporter and confirmed non-presence in brain, in cultured astrocytes.

These results indicate that H_2O_2-induced thymidine incorporation could pass through specific nucleoside transporters, existed in cultured astrocytes.

Fibroblast growth factor 2 modulates purine metabolic enzymes through MAPK pathway in rat spinal cord astrocytes

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Extracellular adenosine (ADO) is mainly produced by metabolism of ATP released from astrocytes in the central nervous system and mediates neuroprotective effect under pathological conditions. In the previous study, we showed that fibroblast growth factor 2 (FGF2) upregulates purine metabolic enzymes, ecto-5'-nucleotidase (CD73) and adenosine deaminase (ADA), in rat spinal cord astrocytes. In this study, we investigated the intracellular signaling pathway of CD73 and ADA modulation by FGF2 in astrocytes.

Cultured astrocytes isolated from rat spinal cord were treated with FGF2. Enzymatic activity for purine metabolism was measured by incubation with artificial cerebrospinal fluid containing AMP or ADO and measurement of those metabolites with high-performance liquid chromatography. The expressions of CD73 and ADA were measured by western blotting.

In cultured astrocytes, FGF2 increased the expression and activity of CD73 and ADA in a concentration- and time-dependent manner. An FGF receptor inhibitor, SU5402 (5 μM), inhibited the increase in the expression and activity of CD73 and ADA by FGF2. FGF2 increased the phosphorylation of ERK and JNK, which is inhibited by SU5402 and MAPK inhibitors. In addition, U0126 (10 μM), a MEK inhibitor, also inhibited the increase in the expression and activity of CD73 and ADA.

These results indicate that FGF2 upregulates the expression and activity of CD73 and ADA through FGF receptor/MAPK pathway. Furthermore, it is suggested that mainly MEK/ERK pathway contribute to the upregulation of CD73 and ADA.

Layer-specific transcriptome analysis of microglia in the medial prefrontal cortex

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Recent studies in mice started to reveal brain region-specific transcriptome and epigenome profiles of microglia. The heterogeneity of microglia might even exist across different layers of the cerebral cortex, since microglia are regulated by various receptors for neurotransmitters that are distributed preferentially in specific layers of the cerebral cortex. We also suspect the layer-dependent heterogeneity of microglia in the medial prefrontal cortex (mPFC), since repeated social defeat stress (R-SDS) induces microglial activation in the mPFC, leading to dendritic shrinkage of pyramidal neurons preferentially in its superficial layer. However, the heterogeneity of microglia across different cortical layers has not been elucidated, perhaps due to technical difficulty. To address this issue, here we developed a technique based on laser microdissection microscopy to perform transcriptome analyses with RNA-seq for microglia in the superficial and deep layers of the mPFC. Our preliminary findings suggest that mPFC microglia in adult mouse brains show layer-specific gene expression profiles even under the resting condition. We are currently investigating whether and how R-SDS affects layer-specific gene expression profiles of mPFC microglia.

Computer chemistry of non-peptide oxytocin agonists.

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Oxytocin is a 9-amino acid peptide that is neurosecreted from the paraventricular nucleus of the hypothalamus and the supraoptic nucleus and enters the blood from the posterior lobe of the pituitary gland. Peripheral effects are smooth muscle contraction and lactation. One kind of oxytocin receptor is Gq of 7-transmembrane G protein. There are three types of Vasopressin, V1, V2, and V3, V1 and V3 are G protein phosphorylated inositol / Ca systems, and V2 is a cAMP system.

Oxytocin nasal spray administration reduces runaway, sting, fear and anxiety. A therapeutic effect on Autistic Spectrum Disorder can be expected. This is why non-peptide compounds of oxytocin are expected. There are commercially available non-peptide oxytocin drugs. We used known data of D.M. Ashworth etal. An analysis by computational chemistry was attempted using the data of the structure-activity relationship report published by them.

The method optimizes the chemical structure of the compound with MOPAC6 in Winmostar and calculates the molecular structure and physicochemical indices. The number of compounds in the paper is 27, but EC50 is measured for 19 from No.9 to No.27. Multivariate analysis , when the dependent variable is EC50 and the independet variable is a physicochemical index in multiple regression analysis (decrease-increase method), the probability p = 0.0182 * is significant.

EC50 = -25.4 * (all E) + 9234.8 * (ionization E) -1473.5 * (field number) -94437.1

Multiple correlation coefficient R = 0.6912

In the molecular image, the frontier electrons of highly active molecules gather in a diazepine ring, and the UV spectrum absorption band is on the shorter wavelength side of 180 nm. In the low activity molecule, the frontier electrons were dispersed in the side chain, and the UV spectrum was shifted to the longer wavelength side of 190 nm. Other calculations method were also interesting results.

Psychiatric-disorder-related behavioral phenotypes and cortical hyperactivity in a mouse model of 3q29 deletion syndrome

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The 3q29 microdeletion is a rare recurrent copy number variant (CNV) leading to an increased risk for neurodevelopmental disorders, such as intellectual disability and autism spectrum disorder (ASD), and a >40-fold increased risk for schizophrenia. However, the neurobiological basis for 3q29 deletion syndrome is currently unknown. In order to investigate the biological changes induced by the microdeletion, we generated a mouse model of human 3q29 deletion syndrome by deleting the orthologous region. 3q29 deletion (Df/+) mice showed reduced body and brain weight. Importantly, Df/+ mice showed deficits in social interaction and prepulse inhibition, which are reminiscent of the phenotypes in patients with 3q29 deletion syndrome. By unbiasedly analyzing the whole-brain neural activity, we found that neuronal activity was abnormally activated in a restricted region of the cortex of Df/+ mice. Furthermore, we found that the expression levels of immediate early genes were increased and that the number of parvalbumin positive neurons was decreased in the cortex of Df/+ mice. Our results suggest that Df/+ mice may provide important clues for understanding the disease-causative molecular and cellular pathology of psychiatric disorders.

The role of prostaglandin E_2 in environmental factors of psychiatric disorders

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We investigated the possibility of prostaglandin E_2 (PGE_2) as one of common molecules associated with vulnerability to neurodevelopmental disruptions induced by environmental factors. PGE_2 levels in whole brain were significantly increased after exposure to viral infection [injection of polyinosinic-polycytidylic acid (polyI:C)], hypoxia (exposure to CO_2), and neglect (separation from the dams) in postnatal day (PD) 2, compared to those after non-exposure. The mice administered polyI:C during PD 2-6 exhibited the impairment of sociality, object recognition memory, and pre-pulse inhibition (PPI) in adult at PD 70, and further, significant decreased spine density of the mPFC in adult mice. Exposure to CO_2 at PD 2 and separation from dams during PD 2-21 exhibited the impairment of PPI and decrease of spine density in adult mice. These behavioral impairments induced by administration of polyI:C were recovered by an inhibition of PGE_2-EP1 (PGE_2 receptor subtype) and of cyclooxygenase (COX). Our findings suggest that PGE_2 is one of potential common molecules associated with vulnerability to neurodevelopmental disruptions induced by environmental factors, and PGE_2 plays a crucial role in the development of behavioral and neuronal impairments, which are associated with activation of PGE_2-EP1.

Nox1 deficiency alleviated behavioral abnormalities in obsessive-compulsive disorder model mice via inhibition of D_2 receptor/β-arrestin pathway-mediated synaptic facilitation

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Obsessive-compulsive disorder (OCD) is a neuropsychiatric disorder characterized by repeated rising concern (obsessions) and repetitive behaviors to get rid of obsessions (compulsions). Considering the high rate of treatment-refractory patients, novel therapeutic strategies are strongly awaited. Recent clinical researches indicate that antioxidants, such as N-acetylcysteine, enhance treatment response. However, pathological roles of reactive oxygen species (ROS) and the mechanisms of action of antioxidants are poorly understood. In this study, we showed that mRNA expression of NOX1, the catalytic subunit of NADPH oxidase, was significantly increased in the striatum of OCD model mice. *Nox1* deficiency or pharmacological inhibition of NOX1 alleviated OCD-like behavioral abnormalities. *Nox1* deficiency also suppressed D_2 receptor/β-arrestin pathway-mediated synaptic facilitation in the indirect pathway medium spiny neurons (iMSNs) in the central part of the striatum (CS) shown in OCD model mice. These results suggest that NOX1-derived ROS enhance synaptic facilitation in the CS iMSNs via modulation of the D_2 receptor/β-arrestin pathway, leading to OCD-like behavioral abnormalities.

Alteration of parvalbumin expression and perineuronal nets formation in the anterior cingulate cortex of *Fabp3* KO mice

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Polyunsaturated fatty acids (PUFAs) are essential for brain development and function. Increasing evidence has shown that an imbalance of PUFAs is associated with various human psychiatric disorders, including autism and schizophrenia. Fatty acid-binding proteins (FABPs), cellular chaperones of PUFAs, are involved in their intracellular trafficking, signal transduction, and gene transcription. Previously, we showed that FABP3 is strongly expressed in the parvalbumin-expressing interneurons (PV neurons) of the mouse anterior cingulate cortex (ACC), which is a component of the limbic cortex and is important for the coordination of cognitive and emotional behaviors. In addition, FABP3 regulates GABA synthesis through transcriptional regulation of *Gad67* in the ACC and that methionine restores normal *Gad67* expression and behaviors in *Fabp3* knockout (KO) mice. In this study, we analyzed the density and the percentage of PV neurons surrounded by perineuronal nets (PNNs) in the ACC of adult *Fabp3* KO mice. PNNs are key components of extracellular matrix that enwrapping PV neurons and regulate synaptic plasticity. PV density increased in the ACC of adult *Fabp3* KO mice, whereas the number of PV-neurons remained unchanged. The density of PNN and the number of PNN-positive PV neurons were significantly increased in the ACC of adult *Fabp3* KO mice. These findings demonstrate that FABP3 is involved in the control of expression of PV and formation of PNNs in the ACC, thus suggesting the importance of PUFA homeostasis in the ACC for maturation of PV neurons.

Yokukansan and keishito ameliorate ASD-like sociability deficits in ovariectomized mice

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Autism spectrum disorder (ASD) is a neurodevelopmental disorder with core symptoms of sociability deficit. We focus on allopregnanolone (ALLO) which is a neurosteroidal positive modulator of the $GABA_A$ receptor. Our previous findings showed that post-weaning social isolation rearing (SI) of male mice induces ASD-like sociability deficits, and these abnormalities are in part due to SI-induced decreases in ALLO contents in the brain. It is also demonstrated that yokukansan (YKS) and keishito (KST), traditional herbal medicines, improved sociability deficits in SI mice. Moreover, we reported that dissection of ovary (OVX) of female mice also induced ASD-like sociability deficits. This study investigated the effects of YKS and KST on the ASD-like behavioral abnormalities of OVX mice. The ovariectomy was conducted on 6-week-old mice. The administration of YKS and KST was started 1 week after surgical operation. After finishing behavioral tests, the ALLO contents in cortex were measured by ELISA. YKS and KST improved the ASD-like sociability deficits induced by OVX without restoring brain ALLO levels, similar to the results of SI mice. These results suggest that YKS and KST are effective for ASD treatment. Currently, we are analyzing the mechanism of YKS and KST for improving sociability deficits.

Involvement of hippocampal alpha2A-adrenoceptors in impulsive-like behaviors induced by Intermittent sleep deprivation in mice

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Attention deficit/hyperactivity disorder (ADHD) is a neurodevelopmental disorder characterized by inattention, hyperactivity, and impulsivity. In this study, we investigated whether intermittent sleep deprivation (SD) caused changes in impulsive-like behaviors and expression levels of alpha2A-adrenoceptors (alpha2A-R) in the hippocampus (HP) and frontal cortex of mice using an elevated plus maze (EPM) test. Mice were deprived of REM sleep intermittently by using the platform method (20 h/day) for 3 days. The % of time spent in the open arm (TOA) and alpha2A-R expression in HP were significantly increased and decreased, respectively, by SD. The increase in the % of TOA was significantly improved by oxymetazoline (OXY, an alpha2A-R agonist), methylphenidate, and atomoxetine, which are clinically used to treat ADHD symptoms. Moreover, these positive effects of OXY were attenuated by yohimbine a selective alpha2-R antagonist and BRL44408 a selective alpha2A-R antagonist. These results suggest that the increase in the % of TOA induced by SD may serve as a model of the impulsivity-like behavior in ADHD. Furthermore, the SD eliciting impulsive behaviors may be linked to alpha2A-R signaling, and as indicated by a decrease in alpha2A-R, particularly in the mouse HP of mice.

Changes in prefrontal cortical myelination in olfactory bulbectomized mice is associated with depressive-like behavior

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Recent study has reported that demyelination is associated with the development of depression. Olfactory bulbectomized (OBX) rodents are a useful experimental animal model for depressive disorder. However, it remains unclear about the change in myelination in the brain of OBX mice. To address this question, we determined quantity of myelin-associated protein such as myelin basic protein (MBP), myelin proteolipid protein, myelin-associated glycoprotein (MAG), myelin-associated oligodendrocyte basic protein, myelin oligodendrocyte glycoprotein and cyclicnucleotide phosphodiesterase (CNPase) in the prefrontal cortex (PFC) and dorsal hippocampus 2 or 3 weeks after surgery when OBX mice begin to exhibit depressive-like behaviors. Then, we also investigated the association with depressive-like behavior and changes in myelin-associated protein. OBX mice showed depressive-like behavior in the tail-suspension test and decreases in MBP, MAG and CNPase in the PFC, but not hippocampus 3 weeks after surgery. Furthermore, linear regression analysis revealed the significant correlations between the changes in prefrontal cortical myelin-associated protein (MBP, MAG and CNPase) and the immobility time 3 weeks after surgery. These findings indicate that OBX-induced demyelination in the PFC is associated with depressive-like behavior.

Differential effects of ketamine metabolites on depression-like behaviors induced by chronic corticosterone injection in mice

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Clinical and preclinical studies have shown that the NMDA receptor antagonist ketamine exerts rapid and long-lasting antidepressant effects. Although ketamine metabolites might also have potential antidepressant properties, controversial results have been reported on (*2R,6R*)-hydroxynorketamine ((*2R,6R*)-HNK) in particular and there is little information on the effects of other ketamine metabolites. Here we aimed to compare the effects of (*R*)-norketamine ((*R*)-NK), (*S*)-NK, (*2R,6R*)-HNK and (*2S,6S*)-HNK in a mouse model of depression induced by chronic corticosterone (CORT) injection. None of these ketamine metabolites at doses up to 20 mg/kg showed antidepressant-like activity in naïve male C57BL6/J mice. Chronic CORT treatment increased immobility in the forced swim test and caused anhedonic-like behaviors in the female encounter test. A single administration of (*R*)-ketamine, but not an SSRI fluoxetine, showed antidepressant-like activity in chronic CORT-treated mice. (*S*)-NK and (*2S,6S*)-HNK dose-dependently reduced the increased immobility at 30 min after injection, while (*R*)-NK or (*2R,6R*)-HNK did not. Additionally, (*S*)-NK and (*2S,6S*)-HNK improved anhedonic-like behaviors at 24 h after injection. These results suggest that (*S*)-ketamine metabolites (*S*)-NK and (*2S,6S*)-HNK have potent acute and sustained antidepressant effects.

The claustrum neuronal ensembles that were activated by social defeat stress mediate anxiety-like behavior through regulating the activation of multiple stress-responsive nuclei

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Acute mental stress induces negative emotional states including anxiety. However, the neuronal mechanisms underlying stress responses remain unknown. Recently, we found that the neuronal activation in the claustrum prominently differs between control mice and mice received stress. Here we examined the role of the claustrum neuronal ensembles that were activated by social defeat stress in anxiety-like behavior and the underlying neuronal networks. We bilaterally co-injected adeno-associated virus vectors expressing the tamoxifen-dependent recombinase $^{ERT2}Cre^{ERT2}$ and Cre-dependent DREADDs into the claustrum. Chemogenetic re-activation of DREADDs-tagged claustrum neurons increased the anxiety-like behaviors and induced neuronal activations of anxiety-related nuclei including the amygdala. Chemogenetic inhibition of these neurons suppressed stress-induced anxiety-like behaviors and prevented neuronal activation by the defeat stress. In addition, the claustral neurons received innervation from stress-responsive neurons in the amygdala and the medial prefrontal cortex. The stress-responsive claustral neurons projects to the cortices including the medial prefrontal cortex. These results suggest that the claustrum neuronal ensembles regulate stress-induced anxiety through orchestrating activations in the multiple brain nuclei.

Involvement of serotonergic system in the antidepressant-like effect of a novel curcumin derivative CUD003 in mice

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Curcumin, a natural polyphenol compound which is contained in turmeric (Curcuma longa), has been reported to exert antidepressant-like effect in various animal models of depression. In this study, we investigated the antidepressant-like effect of a novel synthetic derivate of curcumin CUD003 and the possible mechanism of its action in mice using behavioral tests. Treatment with CUD003 (3 mg/kg, p.o.) decreased the immobility time in the forced swim test (FST) without affecting locomotor activity in the open field test. CUD003 was more effective than curcumin in the FST. The antidepressant-like effect of CUD003 was abolished by the pretreatment with p-chlorophenylalanine (a tryptophan hydroxylase inhibitor), WAY-100635 (a selective 5-HT_{1A} antagonist), ketanserin (a 5-$HT_{2A/C}$ antagonist), or ondansetron (a selective 5-HT_3 antagonist). Moreover, social defeat stress-induced depressive behaviors as evidenced by an increased immobility time in the FST, and a decreased grooming time in the sucrose splash test were attenuated by the treatment of CUD003. These results suggest that CUD003 has more potent antidepressant-like effect than curcumin, which may be mediated by the serotonergic signaling through 5-HT_{1A}, 5-$HT_{2A/2C}$ and 5-HT_3 receptors.

Activating transcription factor 4(ATF4)-dependent activation of the human tryptophan hydroxylase 2 gene is mediated through binding to a CCAAT-enhancer-binding protein (CEBP)-ATF composite site in its promoter

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Tryptophan hydroxylase 2(TPH2) plays a critical role in the regulation of 5-HT neurotransmission and is thus a promising therapeutic target for the treatment of neuropsychiatric disorders. Activating transcription factor 4 (ATF4) has been implicated in various neural functions. ATF4 can form a homodimer, and heterodimers with CCAAT-enhancer-binding proteins (CEBPs). ATF4-CEBP heterodimers bind to DNA sequences called CEBP-ATF composite site to regulate target gene expression. Bioinformatics analysis revealed one potential CEBP-ATF composite site near the transcription start site of the hTPH2 gene. In this study, we examined how the hTPH2 promoter activity changes by ATF4 and CEBPs. Promoter activities were assessed by transfections of reporter plasmids containing a 2-kb of the hTPH2 promoter into RN46A cells. Overexpression studies demonstrated that ATF4-mediated activation of the hTPH2 promoter was further enhanced by co-expression of each of the five CEBPs including CEBPG which lacks all known activation domains. The CEBP-ATF composite site mutations negated the effects of ATF4. A dominant negative ATF4 blocked the effects of ATF4. Functional analysis of N-terminal and internal deletion mutants indicated that ATF4 (aa 1-124) is critical for activation. Moreover, co-expression of endogenous inhibitor proteins, Trib3 or TXLNG attenuated the effects of ATF4. Altogether, these results imply that ATF4 plays a pivotal role in regulating the hTPH2 gene expression, and itself undergoes complex regulation at multiple levels.

The contribution of the NMDA receptors in the bed nucleus of the stria terminalis for the induction of depressive-like behaviors in mice

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The synaptic plasticity in the bed nucleus of stria terminalis (BNST) is induced by the activation of α_1 and β-adrenergic receptors, and/or NMDA receptors. We previously reported the possibility that the synaptic plasticity in BNST contributes to the induction of depressive-like behavior, because the α_1 and β-receptors in BNST regulated the learned despairs in mice. However, neither α_1 nor β-receptors in BNST affected the lipopolysaccharide (LPS)-induced behavioral despair in mice. Therefore, we investigated whether the NMDA receptors in BNST contribute to the induction of LPS-induced behavioral despair. The bilateral intra-BNST pretreatment of MK-801, a NMDA receptor antagonist, 30 min prior to LPS injection, decreased the immobility time during tail suspension test (TST) 24 hours after the LPS challenging. In consistent, bilateral intra-BNST injection of NMDA (24 mg/125 nl/side) slightly shortened the immobility time during TST. However, bilateral intra-BNST co-injection of muscimol (75 ng/125 nl/side), a $GABA_A$ receptor agonist, with NMDA potently decreased the immobility time during TST. Because this dose of muscimol alone did not affect the immobility time of TST, in the present study, we suggest that the activation of NMDA receptors with $GABA_A$ receptors in BNST is important for the induction of depressive-like behavior.

Overgeneralization of context fear memory promotes the despair behavior in PTSD-considered model mouse

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Re-experiencing trauma by overgeneralization is a hallmark of PTSD which is high comorbidity with depression. We thus studied the relation from overgeneralization to depression. Mice were subjected to context A with electric shock as a conditioning which was followed by unconditioned context B using a different, but similar box in 3h on Day1 and was followed by re-exposure to context A (Group ABA) or B (Group ABB) on Day2. Group ABA and ABB mice exhibited the longer freezing in context A and B on Day2. However, the mice which was not exposed to context B on Day1 (Group A-B) showed the shorter freezing time in context B on Day2, indicating that the overgeneralization was induced by experiencing the context B after the conditioning in 3 h. Group ABB mice exhibited the longer immobility time of tail suspension test (TST) than Group ABA and A-B. Freezing time in the first half of the test in context B and immobility time of TST in Group ABB were negatively correlated with the staying time of center zone in the box during the context B on Day1. In contrast, the freezing and immobility time of TST in Group ABA have positive correlation with the time of center zone during context B on Day1. These results suggest that the PTSD and depression may depend on the coping style during unconditioned context within a few hours after the trauma.

The effect of salt intake on the defensive strategies against inescapable innate fear and fear stress-associated learned despair in mice

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A component of fox faces, 2,4,5-trimethylthiazoline (TMT) induced innate fear of mice produced active escape behaviors in inescapable box is followed by freezing as passive escape behavior. The duration of active escape behavior is regulated by the neurons releasing the corticotropin-releasing hormone from the paraventricular nucleus of hypothalamus to the bed nucleus of the stria terminalis. Recently we found that the 2% salt intake for 5 days decreased the duration of TMT-induced active escape behaviors and increased the duration of freezing time in inescapable acryl box (30 cm^3). Against our expectation, the mice with 2% salt intake exhibiting shorter duration of active escape behaviors delayed the induction of learned despair during tail suspension test (TST) and decrease in the duration of the immobility time during TST, because the paradigm of innate fear-induced inescapable behaviors from active to passive coping strategies is similar with that of TST. Excessive daily salt intake is the risk for the high blood pressure, myocardial infarction and stomach cancer etc. In the present study, we suggest that the tasting the adequate amount of salt, but not excessive, may decide the coping style to adapt the psychological stresses and benefit for preventing the despair behavior as enhancing the resilience against daily stresses.

Effects of sunitinib perfusion on vascular function in the rat mesenteric arteries.

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Background: Sunitinib, the multikinase inhibitors (MKIs) is used extensively for treatment of human tumors. It has become a clinical problem with sunitinib-induced hypertension, which is one of most common adverse effects related to MKI treatment. Our previous study have investigated that acute treatment of sunitinib perfusion in the rat mesenteric arteries significantly promoted both vasoconstrictor and vasodilator responses to periarterial nerve stimulation (PNS) and vasoactive agents. However, the detailed mechanisms have been unknown. The aim of this study is to demonstrate the mechanism of increased vascular function caused by sunitinib.

Methods: The mesenteric vascular beds were isolated from pentobarbital-anesthetized rats and perfused with Krebs solution at a constant flow rate of 5 ml/min with a peristaltic pump. Changes in perfusion pressure were measured with a pressure transducer.

Results: In rat mesenteric vascular beds treated with sodium deoxycholate (SD) to remove vascular endothelial cells with active tone, acetylcholine (ACh)-induced vasodilation was markedly inhibited. PNS (1 to 4 Hz) and sodium nitroprusside (SNP) increased adrenergic nerve-mediated vasoconstriction and vasodilation respectively, compared with that of control group. Sunitinib (1 nM) perfusion decreased PNS-induced vasoconstriction, which is inhibited by ruthenium red (1 uM), the transient receptor potential channel Vanilloid 1 (TRPV1) channel blocker, or capsaicin (1 uM) treatment. However, ACh, CGRP, SNP and PNS-induced calcitonin gene-related peptide (CGRP) ergic vasodilations had no change, compare with that of SD-treated group.

Conclusion: These results suggest that sunitinib has facilitatory action on CGRPergic nerve mediated by TRPV1.

Inducible COX-2 expression is regulated by the ARE-binding proteins in inflammatory response in satellite glial cells.

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Satellite glial cells (SGCs) related to primary sensory neurons are altered structurally and functionally under neuroinflammatory conditions. In neuroinflammation expression of cyclooxygenase-2 (COX-2) in peri-sensory neurons results in the production of prostanoids, which affects sensory neuronal activity and responsiveness and causes hyperalgesia. we have shown the facilitated expression of COX-2 by proinflammatory mediators in cultured dorsal root ganglion (DRG) cells. To evaluate the regulatory system of COX-2 expression in the specific cells we investigated the mechanisms using cultured satellite glial cells (cSGCs).

The cSGCs were cultured by dispersing the isolated rat DRG cells and separated by Percoll density gradient centrifugation. mRNA levels were identified with RT-Real time PCR and protein levels were analyzed with Western blotting.

Synergistic expression of COX-2 by interleukin-1beta and bradykinin was observed in the cSGCs. And then the COX -2 transcriptional activities was just increased in an additive manner by a COX-2 promoter luciferase assay. Thus the post-transcriptional regulations might be involved in the COX-2 mRNA levels. Immunoprecipitated HuR, an RNA-binding protein, in the cSCGs contained more COX-2 mRNA than that of the control.

The aberrant control of COX-2 mRNA turnover in SGCs may be implicated in diseases including chronic neuroinflammation, which results in inflammation-derived hyperalgesia occurred around primary sensory neurons.

Dorsal Root Ganglia Homeobox (DRGX) in the DRG neurons is involved in neuropathic pain

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Neuropathic pain is caused by lesions or diseases of the somatosensory system and is less responsive to pain medications. Transcriptomic changes in dorsal root ganglion (DRG) neurons are involved in initiation and maintenance of neuropathic pain. Dorsal Root Ganglia Homeobox (DRGX) is a paired-like homeodomain transcription factor crucial for the development of nociceptive DRG neurons. However, roles for DRGX after development are almost unclarified. Here, we report that DRGX downregulation in DRG neurons due to nerve injury in the post-developmental stage is involved in neuropathic pain in rats. DRGX expression was persistently decreased in DRG neurons in neuropathic pain model rats produced by spinal nerve ligation. DRGX protein was mainly downregulated in nuclei of small-to-medium DRG neurons after the nerve injury. Additionally, DRGX downregulation using an adeno-associated viral vector expressing short hairpin RNA induced mechanical allodynia and thermal hyperalgesia in intact rats, while DRGX overexpression suppressed neuropathic pain. DRGX regulated mRNA expression of matrix metalloproteinase-9 and prostaglandin E receptor 2 in the DRG. These results suggest that DRGX downregulation after development contributes to neuropathic pain through transcriptional modulation of pain-related genes in DRG neurons.

Effects of silybin, flavonolignans, on catecholamine secretion and tyrosine hydroxylase activity in cultured bovine adrenal medullary cells

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Silymarin, a complex of flavonolignans derived from the seeds of the milk thistle (*Silybum marianum*), is a traditional drug and food supplement employed for numerous liver disorders. Silymarin contains mainly silybin, silichristin, silidianin, isosilybin, and taxifolin. Silybin, the principal flavonoid contained in silymarin, showed antioxidant, anti-inflammatory and anticarcinogenic properties. Adrenal medullary cells, the neuroendocrine arm of the sympathetic nervous system, secrete catecholamine to mediate the physiological response to stress. This study was conducted to investigate the effects of silybin on catecholamine secretion and tyrosine hydroxylase activity in cultured bovine adrenal medullary cells to clarify the influence of silybin on a stress reaction. Silybin suppressed catecholamine secretion and $^{45}Ca^{2+}$ influx induced by acetylcholine (ACh), a physiological secretagogue and agonist of nicotinic ACh receptors, in a concentration dependent manner. Silybin had a little effect on catecholamine secretion induced by 56 mM K^+. Silybin also suppressed both basal and ACh-induced tyrosine hydroxylase activity. These findings suggest that silybin inhibits catecholamine secretion and tyrosine hydroxylase activity by suppression of nicotinic ACh receptor-ion channels in bovine adrenal medulla cells. Therefore, silybin may be reduce the activity of catecholamine system elevated by stress stimuli.

Establishment of high HPRT activity *Urat1-Uox* double knockout mouse and the effects of xanthine oxidoreductase inhibitor

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It is known that there are species differences in the purine metabolic system between humans and rodents (e.g. urate oxidase (Uox), and hypoxanthine phosphoribosyltransferase (HPRT), etc.). URAT1 (SLC22A12) is renal urate (UA) reabsorption transporter and the target for UA-lowering therapies. In humans, URAT1 deficiency has a significant UA-lowering effect (ULE), but not in *Urat1*-knokout (KO) mice. The aim of this study is the establishment and urate kinetic profiling of high HPRT activity *Urat1-Uox* double knockout (DKO) mice and the investigation of the effect of xanthine oxidoreductase inhibitor (XOI), topiroxostat in this model mice. Topiroxostat 1 mg/kg (Top) was administered to DKO mice for 7 days by feeding diet. Oxypurines (UA, hypoxanthine and xanthine) and creatinine in plasma and urine were measured by HPLC. DKO mice showed a significant decrease in plasma UA levels, increased fractional excretion of UA (FE_{UA}), and enhanced Top-induced ULEs, compared with *Uox*-KO only. Thus, high HPRT activity seems to be important for producing ULE by URAT1 inhibition. The combination therapy of URAT1 inhibition and XOI showed an effective ULEs, suggesting that it is useful for the treatment of hyperuricemia.

Inhibition of renal tubular cells by novel anti-HIV therapeutic agents and interaction with organic anion transporters

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[Background]EFdA (4'-ethynyl-2-fluoro-2'-deoxyadenosine) is a novel anti-HIV therapeutic agent. It has a unique structure and high antiviral activity, and is expected as a revolutionary new drug. TDF (Tenofovir disoproxil fumarate) and TAF (Tenofovir alafenamide) are nucleoside reverse transcriptase inhibitors that have already been clinically applied. TDF is known to cause renal damage after long-term administration. hOAT1 and hOAT3 are organic anion transporters expressed on the proximal tubule of the kidney and act as a cellular uptake pathway for the secretion of drugs. As one of the onset mechanisms of drug-induced renal damage, it is considered that drugs taken into proximal tubular cells via these transporters cause damage by accumulating in the cells. In this study, we investigated the effect of EFdA on renal tubular cells and the possibility of EFdA being taken into the cells via these transporters.

[Results] In the cell viability assay, EFdA, TDF, and TAF showed cell growth inhibition, after 72 hrs. However, no inhibition of substrate uptake by these compounds was observed in S2-hOAT1 cells and S2-hOAT3 cells.

[Discussion] EFdA can cause damage to proximal tubular cells to the same extent as TDF and TAF under pharmacological doses. However, other reports show that EFdA exhibits high antiviral activity at very low concentrations, and it is unlikely that the blood concentration will be high enough to cause kidney damage in clinical practice. It was considered that hOAT1 and hOAT3 are less involved in the damage of EFdA, TDF, and TAF to proximal tubular cells.

Screening of endogenous compound potentially applicable for estimation of effective renal plasma flow

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Renal plasma flow (RPF) is one of effective biomarkers for estimation of renal function. *p*-Aminohippuric acid (PAH) is clinically used to test effective RPF, but intravenous administration of PAH is required to use this biomarker. In addition, efficient renal secretion of this compound may not be suitable for renal failure patients. Thus, endogenous compound may be more practical for estimation of RPF, but has not yet been fully optimized. The aim of the present study is to identify candidate for endogenous RPF biomarker. PAH was intravenously infused in Sprague Dawley rats for 90 min, and plasma was collected from both femoral artery and renal vein at the end of the infusion. These samples were then subjected to untargeted metabolome analysis using LC-TOFMS to identify ions showing different intensity between circulating and renal venous plasma. In another experiment, renal arteries were ligated after start of the infusion, and plasma was collected at 60 and 90 min to pick up the ions showing increased peak intensity by the ligation. PAH and other 13 ions showed significantly higher peaks in circulating plasma and were increased by the ligation. Among them, *N*-methylnicotinamide and hippuric acid were identified, the former being previously used to estimate RPF in rats. Further studies are ongoing to validate them as RPF marker.

Repeated ischemia/reperfusion leads to acceleration of AKI to CKD progression in rats

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Ischemia/Reperfusion (I/R) injury contributes to acute kidney injury (AKI) and subsequent chronic kidney disease (CKD). I/R injury model by single clamping renal artery is usually used. However, the mortality is high if the renal damage is severer and the AKI to CKD transition is not occurred if the damage is mild. The aim of study is to produce the new model of AKI to CKD transition with high survival rate and high reproduce. The rats were divided four groups, sham, single I/R, twice I/R and three time I/R. Animals were anesthetized by medetomidine, midazolam and butorphanol and the left artery and vein were clamped for 45 minutes 2 weeks after contralateral nephrectomy. Animals were sacrificed third I/R or sham-operation after 8 weeks and we measured renal functional parameters. Rats of all groups were alive. Urinary excretion of protein was progressively increased in second and third I/R although the other renal functional parameters were not changed. There was not significant change of renal functional parameters in sham and single I/R. These findings suggest that repeated I/R leads to glomerular injury. In conclusion, repeated I/R-injury model is possibly useful for investigation of AKI to CKD transition.

Production of hydrogen sulfide in mammalian cells

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Hydrogen sulfide (H_2S) has been recognized as a signaling molecule as well as a cytoprotectant. H_2S modulates synaptic activity by enhancing the activity of *N*-methyl-D-aspartate receptors in neurons and by activating astrocytes that surround the synapse. It protects neurons from oxidative stress by recovering glutathione levels, scavenging ROS and suppressing intracellular Ca^{2+} concentrations. H_2S is known to be produced from L-cysteine by two pyridoxal 5'-phophate (PLP)-dependent enzymes, cystathionine beta-synthase (CBS) and cystathionine gamma-lyase (CSE). Recently, 3-mercaptopyruvate sulfurtransferase (3MST) has emerged as the third H_2S-producing enzyme. 3MST produces H_2S from 3-mercaptopyruvate (3MP), an achiral keto acid, which is generated by PLP-dependent cysteine aminotransferase (CAT) from L-cysteine and alpha-ketoglutarate. In addition to these enzymes, we found an additional pathway to produce H_2S from D-cysteine. D-Cysteine is metabolized by D-amino acid oxidase (DAO) to 3MP, which is a substrate for 3MST. Unlike the L-cysteine pathway, this D-cysteine pathway operates predominantly in the cerebellum and the kidney. The activity to produce H_2S from D-cysteine is greater than that from L-cysteine. Exploring sources of D-cysteine may lead to a new insight into the physiological role of H_2S.

Prenatal glucocorticoid administration accelerates kidney development in the fetal rats.

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The hypothesis of the "development origins of health and diseases" addresses the risk of chronic kidney disease in adulthood. Although prenatal glucocorticoid (GC) therapy has shown to prevent infant respiratory injury in the neonate, the effects of the kidney functions remains unknown. This study aimed to investigate whether prenatal GC administration is associated with fetal kidney maturation.

Dexamethasone (DEX) was administered to pregnant rats for 2 days on days 17 and 18 or days 19 and 20 of gestation, and the kidney tissues of 19- and 21-day fetuses and 1-day-old neonates were analyzed. The expression of kidney development-related markers (alpha-SMA, aquaporin1and 2) were evaluated by immunohistochemistry and glomeruli and tubules numbers were calculated by H-E staining in the fetal and neonatal rats with prenatal GC.

The expression of kidney development-related markers (alpha-SMA, aquaporin1and 2) were evaluated by immunohistochemistry and glomeruli and tubules numbers in the kidney tissue were measured by H-E staining in the fetal and neonatal rats with prenatal GC. In non-treated group, a kidney size in the neonate was significantly increased compared with that of the fetus. DEX did not changed a kidney size in the fetal and the neonate. The glomerular number was not changed by DEX, but tubular number was significantly increased in 19-day and 21-day fetal kidney tissues.

The expression of alpha–SMA which decrease with growth of mesangial cells was significantly decreased in the fetuses with DEX. Furthermore, DEX increased the expression of aquaporin 1 and 2 in tubuli of the fetal kidney.

In conclusion, these results indicate that prenatal GC administration may contribute to the development of glomeruli and tubules in the immature fetal rat.

Interaction of Osp94, a hypertonic stress sensitive molecular chaperone, with mRNAs of tau alternative splicing variants

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In our previous studies, we reported the decreased microtubule transport velocity and mRNA level of 3R-tau, an alternative splicing variants of tau, in molecular chaperone Osp94 (Osmotic stress protein 94 kD)-knocked down Neuro2a cells. To clarify the function of Osp94 in neuronal cells, we investigated an interaction of Osp94 with 4R- and 3R-tau mRNAs of tau alternative splicing variants. In Osp94-siRNA treated Neuro2a cells, 4R-/3R-tau mRNAs and its proteins expressions were analyzed by Real-time PCR and Western blotting, respectively. Additionally, RNA immunoprecipitation were used for demonstrating an interaction of Osp94 with 4R-and/or 3R-tau mRNAs. Real-time PCR and Western blotting revealed marked decreases in 3R-tau mRNA and protein in cells exposed to Osp94-siRNA for 72 h. RNA immunoprecipitation using anti-Osp94 antibody showed amplified PCR products derived from 4R-and 3R-tau mRNAs in nuclear and cytosol fractions. No obvious difference in the ratio of 4R-tau/3R-tau mRNAs bound to Osp94 protein was observed between nuclear and cytosol fractions. The present study indicates that molecular chaperon Osp94 binds to 4R-tau and 3R-tau mRNAs in nucleus and cytoplasm of Neuro2a cells.

Interaction of AGN-1 protein, which binds to protein phosphatase 6, with tau alternative splicing variants mRNAs

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We cloned AGN-1 that interacted with protein phosphatase 6 as a molecule highly expressed in nephritic rat kidney. Our previous studies also revealed that AGN-1 was co-localized with U1snRNP and splicing factor SC35 participating in the synthesis of tau alternative splicing variants, 4R-tau and 3R-tau. In addition, we showed that AGN-1-siRNA treatment altered the expression ratio of 4R-tau/3R-tau mRNA in Neuro2a cell and that AGN-1 may localize with 4R-tau and 3R-tau mRNAs in U1 spliceosome. In this study, we investigated an interaction of AGN-1 protein with tau alternative splicing variants mRNAs. By using nuclear and cytosol fractions prepared from Neuro2a cells, RNA immunoprecipitation analysis was carried out with anti-AGN-1 antibody, followed by RT-PCR to detect 4R-tau and 3R-tau mRNAs bound to AGN-1protein. RNA immunoprecipitation with nuclear fraction showed PCR fragments derived from 4R-tau and 3R-tau mRNAs, accompanied with an equal binding level of 4R-tau and 3R-tau mRNAs. However, in cytosol fraction, 4R-tau mRNA amount bound to AGN-1 protein was higher than 3R-tau mRNA. This result suggests that AGN-1 protein interacts with 4R-tau and 3R-tau mRNAs, and may involve in the differential regulation of a metabolism of 4R-tau and 3R-tau mRNAs.

Membrane proteomics for sex differences in renal proximal tubules using Sry gene-modified mice

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It has been shown that the biological sex difference lies not only in gonadal functions but also in physiological or pathophysiological aspects of whole body, including drug efficacy, adverse effects, and pharmacokinetics. Particularly, women experience more adverse events than men. Although it is plausible that the higher risks of adverse events in women could be partly explained by lower renal excretion of drugs, the underlying mechanisms on the sex differences have not been elucidated yet. It is known both hormonal and sex chromosome effects make sex-specific biological factors. Thus, in order to segregate hormonal effects and sex chromosome effects, we employed a murine model system in which the *Sry* gene was moved from the Y-chromosome to an autosome. The renal brush border membrane vesicles (BBMV) were isolated from murine kidney, and were subjected to quantitative membrane proteome analysis using LC/MS/MS (Q Exactive, Thermo Fisher Scientific). We have identified and quantitated 4309 molecules in BBMV and narrow it down to 736 sex specific molecules (80 transporters included). The results of the pathway analysis suggested that gonadal type influences membrane transports and sex chromosome complement influences cell metabolisms, implying that both sex chromosome complement and gonadal sex influence renal excretion of drugs.

Establishment of chronic obstructive pulmonary disease model using intratracheal mist spray

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When chronic obstructive pulmonary disease (COPD) develops, it is difficult to completely recover. The number of patients dying of COPD is increasing year by year. Researches on degenerative medicines of pulmonary diseases, including evaluation with animal models, are being advanced. A spray, which had been used for intratracheal administration, is currently unavailable. Therefore, we aimed to establish a COPD mouse model using a new intratracheal spray.

Using KN-34700 Natsume Aerosol Sprayers, elastase was administered intratracheally to male C57BL/6J mice at 10 weeks of age. LPS was administered intratracheally 3 weeks after the elastase administration. Bronchoalveolar lavage fluid was collected 3 days after the LPS administration. Respiratory function was measured 3, 6, and 12 weeks after the elastase administration. The lungs were isolated 3, 6, and 12 weeks after the elastase administration and examined histopathologically.

Pulmonary emphysema was confirmed to have developed 3 weeks after the elastase administration. BALF and respiratory function are being analyzed.

Effects of topical dosed anti-coagulant on LPS-induced exacerbation in asthma model mice

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RNA virus and bacterial infections induce exacerbation and steroid insensitive airway inflammation in patients with asthma. We have previously demonstrated that dabigatran inhibited steroid insensitive airway inflammation. The aim of this study is to evaluate the effects of anti-coagulant on LPS-induced steroid insensitive airway inflammation in asthma model mice. OVA-sensitized A/J mice were exposed to OVA every other day, then were exposed with LPS intranasally twice daily for 3 days. Fluticasone propionate (FP), dabigatran (Dabi; thrombin inhibitor) and edoxaban (Edo; factor Xa inhibitor) were administered intranasally at 2h before each LPS exposure. BALF was collected at 24 h after the last LPS exposure and eosinophils and neutrophils were quantified by FACS analysis. The level of CXCL1, TNF-α (inflammatory cytokine) and D-dimer, PAI-1 (blood coagulation/fibrinolysis system associated factors) in BALF were measured by ELISA. LPS exposure showed significant increase in eosinophils and neutrophils in BALF. Neither FP nor Edo inhibited inflammatory cells, while Dabi was inhibited in LPS-exposed asthma model mice. In addition, Dabi inhibited increased production of CXCL1, TNF-α, D-dimer and PAI-1 in BALF induced by LPS exposure. This profile provides new insights into steroid insensitive airway inflammation and future treatment.

Protective effect of a metallothionein inducer on cadmium-induced lung epithelial injury.

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Exposure to cadmium has been reported to cause respiratory disease in humans by inducing oxidative stress-dependent cellular injury. Metallothioneins are intracellular, cysteine-rich, metal-binding proteins that have a detoxifying action on heavy metals such as cadmium in various organs. In addition, expression of metallothioneins is induced by metals with low biological toxicity, such as zinc. Therefore, in this study we examined whether polaprezinc, a chelate compound consisting of carnosine and zinc, can suppress cadmium-induced lung epithelial cell death. We found that cell viability and cytotoxicity were decreased and increased, respectively by cadmium treatment; however, polaprezinc significantly reversed these changes. Moreover, cadmium-dependent endoplasmic reticulum stress responses were suppressed by polaprezinc treatment. Cadmium induced the production of reactive oxygen species (ROS) in A549 cells in a dose-dependent manner and polaprezinc significantly suppressed this cadmium-induced ROS production. Finally, we found that polaprezinc dose-dependently induced metallothioneins using real-time RT-PCR, ELISA, and western blotting analyses. These results indicate that polaprezinc can suppress cadmium-induced lung epithelial cell death and oxidative stress by inducing metallothioneins. We therefore suggest that polaprezinc may have therapeutic effects against respiratory diseases, such as chronic obstructive pulmonary disease and idiopathic pulmonary fibrosis.

A pharmacological study on Asthma-COPD overlap (ACO) model in mice

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Asthma-COPD overlap (ACO) rapidly deteriorates the respiratory function and is steroid resistant, and its effective treatment has not been established. The effects of dexamethasone (Dex) and roflumilast (Rof) on the asthma model sensitized and challenged by ovalbumin (OVA), and the ACO model exposed to OVA and cigarette smoke (CS) in BALB/c mice were examined.

In the OVA + CS group, the peak expiratory flow, tidal volume, Newtonian resistance (Rn, central airways resistance) and airway reactivity to methacholine were decreased, and the quasi-static compliance and airway neutrophils were increased when compared with the OVA group. Dex inhibited the specific airway resistance, airway reactivity, airway eosinophils and lymphocytes, and goblet cell hyperplasia in the OVA group. In the OVA + CS group, Dex reduced the airway eosinophils and goblet cells but increased the airway neutrophils and did not affect the respiratory function. Rof decreased the Rn in the OVA group, but did not affect other parameters in the OVA and OVA + CS groups.

Based on these results, the therapeutic effect of Dex was lower in the ACO model than in the asthma model, and the effect of Rof was not clear in both the ACO and asthma models.

Analysis of oral pathogenic bacteria in the patients of eosinophilic esophagitis

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Background&Aim: We have reported that oral pathogenic bacteria including *Porphyromonas gingivalis* (*P.g*) and *Streptococcus mutans* (*S.m*) accelerates systemic diseases. In our present study, we focused on eosinophilic esophagitis (EoE), a refractory rare disease, and analyzed oral bacteria in EoE patients to find relationship between EoE and infection of bacteria.

Method: Twenty four healthy control volunteers, 52 EoE subjects who were diagnosed by Shimane university and 22 subjects of reverse esophagitis (RE) were recruited. Oral bacteria were collected by mouse washing with 1.5 mL of distilled water. Bacterial DNA was extracted and analyzed by next generation sequencer. In addition, popular pathogenic oral bacteria including *P.g* and *S.m* were detected by PCR.

Resutls: Population of *Prevotella* genus was smaller in EoE and RE compared to that in healthy control. Same tendency was observed in four *Prevotella* spieces. On the other hand, *S.m* and *S.m*-derived collagen-binding protein (*cnm),* through which *S.m* induces dysfunction of tissues or organs, were detected more frequently both in EoE and RE than healthy control.

Conclusion: Oral bacterial flora in EoE subjects are likely to be different from that in healthy subjects, and infection to *S.m* is correlates with prevalence of EoE.

Influence on the rat fetal liver maturation with antenatal glucocorticoid administration.

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Purpose : The fetal liver is immature, and physiological jaundice often occurs. The symptom of jaundice will become more severe in premature infants. To investigate the maturation and the function in the liver of premature infants is necessary. Antenatal glucocorticoid (GC) administration is the standard of care for women at risk of a preterm birth. The purpose of this study was to examine whether GC administration acts on maturation factors in the fetal liver for development.

Methods : Dexamethasone were administered to pregnant rats for 2 days and the livers of 19-day-old fetuses, 21-day-old fetuses and 1-day-old neonates were analyzed. We evaluated mRNA levels of HNF4α, Ki-67 and Cyclin B as liver maturation factors and UGT1A1 as bilirubin metabolism-related factor by real-time PCR. In histochemistry, cell size of a hepatocyte was confirmed H-E staining.

Results and Discussion : The mRNA levels of HNF4α and UGT1A1 increased with growth. The mRNA expressions of HNF4a and UGT1A1 were increased in fetal liver with antenatal dexamethasone administration. Cell size of a hepatocyte was enlarged with growth, which is accelerated with antenatal GC administration. These results suggest that antenatal GC administration may accelerated maturation, and may increases bilirubin metabolism in the liver of premature infants.

Identification of B38-CAP as an ACE2-like enzyme to suppress hypertension and cardiac dysfunction in mice.

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Angiotensin-converting enzyme 2 (ACE2) is a negative regulator of the renin-angiotensin system, critically involved in blood pressure regulation, heart function, lung injury, or fibrotic kidney disease. Recombinant human ACE2 protein (rhACE2), currently clinically evaluated to treat acute lung failure, is a glycosylated protein, requiring time- and cost-consuming protein production in mammalian cells. Here we show that the B38-CAP, a carboxypeptidase derived from *Paenibacillus sp.* B38, is a novel ACE2-like enzyme to decrease angiotensin II levels in mice. Comparative analysis of protein 3D structures revealed that B38-CAP homologue shares structural similarity to mammalian ACE2 without any apparent sequence identity, containing the consensus HEXXH amino acid sequence of the M32 peptidase family. In vitro, recombinant B38-CAP protein catalyzed the conversion of angiotensin II to angiotensin 1-7, as well as other known ACE2 target peptides, with the same potency and kinetics as human ACE2. Treatment with B38-CAP reduced plasma angiotensin II levels and suppressed angiotensin II-induced hypertension, cardiac hypertrophy and fibrosis in mice. Moreover, continuous infusion of B38-CAP inhibited pressure overload-induced pathological hypertrophy, myocardial fibrosis, and cardiac dysfunction in mice, without any overt toxicity of liver and kidney. Our data identify the bacterial B38-CAP as an ACE2-like carboxypeptidase, which exhibits ACE2-like functions in vitro and in vivo. These results indicate that evolution has shaped a bacterial carboxypeptidase to a human ACE2-like enzyme. Bacterial engineering could be utilized to design improved protein drugs for hypertension and heart failure.

Involvement of mitochondrial Na^+/Ca^{2+} exchanger in hypoxia-induced pulmonary arterial hypertension.

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Pulmonary arterial hypertension (PAH) is characterized by pulmonary artery remodeling and inappropriate vasoconstriction, which results in a marked increase in pulmonary arterial pressure and right ventricular hypertrophy. Because a multiple factor participates in the pathogenesis of PAH, elucidation of the further mechanism is needed. Mitochondrial dysfunctions have been reported in the pathogenesis of PAH. In this study, to investigate the involvement of mitochondrial Na^+/Ca^{2+} exchanger (NCLX) in the development and progression of PAH, we generated a mouse model of hypoxia-induced PAH using NCLX knockout (NCLX-KO) mice, vascular smooth muscle-specific NCLX transgenic (VSM-NCLX-Tg) mice and wild-type (WT) mice. Increase in right ventricle systolic pressure (RVSP) induced by chronic hypoxia was significantly reduced in NCLX-KO mice compared with WT mice, whereas it was markedly augmented in VSM-NCLX-Tg mice. Moreover, administration of CGP37157, a selective NCLX inhibitor, to WT mice with chronic hypoxia significantly attenuated the increase in RVSP. These results suggested that vascular smooth muscle NCLX is involved in the pathogenesis of chronic hypoxia-induced PAH.

Expression analysis of mast cell-related genes in fetal and neonatal periods of spontaneously hypertensive rats (SHR)

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Mast cells are not only responsible for immune functions, but are also involved in the development of various diseases. Previously, we analyzed and reported about the appearance pattern of mast cells in the early stages of development in normal Wistar rat strain. It has been also reported that chymase accumulated in granules of mast cells is involved in the development of hypertension. In this study, we analyzed the relationship between mast cell expression patterns in the early stages of development and the onset of hypertension using model animals.

We used three strains of rats, SHR/NCrj, WKY/Ncrj rats and Wistar/Slc rats. They were planned to become pregnant, and fetuses at 9.5, 11.5, 13.5, 15.5, and 18.5 days of gestation and neonates at 1, 3, 7, and 14 days after birth were obtained. mRNA was purified from whole embryos or several tissues at each stage. RT-PCR was performed using primers for six molecules, i.e., c-kit, FceRI, rMCP-I, rMC-CPA, VEGF, and TNFalpha.

As a result, there was almost no difference in the expression timing and localization of rMCP-I among strains. However, there were significant differences in the expression patterns of c-kit and VEGF during embryonal stages. The difference between Wistar and WKY was greater than Wistar and SHR. It was considered that the development of hypertension is associated with the maintenance or differentiation of blood stem cells and the development of vascular system rather than the expression pattern of mast cells. It was also suggested that WKY rats may have already been committed to the development of hypertension.

Serum Indoxyl Sulfate as a Potential Independent Biomarker of Arterial Stiffness in Patients with Coronary Artery Disease

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Indoxyl sulfate (IS) is a low molecular weight metabolite and a uremic toxin which induces oxidative stress in myocardial, vascular smooth muscle cells, vascular endothelial cells and also involves in cardiovascular (CV) diseases. Therefore, we investigated the association between serum IS levels and aortic stiffness in coronary artery disease (CAD) patients. A total of 144 CAD patients were recruited. Carotid-femoral pulse wave velocity (cfPWV) was measured by the SphygmoCor system and the value over 10 m/s was classified as the arterial stiffness group. Serum IS levels were determined by liquid chromatography-mass spectrometry. Fifty patients (34.7%) had arterial stiffness and higher percentages with diabetes, elderly, higher systolic blood pressure, blood urea nitrogen, creatinine, serum IS level, lower estimated glomerular filtration rate compared with the control group. After adjustment of the factors by multivariable logistic regression analysis, the serum IS levels revealed significantly correlated with arterial stiffness (odds ratio = 3.834, P = 0.031), and has potential to be an independent predictor of arterial stiffness in CAD patients. In addition, the serum IS levels (β = 0.167, adjusted R2 change: 0.026, P = 0.027) were significantly positively correlated with cfPWV values in CAD patients in multivariable forward stepwise linear regression analysis. Our results suggest that serum IS has potential as an independent biomarker for aortic stiffness in CAD patients.

Angiotensin II type 1 receptor antagonist restores dysfunction of vascular reactivity independent of perivascular adipose tissue-mediated mechanisms in rats with metabolic syndrome

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Perivascular adipose tissue (PVAT) regulates vascular tone. We demonstrated that PVAT masks impaired vasodilation in the mesenteric arteries of SHRSP.Z-*Lepr*^{fa}/IzmDmcr rats (SHRSP.ZF) with metabolic syndrome (MetS); however, this enhanced vasodilation caused by PVAT disappears at around 23 weeks (wks) of age. Therefore, we investigated whether an angiotensin II type 1 receptor antagonist, azilsartan, protects against the deterioration in PVAT compensatory vasodilator function that occurs with aging in MetS.

SHRSP.ZF rats at 13 wks were orally administered azilsartan once daily for 10 wks. The vasodilation response in the superior-mesenteric arteries was determined in the presence or absence of PVAT, using organ bath methods. Azilsartan preserved both acetylcholine- and sodium nitroprusside-induced vasodilation independent of the presence or absence of PVAT, and did not improve the dysfunction in PVAT-mediated modulation of vascular tone in SHRSP.ZF rats.

This study demonstrated that the protective effect of azilsartan is mediated by restoring the endothelium- and vascular smooth muscle-mediated mechanisms, and not by improving PVAT dysfunction in MetS.

Effects of allopurinol, a xanthine oxidase inhibitor, on TNF-α-induced endothelial cells

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Tumor necrosis factor (TNF) is a kind of cytokine involved in infection protection and antitumor action by expressing cell adhesion molecules such as vascular cell adhesion protein-1 (VCAM-1), and by inducing apoptosis and inflammatory mediators. VCAM-1 is known to exacerbate cardiovascular disease and is a risk factor for cardiovascular disease events. Gout is not just a disease of the joints, it causes inflammation throughout the body and affects various organs. Since TNF-α is induced under inflammatory conditions such as hyperuricemia, we investigated an effect of allopurinol, a treatment agent for hyperuricemia, on VCAM-1. Human Umbilical Vein Endothelial Cells (HUVEC) were cultured confluently. Allopurinol (0.1-100 μM) was treated 20 minutes before TNF-α (10 ng/mL) exposure. The amount of VCAM-1 induced by TNF-α was evaluated using Western blotting. VCAM-1 protein levels in cultured HUVEC increased 24 hours after TNF-a exposure, which allopurinol suppressed the significantly ($p < 0.05$, $n = 4$). Allopurinol is thought to inhibit the induction of VCAM-1 by TNF-α and may decrease cardiovascular disease events.

Febuxostat attenuates the induction of vascular cell adhesion protein 1 by TNF-α in Human Umbilical Vein Endothelial Cells.

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In CARES clinical trial, febuxostat, a non purine xanthine oxidase inhibitor, was proved non-inferiority the rate of adverse cardiovascular events in patients with gout and major cardiovascular coexisting conditions. In this study, we evaluated the effect of febuxostat on the vascular cell adhesion protein 1 (VCAM-1) induction cultured Human Umbilical Vein Endothelial Cells (HUVEC) were exposed to 24-hour TNF-α (10 ng/mL) treatment. Febuxostat (0.1-100 mM) or solvent was added to the bath medium 20 minutes before TNF-α treatment. VCAM-1 protein levels in HUVEC increased after 24 hours TNF-atreatment ($n = 4$). Febuxostat significantly suppressed VCAM-1 induced by treatment with TNF-a in a dose-dependent manner ($p < 0.05$, $n = 4$). This finding suggests that treatment with Febuxostat on cardiovascular events may associate with the protection for the infiltration of lymphocyte or monocyte through the VCAM-1 induction in the inflamed-endothelial cells such as arterial sclerosis.

Impaired endothelium-dependent vasodilator responses of retinal blood vessels in adult rats with a history of retinopathy of prematurity

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Retinopathy of prematurity (ROP) is the leading cause of childhood blindness. We reported that short-term interruption of retinal vascular development with blockade of vascular endothelial growth factor (VEGF) signaling pathway in neonatal rats induces ROP-like retinal blood vessels, such as aggressive angiogenesis and tortuous arteries. Using this ROP model rat, we examined whether a history of ROP affects retinal vasodilator responses in adulthood. ROP was induced in rats by the subcutaneous injection of the VEGF receptor tyrosine kinase inhibitor KRN633 on postnatal day (P) 7 and P8. Tortuous arteries were observed in retinas of P56 KRN633-treated (ROP) rats. Retinal vasodilator responses to endothelium-dependent vasodilators (acetylcholine and GSK1016790A [an activator of TRPV4 channels]) were smaller in P56 ROP rats than age-matched control rats. No diminishment of acetylcholine-induced retinal vasodilator response was observed in P56 ROP rats treated with L-NAME, an inhibitor of NO synthase. Retinal vasodilator responses to NOR3, an NO donor, and salbutamol, a β_2 receptor agonist, were unaltered. These results suggest that the production and release of NO in retinal blood vessels are impaired in adult rats with a history of ROP. A history of ROP may increase the risk of the onset of retinal vascular diseases in adulthood.

Menaquinone-4 accelerates calcification of human aortic valve interstitial cells in high-phosphate medium through PXR

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Recently, we confirmed that menaquinone-4 (MK-4), the most common form of vitamin K_2 in animals, induced the calcification of human aortic valve interstitial cells (HAVICs) isolated from aortic valve stenosis (AVS) patients in high inorganic phosphate (high-Pi) medium via BMP2-ALP pathway. However, the mechanism of MK-4-induced BMP2 expression is unclear. There is a report that MK-4 can enhance collagen accumulation through pregnane X receptor (PXR) resulting in bone formation. So, the involvement of PXR in MK-4-induced calcification of HAVICs and BMP2 gene expression was investigated. HAVICs from AVS patients were cultured in α-MEM containing 10% FBS, and when the cells reached 80% confluence, they were further cultured in the presence or absence of MK-4 for 7 days in high-Pi medium (3.2 mM Pi). MK-4 dose-dependently accelerated PXR activity (EC_{50} 6.2 nM). MK-4-induced calcification was potently suppressed by two PXR inhibitors, ketoconazole and coumestrol. In physiological-Pi medium, MK-4 alone also increased BMP2 gene expression, which was significantly suppressed by coumestrol. These results suggested that MK-4 accelerates the calcification of HAVICs from AVS patients through the PXR-BMP2-ALP pathway.

Pressure stress delays the transient cyclooxygenase-2 expression by interleukin-1β stimulation in cultured human pulmonary artery smooth muscle cells.

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Pulmonary artery smooth muscle cells (PASMCs) play an important role in a sequence of events leading to the formation of pulmonary artery hypertension (PAH). Nevertheless, little is known about the direct effects of high pressure on the function and the intercellular signaling pathways of PASMCs. The aim of this study was to evaluate the effect of pressure stress which simulates PAH on interleukin-1β (IL-1β)-induced cyclooxygenase-2 (COX-2) expression in cultured human PASMCs. To investigate the effect of PAH on PASMCs, either 20 or 60 mmHg of an atmospheric pressure was given to PASMCs by a pressure-loading apparatus. Protein expression and phosphorylation were analyzed by Western blotting. IL-1β-induced the transient COX-2 protein expression peaking at 6 h in non-pressurized cells, whereas the COX-2 expression was delayed, peaking at 12 h, in the pressurized cells. The pressure stress also delayed the peak time of IL-1β-induced mitogen-activated protein kinases (MAPKs) phosphorylation, i.e., extracellular signal-regulated kinase, p38 MAPK, and c-jun N-terminal kinase. These results suggest that the pressure stress apparatus enable to simulate PAH, and delays in IL-1β-induced COX-2 expression occurs via late activation of MAPKs in PASMC.

Nitric oxide-insensitive soluble guanylate cyclase-mediated vasorelaxation in smokers

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Cigarette smoking is known to be accompanied with a decrease in nitric oxide (NO) bioavailability in the vascular system. A shift of soluble guanylate cyclase (sGC) from the NO-sensitive form to the NO-insensitive form could decrease NO bioavailability. Therefore, this study investigated whether NO-insensitive sGC-mediated relaxation is augmented in smokers. The right gastroepiploic artery was isolated from patients undergoing gastrectomy or coronary artery bypass grafting. BAY 60-2770 (NO-insensitive sGC stimulant) evoked a concentration-dependent relaxation of the artery, which was not different between non-smokers and smokers. In addition, there was also no significant difference in the concentration-response curve for sodium nitroprusside (NO-sensitive sGC stimulant). These findings suggest that NO-insensitive sGC-mediated vascular tone regulation is not affected by cigarette smoking. It is considered that the balance between the two forms of sGC is maintained even in the blood vessels damaged by cigarette smoking.

Involvement of Na^+ / Ca^{2+} exchanger in the spontaneous Ca^{2+} Transients of Guinea-Pig Pulmonary Vein Cardiomyocytes

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Pulmonary veins contain a myocardial layer, whose electrical activity is considered to be involved in the genesis and maintenance of atrial fibrillation.

To obtain insight into the automaticity of the pulmonary vein myocardium, we studied the spatio-temporal pattern of the rise in Ca^{2+} during the early phase Ca^{2+} transient of the isolated guinea pig pulmonary vein cardiomyocytes were studied with confocal microscopy.

On induction of Ca^{2+} transients by electrical field stimulation of the pulmonary vein cardiomyocytes, the rise in Ca^{2+} concentration first occurred at the subsarcolemmal region and then spread to the cell interior; this phenomenon was similar to that of atrial but not ventricular cardiomyocytes.

In pulmonary vein cardiomyocytes showing spontaneous activity, the Ca^{2+} transients were preceded by increased firing of Ca^{2+} sparks, which means Ca^{2+} release from sarcoplasmic reticulum. SEA0400, an inhibitor of the Na^+/Ca^{2+} exchanger, decreased the frequency of the Ca^{2+} transients and eventually inhibited the Ca^{2+} transients completely without decreasing the firing of Ca^{2+} sparks.

In conclusion, the guinea-pig pulmonary vein myocardium has a tendency to show spontaneous electrical activity, which is mediated by Ca^{2+} released from the sarcoplasmic reticulum and the resulting activation of the Na^+/Ca^{2+} exchanger.

Cardiac effects of a specific I_f channel blocker ivabradine in anesthetized rabbits: simultaneous assessment of the atrial and ventricular automaticity

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We simultaneously assessed effects of a specific I_f channel blocker ivabradine on the atrial and ventricular automaticity in anesthetized rabbits. Under isoflurane anesthesia, the atrioventricular node of NZW rabbits (n=3) was ablated by application of radiofrequency energy, and stable idioventricular escaped rhythm was observed. The surface lead II electrocardiogram was measured to monitor changes in the atrial rate (AR) and ventricular rate (VR). The monophasic action potential (MAP) was recorded from the right ventricle to assess the MAP duration (MAP_{90}). Intravenous administrations of ivabradine hardly affected the AR or VR at 0.01 and 0.1 mg/kg. Additional administration of ivabradine at 1.0 mg/kg decreased both AR and VR by 45 and 51 beats/min, respectively. Moreover, the MAP_{90} was prolonged with decrease of the VR, and torsade de pointes was induced in one animal. These results suggest that ivabradine affects the ventricular as well as the atrial pacemaker activity with a similar potency.

Establishment of the diet-induced obese rat model for atrial fibrillation.

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Background: Obesity is a risk factor for atrial fibrillation (AF). However, the mechanisms underlying AF in obesity remain unclear. In this study, we established the diet-induced obese-rat model with high AF inducibility and evaluated the relationship between AF inducibility and cardiac function.

Methods: Male Sprague-Dawley rats were fed with normal chow diet + normal drinking water (NCD) or high-fat diet + 30% fructose in drinking water (HFFr) for 12 weeks. These rats were subjected to hemodynamic measurements, echocardiography to assess the evaluation of the cardiac structure and function., and transesophageal burst atrial pacing for the induction of AF.

Results: HFFr-fed rats were divided into 2 groups: obese and non-obese HFFr-fed rats. Compared with NCD-fed rats, the inducibility of AF significantly increased in obese HFFr-fed rats, but not in non-obese HFFr-fed rats. On echocardiography, LVEF (an indicator of LV systolic function), and E/A ratio (a marker of LV diastolic function) didn't change among these rats. For hemodynamic measurements, LVSP, dP/dtmax, and heart rate increased in obese HFFr-fed rats.

Conclusion: We established the obese rat model with high AF inducibility while maintaining normal cardiac function. This model would be useful to elucidate the mechanisms of obesity-related AF.

Effects of thrombospondin-4 on voltage-gated ion channels in rat ventricular myocytes

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Thrombospondin (TSP)-4, a matricellular protein, is highly expressed in heart tissues of various cardiac disease models. Although TSP-4 is known to regulate voltage-dependent calcium channel activity in dorsal root ganglionic neurons, it remains to be clarified whether it affects electrical activity in cardiomyocytes. We examined the effects of TSP-4 on voltage-gated ion channels in rat ventricular myocytes. Ventricular myocytes isolated from male Wistar rats were seeded on a glass plate coated with laminin. Recombinant mouse TSP-4 (5 nM) or its vehicle was treated for 4 hours. L-type calcium channel (LTCC) current, voltage-gated potassium channel (VGKC) current, and action potential duration (APD) were measured by a whole-cell patch-clamp method. TSP-4 inhibited both currents of LTCC and VGKC. TSP-4 tended to prolong APD_{50} and APD_{90}. This study for the first time demonstrated that TSP-4 inhibits the activity of LTCC and VGKC, which consequently leads to APD prolongation. The APD prolongation might be partly due to the suppression of VGKC activity because the inhibition of LTCC should lead to an APD shortening. It is suggested that TSP-4 might be related to the ventricular arrhythmia via regulating voltage-gated ion channels.

Drug-induced arrhythmia prediction method based on voltage-dependent I_{CaL}block

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Drug-induced arrhythmia can occur under prolonged action potential duration (APD) due to block of I_{Kr}. Therefore, I_{Kr} block and APD prolongation have been used for predicting drug induced arrhythmia.However, I_{Kr} blockers have difference in risk for drug-induced arrhythmia. One of the reasons is that the occurrence of drug-induced arrhythmia under bradycardia is initiated by early afterdepolarization (EAD) at the repolarization phase in prolonged action potential. For example, terfenadine, which prolongs APD and cause EAD, is considered as a drug with a high risk for drug-induced arrhythmia. On the other hand, amiodarone, which prolongs APD but does not cause EAD in clinical practice, has been considered as a relatively safe antiarrhythmic drug. Therefore, there is a possibility that EAD occurrence can account for the difference in the risks among I_{Kr} blockers. To study the mechanisms underlying different occurrence of EAD, we examined the effects of voltage-dependent I_{CaL} block property on EAD. In the present study, we used a mathematical model of human ventricular action potential. The results showed that amiodarone-like I_{CaL} block model suppressed EAD. But, I_{CaL} block models of terfenadine-like and bepridil-like increased EAD occurrence. The different effects on EAD were accounted for by difference in voltage-dependent block of I_{CaL}, as weak I_{CaL} block in hyperpolarized potential increased the occurrence of EAD. Therefore, to predict drug-induced arrhythmia, not only APD prolongation but also voltage-dependent property of I_{CaL} block should be checked.

A simple and dual expression plasmid system in prokaryotic (*E. coli*) and mammalian cells.

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We introduce a simple and universal cloning plasmid system for gene expression in prokaryotic (*Escherichia coli*) and mammalian cells. This novel system has two expression modes: the (subcloning) prokaryotic and mammalian modes. This system streamlines the process of producing mammalian gene expression plasmids with desired genes. The plasmid (prokaryotic mode) has an efficient selection system for DNA insertion, multiple component genes with rare restriction sites at both ends (termed "units"), and a simple transformation to mammalian expression mode utilizing rare restriction enzymes and re-ligation (deletion step). This system is highly efficient for the subcloning of blunt-end fragments, including PCR products. After the insertion of the desired gene, protein encoded by the desired gene can be detected in *E. coli* with IPTG induction. Then, the lac promoter and operator are readily deleted with 8-nucleotide rare-cutter blunt-end enzymes (deletion step). Following re-ligation and transformation, the plasmid is ready for mammalian expression analysis (mammalian mode). This idea (conversion from prokaryotic to mammalian mode) can be widely adapted. With pgMAX system, we made epitope-library of the calcium channel alpha1 subunit (CaV1.2) and found a novel binding site to calcium channel beta2 subunit. The pgMAX system could be widely adopted for simple expression analyses.

Metalloprotease nardilysin controls heart rate through the transcriptional regulation of ion channels critical for sinus automaticity.

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Nardilysin (NRDC; N-arginine dibasic convertase) is a metalloprotease of the M16 family. We reported that NRDC is a protease having localization-dependent multiple functions; an enhancer of ectodomain shedding in the extracellular space and a transcriptional coregulator in the nucleus. NRDC-deficient mice (Nrdc-/-) showed wide range of phenotypes such as hypomyelination, hypothermia, and bradycardia. In this study, we have explored a role of NRDC in the regulation of heart rate, and obtained the following results. (1) Pharmacological blocking of autonomic nervous system revealed that an intrinsic heart rate of *Nrdc-/-* was significantly reduced compared with that of wild-type mice. (2) In *Nrdc-/-* hearts, mRNA levels of Cav3.1 and HCN1/4, ion channels responsible for sinus automaticity, were significantly reduced. (3) Funny (If) current and T-type Ca current measured in the sinus node cells were markedly reduced in *Nrdc-/-* cells, indicating that the functions of Cav3.1 and HCN1/4 are impaired. (4) Gene knockdown of NRDC in primary rat ventricular myocyte led to the reduction of mRNA level of HCN1/4. (5) Chromatin immunoprecipitation-PCR analysis showed that NRDC binds to the promoter region of Cav3.1 and HCN1/4, suggesting the direct involvement of NRDC in transcriptional regulation of these ion channels. (6) Atrium-specific *Nrdc-/-* (obtained by mating *Nrdc* floxed mouse with Sarcolipin-Cre mouse) showed mild bradycardia and reduced Cav3.1 mRNA expression. (7) In silico simulation model of human iPS cell-derived sinus node cells recapitulated the bradycardia in NRDC-deficient cells. Together, our results indicated that NRDC in cardiomyocyte controls heart rate through the transcriptional regulation of ion channels critical for sinus automaticity.

Utility of isoflurane-anesthetized guinea pigs for the assessment of the QT-interval prolongation induced by drugs with positive chronotropic action

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[Background] The QT interval can be shortened by tachycardia, which may underestimate risks of the drug-induced QT-interval prolongation in the safety pharmacology studies. To investigate utility of guinea pigs for the assessment of the QT-interval prolongation, we assessed cardiac effects of suspect drugs prolonging QT interval, sulpiride and aripiprazole, both of which exerted positive chronotropic actions in dogs.

[Methods] Under isoflurane-anesthesia, electrocardiogram and monophasic action potential (MAP) of right ventricle were continuously recorded from guinea pigs to measure the heart rate (HR) and the MAP duration (MAP_{90}), respectively. Sulpiride (2, 20, and 60 mg/kg) or aripiprazole (0.03, 0.3, and 3 mg/kg) were administered intravenously over 10 min.

[Results] Sulpiride at 2 mg/kg did not affect HR or MAP_{90}, and increased MAP_{90} with decrement of HR at 20 and 60 mg/kg. Aripiprazole at 0.03 and 0.3 mg/kg did not affect HR or MAP_{90}, and increased MAP_{90} with decrement of HR at 3 mg/kg. Meanwhile, positive chronotropic actions of sulpiride and aripiprazole were not observed.

[Conclusions] Since sulpiride and aripiprazole have been clinically reported to hardly induce tachycardia, anesthetized guinea pigs are useful for screening of drug-induced QT interval prolongation for safety pharmacology studies.

Evaluation of atherosclerotic lesions by BCR/ABL1 tyrosine kinase inhibitor effects in a familial type Ⅱ$_a$ model mouse.

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[Introduction] BCR/ABL1 tyrosine kinase inhibitors (TKIs) have improved the treatment of chronic myeloid leukemia. However, it becomes widely known that treatment with TKIs increases vascular adverse events (VAEs) and the detailed mechanism for VAEs is unknown. We hypothesized that TKIs accelerate atherosclerotic lesions and studied atherosclerotic lesions by TKIs in a familial type II a model mouse.

[Methods] In order to evaluate atherosclerotic lesion by TKIs, *Ldlr*$^{-/-}$and *Apobec1*$^{-/-}$($L^{-/-}/A^{-/-}$) mice were used. $L^{-/-}/A^{-/-}$ mice have a high plasma LDL levels and more pronounced development of atherosclerosis. 8-week-old male $L^{-/-}/A^{-/-}$ mice were randomized in 4 groups (n=10 per group) and received oral gavage with DMSO, imatinib(50mg/kg), nilotinib(45mg/kg), ponatinib(10mg/kg) for 16 weeks. Thereafter, mice were sacrificed to evaluate atherosclerotic lesions and plasma cholesterol levels.

[Results] There were no significant differences in atherosclerotic lesions and plasma cholesterol levels between 4 groups.

[Discussion] We could not find an association between atherosclerosis and TKIs in this study. The onset of VAEs by TKIs is very complex and it may be difficult to explain solely by atherosclerosis.

Histidine-rich glycoprotein regulates neutrophil condition in sepsis

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Reactive oxygen species (ROS) play important roles in the progression of septic pathogenesis. Recent studies revealed that neutrophil adhesion on vascular wall followed by neutrophil extracellular traps (NETs) release may trigger platelet aggregation and immunothrombus formation in septic organ failure. Additionally, the adherent neutrophils-produced ROS induce the immunothrombus formation and tissue damage. Our previous study indicated that plasma histidine-rich glycoprotein (HRG) levels significantly decreased in cecal ligation and puncture (CLP) septic mice model and administration of HRG dramatically improved the survival rate of CLP mice. However, the role of HRG on neutrophil ROS production and immunothrombosis in septic condition was poorly understood. In this study, we showed that HRG inhibited immunothrombus formation in pulmonary vasculatures by keeping neutrophils quiescent morphologically and functionally using immunohistochemical staining and in vivo imaging methods and suppressed excess extracellular ROS production from neutrophils using isoluminol method. These results suggested that HRG may regulate the uncontrolled activation of circulating neutrophils in septic condition.

Regulation of erythropoiesis in zebrafish model by the kinase of ribosomal protein S19

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In Diamond-Blackfan anemia (DBA), about half of the patients have mutations in one of several ribosomal protein (RP) genes. The most frequently mutated gene (～25%) is the ribosomal protein S19 (*RPS19*), in which a hot spot for mutations between residues 52 and 62 has been reported. However, it is not clear why mutations in the ubiquitously expressed *RPS19* gene specifically affect erythropoiesis. We previously showed in vitro that the 59th serine residue (Ser59) of RPS19 is phosphorylated by PIM1 kinase. Here we study the involvement of RPS19 and PIM1 in erythropoiesis using zebrafish to determine whether phosphorylation could affect red blood cells production. We generated the *rps19* knockdown zebrafish by injection of morpholino antisense-oligo (MO) at the one-cell stage. The rps19-deficient embryos (morphants) showed abnormal morphologies and a decreased number of red blood cells. Although *in vitro* synthesized *rps19* mRNA rescued the aberrant phenotypes in morphants, the recuperation was not shown by substitution of Ser59 residue with alanine or aspartic acid. These observations suggest that reversible phosphorylation of Ser59 is important for the function of rps19. Therefore, we injected the MO against *pim1*, which phosphorylates Ser59 of rps19. The pim1 morphants showed abnormal head and tail, and a decrease in the number of red blood cells. Co-injection with synthetic *pim1* mRNA restored morphology and red blood cell count. These findings suggested that pim1 was related to erythropoiesis. Further consideration will be needed to yield any findings about the relationship between phosphorylation and erythropoiesis by using pim1 deficient fish.

Sairei-to, a traditional Japanese herbal medicine, inhibits UVB-induced skin inflammation

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[Introduction] Ultraviolet (UV) radiation, especially UVB (280-320 nm) from sunlight, is one of major environmental hazard to induce skin damage. A single high dose UVB exposure on the skin causes the acute inflammatory reaction which is characterized by erythema, increasing vascular permeability and edema formation. The single UVB-induced damage could be accumulated by chronic UVB irradiation. The accumulated skin damage could lead to skin aging and raise the risk of skin cancer. Therefore, the inhibition of single UVB-induced acute inflammation is important target for protecting skin from UVB exposure. Sairei-to (SRT), a traditional Japanese herbal medicine, has been used for inflammation treatment. Recently, it has been clinically evidenced to inhibit edema formation caused by radiotherapy. However, the effect of SRT on UVB-induced skin inflammation is poorly understood. In this study, we investigated the protective effect of SRT against the acute UVB-induced skin damage in hairless mice.
[Methods] Five-week-old male HR-1 hairless mice were treated with SRT suspension (625 or 1250 mg/kg orally) for 3 weeks (5 days/week). After 3 weeks administration, dorsal skins of the mice were exposed to UVB radiation at a dose of 250 mJ/cm^2. The change of skin erythema index (EI) and transepidermal water loss (TWEL) were measured every 24 h after UVB irradiation for 3 days. The dorsal skin tissues were collected to evaluate skin thickness, collagen fibers and infiltration of macrophage and neutrophil.
[Results] SRT significantly attenuated UVB-induced increase of EI and TWEL, and suppressed dermal thickening, collagen loss and macrophage and neutrophil infiltration, compared with UVB radiation alone group.
[Conclusion] These results indicated that SRT had preventive effect against UVB-induced skin damage and suggested that it might be a useful agent for protecting UVB-induced inflammation.

Effect of Jumihaidokuto on UVB-induced skin damage

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Background: Jumihaidokuto (JHT), a traditional Chinese medicine, has been clinically prescribed for the treatment of patients with skin disorder with redness and swelling, such as atopic dermatitis or acne vulgaris. However, it remains unknown how JHT ameliorates the skin disorders. In this study, we investigated the effect of JHT on UVB-induced skin inflammation model.

Methods: Male hairless mice (HR-1) were treated with 1000 mg/kg JHT for 3 weeks. After the last injection, mice were received single dose of 250mJ/cm^2 UVB. Before and after irradiation, we measured following factors; skin moisture content in epidermis and dermis, skin erythema dose, and transepidermal water loss (TEWL). We also evaluated the effect on skin thickness and the infiltration of neutrophil and macrophage by HE staining and immunohistochemical (IHC) analysis, respectively.

Results: UVB irradiation decreased the skin moisture content in both skin layers, and increased skin erythema dose and TEWL. Pretreatment with JHT significantly improved the skin moisture content loss and TEWL increment, but not skin erythema.

In addition, HE staining revealed UVB irradiation leaded to edema in dermis and hyperplasia in epidermis. Furthermore, IHC analysis also revealed UVB irradiation facilitated the infiltration of neutrophil and macrophage into dermis. On the other hand, pretreatment with JHT inhibited the edema, hyperplasia and the infiltration of neutrophil and macrophage induced by UVB irradiation.

Conclusion: These results suggest JHT could protect from UVB-induced skin moisture loss and inflammation.

Role of Prostaglandin D_2 for delayed wound healing in Streptozotocin-induced diabetic mice

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Delayed wound healing is a major problem in patients with diabetes, which significantly impairs their quality of life. Prostaglandin (PG) D_2 is a major inflammatory lipid mediator synthesized by hematopoietic PGD_2 synthase (H-PGDS) from PGH_2, a common precursor of all of PGs. In the present study, we investigated the role of PGD_2 in cutaneous wound healing in streptozotocin (STZ)-induced diabetic mice. C57BL/6 mice were injected with 50 mg/kg of STZ intraperitoneally daily for 5 days. Four weeks after the injection of STZ, a full thickness wound was created with an 8-mm diameter biopsy punch on the dorsal of mice. Wound healing was significantly decelerated and cutaneous H-PGDS mRNA was significantly increased in diabetic mice compared with non-diabetic mice. Daily administration of H-PGDS inhibitor for 14 days was significantly promoted wound healing in diabetic mice. These results suggest that PGD_2 involved in delayed wound healing in STZ-induced diabetic mice.

Establishment of a molecular targeting therapy for dog bladder cancer by using dog bladder cancer organoid culture

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【Background】

- Since dog bladder cancer is usually muscle-invasive and the malignant level is quite high, the proper treatment has not been established in a veterinary clinic. In the previous study, we generated a dog bladder cancer organoids, which could reproduce the cancer microenvironment in vivo and could be applied to the anti-cancer drug sensitivity test for each patient. However, it has not been revealed whether molecular targeting drugs are effective in the inhibition of the survival of the organoids.

【Object】

-The purpose of this study is to identify the effective molecular targeting drugs against dog bladder cancer by using dog bladder cancer organoids.

【Method】

-Dog bladder cancer organoids were treated with 14 molecular targeting drugs for 72hours. The survival rate of organoids was evaluated by an alamarblue cell viability reagent. The effects of drugs on the activation and expression of intracellular signal molecules were investigated by performing western blotting.

【Result】

-Among 14 drugs, treatment of gefitinib, erlotinib, trametinib, and afatinib inhibited the cell viability of organoids in a dose-dependent manner. Furthermore, EGFR, and ERK, and CD44 expression were suppressed by erlotinib treatment.

【Conclusion】

-These results suggest that EGFR inhibitors and a MEK inhibitor might suppress the growth of dog bladder cancer organoids through suppression of CD44 expression. This result is expected to be useful for the development of molecular targeting therapy for bladder cancer diseased dog.

The role of a novel hyaluronan depolymerization factor, HYBID, on glioma

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HYBID (hyaluronan binding protein involved in hyaluronan depolymerization) is a novel factor associated with hyaluronan depolymerization. HYBID facilitates the several tumor progression and the expression level of HYBID is helpful as a predictor of tumor progression including colon and pancreatic tumor. Though HYBID is important for hyaluronan metabolism in brain, there is no report on glioma. Therefore, we evaluated the role of HYBID and hyaluronan on glioma using *in vitro* and *in vivo* glioma models.

First, we evaluated the cell proliferation, migration, and the expression of some related proteins after knock of *hybid* by using siRNA in U251 human glioma cell. Moreover, we evaluated the tumor size by using the *in vivo* glioma model with HYBID KO and WT mice. Murine glioma model was estimated by hematoxylin and eosin staining.

Hybid knock down suppressed the glioma cell proliferation, migration and Wnt/β-catenin signal related protein. HYBID may promote the glioma progression *via* Wnt/β-catenin signal. Moreover, tumor size in HYBID KO mice were smaller than that in HYBID WT mice. This result indicates that host derived HYBID is contributed to glioma progression.

In conclusion, these findings indicate that HYBID was an important factor for glioma progression.

Inhibitors of H_2S-generating enzymes reduce the survival of human multiple myeloma-derived KMS-11 cells with resistance to bortezomib, a proteasome inhibitor

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H_2S is endogenously produced by cystathionine-γ-lyase (CSE), cystathionine-β-synthase (CBS) or 3-mercaptopyruvate sulfurtransferase (3-MST). In the present study, we examined the role of endogenous H_2S in the survival of human multiple myeloma (MM)-derived KMS-11 cells and KMS-11/BTZ cells that acquired resistance to bortezomib (BTZ), a proteasome inhibitor. BTZ significantly decreased the viability of KMS-11 and KMS-11/BTZ cells at 10-1000 and 100-1000 nM, respectively. Both Na_2S, an H_2S donor, and GYY4173, a long-lasting H_2S releaser, slightly increased the viability of those cells. Aminooxyacetic acid (AOAA), a CBS inhibitor, strongly suppressed the viability of KMS-11 and KMS-11/BTZ cells, regardless of the presence of BTZ, and DL-propargylglycine (PPG), a CSE inhibitor, exhibited relatively minor cytotoxicity. In contrast, a 3-MST inhibitor had little or no such effect. GYY4173 significantly reversed the cell toxicity of PPG or AOAA in the presence of BTZ. BTZ treatment at 10 nM for 24 h markedly increased protein levels of CBS among three H_2S-generating enzymes in KMS-11, but not KMS-11/BTZ, cells. These data suggest that H_2S generated mainly by CBS promotes the survival of both KMS-11 and KMS-11/BTZ cells, regardless of the presence of BTZ, and that CBS inhibitors are useful to treat BTZ-resistant MM.

Combined effect of anti-PD-L1 antibody with low molecular weight compound in the tumor-bearing mouse model

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Immune checkpoint inhibitors such as anti-PD-1 antibody and anti-PD-L1 antibody have recently been approved for the treatment of melanoma or non-small cell lung cancer. In this study, we examined the anti-tumor effect of anti-PD-L1 antibody with low molecular weight compound.

Mice were subcutaneously inoculated with a mouse cancer cell line. They were allocated into the control, anti-PD-L1 antibody, low molecular weight compound and their combination treatment groups. Anti-PD-L1 antibody was administered intraperitoneally twice a week for two weeks. The low molecular weight compound was administered orally once a day for 14 days. The tumor diameters were measured to calculate the tumor volumes. Observation and measurement were performed for 14 days after the initiation of administration. The tumor was excised and dispersed to analyze tumor-infiltrating lymphocytes (TILs) . The dispersed cells were stained with fluorescent-labeled antibodies and analyzed using the flow cytometer.

As a result, a proportion of TILs subsets including regulatory T cells (Treg), $CD8^+$ T cells, tumor-associated macrophages (TAM) and myeloid-derived suppressor cells (MDSC) were altered by administration of drugs. It suggested that the evaluation system described above is useful for combined efficacy study of anti-PD-L1 antibody with low molecular weight compound in the tumor-bearing mouse model.

Combination therapy of liposome-entrapped muramyl tripeptide phosphatidylethanolamine (L-MTP-PE) against syngeneic tumors in mice

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Antitumor activities of L-MTP-PE (Liposome-entrapped myuramyl tripeptide phosphatidylethanolamine) in the combination treatment with chemo- or immune-therapeutic antitumor agents against various syngeneic tumors were tested. Against liver metastasis model of M5076 carcinoma, L-MTP-PE showed a tendency of elongation of survival days by its single treatment, however, elongation with statistical significance was observed in the combination treatment with 5-FU. Against Meth A fibrosarcoma system, L-MTP-PE showed a significant elongation of survival days in spite of its non-effect on tumor growth, when combined with 5-FU. L-MTP-PE also enhanced antitumor effect of OK-432 (picibanil), a bacterial immunotherapeutic agent against MM46 mammary carcinoma. In parallel with enhanced antitumor activity, TNF production induced by OK-432 was potentiated when primed with L-MTP-PE. These data suggest that L-MTP-PE seems to elongate the survival days of solid tumor bearing mice due to its saving effect on chemotherapeutic drug-induced immunosuppression and that L-MTP-PE also may potentiate the antitumor effect of immunotherapeutic agent OK-432 by the enhanced production of TNF.

15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 inhibits cell migration of renal cell carcinomas independently of PPARγ and CRTH2

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Renal cell carcinoma (RCC) accounts for 2–3% of all malignant tumors. Even with oncologic removal, about 40% of patients will develop metastases after surgical resection. The five-year survival probability of patients with metastatic renal cell carcinoma is less than 10% because of the cancer's resistance to chemotherapy and radiotherapy. Thus, there is an urgent need to establish novel therapeutic approaches for metastatic RCC treatment. The metastatic cascade has been reported to be modulated by an endogenous carcinostatic 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (15d-PGJ_2). A nuclear receptor of 15d-PGJ_2 is peroxisome-proliferator activated receptor γ (PPARγ), and its membrane receptor is chemoattractant receptor-homologous molecule expressed on Th2 cells (CRTH2). 15d-PGJ_2 has also been reported to reduce cell migration, stimulate focal adhesion disaggregation, and induce filamentous actin realignment. In the present study, we evaluated the effects of 15d-PGJ_2 on the migration of Caki-2 RCC cells. Although treatment with low concentrations of 15d-PGJ_2 did not cause apoptosis, it did decrease the migration of Caki-2 cells. PPARγ and CRTH2 did not mediate the inhibitory effect of 15d-PGJ_2 on the migration of Caki-2 cells. Our present study proposes the therapeutic potential of 15d-PGJ_2 for prevention of RCC metastasis.

Differentiation-inducing factor-1 exhibits anti-metastatic effects by inhibiting cellular motility and adhesion in malignant melanoma cells

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We reported that differentiation-inducing factor-1 (DIF-1) inhibited the proliferation of various cancer cells including malignant melanoma and that DIF-1 prevented lung colony formation in a mouse model of metastatic melanoma. However, the mechanisms of this action remain to be elucidated. In the present study, we investigated the anti-metastatic effects of DIF-1 in human malignant melanoma A2058 cells. Activities of cell migration and invasion were measured by the wound healing assay and cell invasion assay, respectively. Activities of cell adhesion to extracellular matrix (ECM) and vascular endothelial cells were measured by using ECM-coated plate and human umbilical vein endothelial cells (HUVECs). Expression levels of signaling molecules were measured by Western blotting. DIF-1 suppressed the phosphorylation levels of signal transducer and activator of transcription 3 (STAT3) and subsequently reduced a variety of genes related to cell migration and invasion such as matrix metalloproteinase-2, vimentin, N-cadherin and twist, resulting in the inhibition of cell migration and invasion. Further, DIF-1 inhibited the melanoma cell adhesion to ECM and HUVECs. These results suggested that DIF-1 suppresses the detachment of cancer cells from the primary tumor by inhibiting cell migration and invasion, and also prevents circulating cancer cells from adhering to vascular endothelial cells. Therefore, DIF-1 may have potential to be a lead chemical compound for developing a novel anti-metastatic agent against malignant melanoma.

Effect of probenecid on 3D-cultured prostate cancer spheroids

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Probenecid is a well-known uricosuric agent used for gout treatment through blockage of urate transporter in renal tubules. On the other hand, probenecid can also inhibit multiple channels and transporters including multidrug resistance protein-1 (MRP-1). Therefore, probenecid might support the effect of anti-cancer drugs via inhibition of efflux of these drugs from tumor cells.

Conventionally, cancer cell lines have been cultured as two-dimensional (2D) monolayer. However, such condition does not reflect the real tumor situation in vivo. Recently, 3D culture techniques have developed to examine the cancer cell lines with more natural tumor property. In this study, we evaluated the effect of probenecid on prostate cancer cell lines which cultured as multicellular tumor spheroids by culturing in ultra-low attachment plate.

Prostate cancer cell line 22Rv1 cultured as spheroid showed lower sensitivity against anti-cancer drug doxorubicin, compared to cells cultured as monolayer. Probenecid treated-spheroid was more sensitive against doxorubicin. Interestingly, we found that probenecid itself has anti-tumor activity in concentration dependent manner. Probenecid was more effective to 3D cultured spheroid than 2D cultured monolayer. In this presentation, we also show the result of other prostate cancer cell lines and discuss the anti-cancer mechanism of probenecid.

Development of new therapeutic drugs for glioma targeting choline transporter-like protein 1

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Choline is an organic cation that plays a critical role in the structure and function of biological membranes. Intracellular choline accumulation through choline transporters is the rate-limiting step in phospholipid metabolism, and it is a prerequisite for cell proliferation. In this study, we examined the functional characterization of choline transporters in U251MG glioma cells. Furthermore, we searched for compounds that inhibit choline uptake as well as cell proliferation in a plant-derived natural organic compound library. Choline transporter-like protein 1 (CTL1) and CTL2 mRNA are highly expressed. CTL1 and CTL2 were located in the cell membrane and intracellular compartment, respectively. [^{3}H]Choline uptake was mediated by a single Na^+-independent, intermediate-affinity transport system. We found two hit compounds that inhibit choline uptake and cell proliferation from 500 plant-derived natural organic compounds. These hit compounds reduced cell survival and enhanced caspase-3/7 activity. One of the compounds inhibited tumor growth in U251MG cell xenograft model mice. These results suggest that CTL1 is functionally expressed in glioma cells and are also involved in abnormal proliferation. Identification of this CTL1-mediated choline transport system provides a potential new target for glioma therapy.

Pyridinium fullerene derivative inhibits cell growth by suppression of Wnt signaling in virus-infected non-Hodgkin's B-cell lymphoma cell.

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Primary effusion lymphoma (PEL) is defined as a rare subtype of non-Hodgkin's B-cell lymphoma which is caused by Kaposi's sarcoma-associated herpesvirus (KSHV) in immunosuppressed patients. PEL is frequently resistant to conventional chemotherapies such as CHOP. Therefore, novel therapeutic options for PEL have been expected. We had reported that a pyrrolidinium fullerene derivative induces apoptosis via Akt suppression in PEL.

Here, we have synthesized eight pyridinium-type cationic fullerene derivatives and evaluated cytotoxic effects of them against PEL. The pyridinium fullerenes decreased the cell viability of PEL compared with KSHV-uninfected B-lymphomas. The most potent derivative suppressed Wnt signaling by β-catenin downregulation in PEL cells, whereas it did not affect MAPKs, NF-kB and Akt signaling. The fullerene derivative decreased not β-catenin mRNA, but β-catenin protein in PEL cells. NF-kB, MAPK, and Wnt pathways are constitutively activated in PEL, and these activations are thought to be necessary for cell survival and growth of PEL. We consider that the pyridinium fullerene exerts an anti-PEL activity by disrupting Wnt signaling. Now, we are attempting to elucidate the mechanism of β-catenin downregulation by the fullerene derivative.

Establishment and characterization of a novel murine model for head and neck cancer cachexia

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Cancer cachexia is the metabolic wasting syndrome that the cancer will release a lot of cytokines and result in metabolic abnormalities and anorexia. The rate of the cancer death has been revealed almost 30% in the cachexia patients. Cancer cachexia can vary according to tumor type, site, mass, and host genotype. Clinical studies showed that more than 60% of head and neck cancer (HNC) patients might develop cancer cachexia. Several animal models have been established to elucidate the importance of pro-cachectic cytokines, such as TNF-α and interleukin 6 (IL-6) in the pathogenesis of cancer cachexia. Unfortunately, the pathogenesis of HNC cachexia is still unknown. Our preliminary results demonstrated that IFIT2, an interferon-induced protein with tetratricopeptide repeat 2 (IFIT2) depletion enhances expression of TNF-α, a well-known cancer-cachexia related cytokine in HNC cells. Thus, this study aims to explore the effect of IFIT2 depletion on HNC cachexia. To the end, a murine model was established by injecting the IFIT2 depleted HNC cells. Moreover, the body weight and survival rate were significantly decreased in IFIT2-depleted cells bearing mice as compared to control mice. The quadriceps had a 28.6% reduction in cachectic mice. Similarly, the gastrocnemius had a 33.3% reduction in cachectic mice. These results suggest that IFIT2-depleted HNC cells bearing mice may act as a model for studies on HNC cachexia.

QSAR analysis of tumor-specificity of newly synthesized 3-styrylchromone derivatives against human oral squamous cell carcinoma cell lines

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Introduction: Chromone ring constitutes basic skeleton of secondary metabolites in various plants. We have previously investigated 16 groups of chromone derivatives (239 compounds) for their tumor-specificity against human oral squamous cell carcinoma (OSCC) cell lines. Since 3-styrylchromone derivative showed prominent tumor-specificity, we performed here QSAR analysis with 14 newly synthesized 3-styrylchromones. ***Method:*** Tumor-specificity (TS) was calculated by dividing the mean 50% cytotoxic concentration (CC_{50}) for three human oral normal cells (gingival fibroblast, periodontal ligament fibroblast, pulp cell) (A) by that for four OSCC cells (Ca9-22, HSC-2, HSC-3, and HSC-4) (B) (T =A/B). PSE value that ☒☒reflects both tumor-specificity and cytotoxicity against cancer cells were calculated as follow: PSE= TS×100/B. Induction of apoptosis was evaluated by cell sorter. QSAR analysis was performed to determine the correlation between cytotoxicity and tumor-specificity of test compounds with 3,167 chemical descriptors, calculated from the most stabilized structure of 3-styrylchromone derivatives. ***Results and Discussion:*** Two compounds [**7, 14**] showed higher tumor-specificity (TS = 301, 182; PSE = 49842, 27898) than doxorubicin (TS = 55, PSE = 24954) and 5-FU (TS = 16; PSE = 26). When the 6 and 7th positions of chromone ring was H and OCH3 group, respectively, higher tumor-specificity was observed. Tumor-specificity was not increased, by introduction of OH, OCH3, Cl, or F into the 3, 4, 5 positions of the benzene ring. Treatment of HSC-2 cells with [**7,14**] induced the accumulation of HSC-2 cells in the subG1 and G2/M phases, suggesting the induction of apoptosis. The tumor-specificity of 3-styrylchromone derivatives were most correlated with descriptors for molecule shape and electronic charge. The present study suggests the applicability of 3-styrylchromone derivatives as seed compounds for exploring new anticancer drugs.

Identification of peptides inhibiting the specific binding between AGEs and RAGE

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Advanced glycation end products (AGEs), produced by non-enzymatic glycation between sugar (or its metabolites) and the amino residues on biomolecules, exert the inflammatory response via the stimulation of some pattern recognition receptors including RAGE. AGEs are known to increase in various age-related diseases, suggesting their involvement in chronic inflammation and tissue remodeling. Therefore, it is likely that the regulation of AGEs signaling will be the potent therapeutic target for prevention and treatment of age-related diseases. Previously, we purified the AGEs-binding factor using affinity gel with AGEs as specific ligand. In this study, we analyzed its properties, and tried to identify the peptides antagonizing the AGEs-RAGE binding. By in vitro AGEs-RAGE binding assay, AGEs-affinity chromatography and MALDI-TOF mass analysis, AGEs-binding factor with molecular mass of 70 kD was isolated. This factor inhibited AGEs-RAGE binding in concentration-dependent manner, and it was revealed that the inhibitory region existed near the N-terminus by the analysis using overlapping-peptides. Additionally, two minimal peptides exhibiting the inhibitory activity were identified. These findings suggested that AGEs-binding factor and its derived inhibitory peptides will have the potential usefulness for regulating chronic inflammation and tissue remodeling.

15-keto-prostaglandin E_2, the metabolite of prostaglandin E_2, may work as biased agonist for EP2 and EP4 receptors.

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Prostaglandin E_2 (PGE_2) are known to be involved in inflammation and cancer. There are four subtypes of E-type prostanoid (EP) receptors, EP1 to EP4, for PGE_2. Among them, EP2 receptor and EP4 receptor are frequently confused because they are both coupled with Gs-protein. Although, we have previously shown that EP4 receptor is additionally coupled with Gi-protein. PGE_2 is metabolized to 15-keto-PGE_2 by the action of 15-hydroxy prostaglandin dehydrogenase. 15-keto-PGE_2 has been considered as an inactive form of PGE_2. However, we thought 15-keto-PGE_2 may activate EP receptor subtypes as biased agonist, since the only difference between PGE_2 and 15-keto-PGE_2 is a hydroxyl or a carbonyl functional group at position 15. Here we found that 15-keto-PGE_2 acts as a full agonist for EP2 receptor, while acting as a partial agonist for EP4 receptor. In addition, when compared to the affinity and efficacy, it was found that PGE_2 is tend to activate EP4 receptor, but when it is metabolized to 15-keto-PGE_2, it prefers to activate EP2 receptor. Thus, 15-keto-PGE_2 may not be just an inactive form of PGE_2, but may involve in the biological and physiological roles that need to be elucidated.

Comparison of cytoprotective effects of piceatannol and resveratrol through SIRT1 activation

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The structure of piceatannol (PIC) is similar to that of a polyphenol resveratrol (RSV), an activator of an NAD^+-dependent protein deacetylase SIRT1. However, whether the PIC exhibit cytoprotective effect through the SIRT1 activation remains unclear. Here we compared to the cytoprotective effect through SIRT1 activation of PIC and RSV.
[Results and Methods] We used the C2C12 myoblasts in this experiment. Treatment with antimycin A, an inhibitor of complex III that induces reactive oxygen species (ROS), fluorescence of MitoSOX Red, an indicator of ROS, was increased 12-fold compared with control, but treatment with PIC or RSV suppressed AA-induced ROS reduced by 72% and 26%, respectively.
Treatment with AA significantly increased apoptosis and necrosis, but treatment with PIC or RSV significantly decreased AA-induced apoptosis and necrosis. In SIRT1 knockdown cells with siRNA, the anti-apoptotic effect of RSV was completely inhibited, whereas, the anti-apoptotic effect of PIC was partially retained.
RSV and PIC similarly decreased acetyl-histone H3 levels, suggesting SIRT1 activation.
RSV increased the expression of antioxidative enzymes such as SOD2 and catalase. On the other hand, PIC elevated catalase, but not SOD2. In the presence of deacetylase inhibitors, neither RSV nor PIC changed the acetyl-histone H3 level and antioxidant expression levels.
[Conclusion]
These results suggest that PIC has unknown cytoprotective mechanisms via SIRT1 activation independent pathway, unlike RSV.

TRPM2 channel regulates tumor angiogenesis via interacting with Stat3

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The tumor microenvironment is a complex tissue which is described as the accumulation of various stromal cells, sustaining angiogenesis and redox imbalance. Especially, tumor-associated macrophages (TAMs) are one of the major components of tumor tissues, and they play pivotal roles in prompting the various tumor growths by producing growth factors. Previously, we have reported that Transient receptor potential melastatin 2 (TRPM2), a ROS-sensitive Ca^{2+} channel, is abundantly expressed in macrophages and regulate immune responses by tuning various gene expressions. Here, we found that deletion of TRPM2 gene inhibited tumor growth, and the tumors developing in these conditions were characterized by a high density network of immature vessels, severe haemorrhage and increased hypoxia due to non-productive angiogenesis. In addition, TAMs isolated from TRPM2 knock out mice showed strong expression of proangiogenic factor VEGF according to the enhanced activity of transcription factor Stat3. Importantly, the intratumoral injection of angiostatic soluble VEGFR-1 in tumor-bearing TRPM2 knockout mice led to a rescue of tumor growth. We also found that the activation of TRPM2 channel induced by H_2O_2 suppress the activity of Stat3. TRPM2 protein showed physical interaction with Stat3 protein, and their complex was degraded gradually in the presence of H_2O_2. Together, our results suggest that TRPM2-Stat3 complex promotes functional blood vessel formation by controlling the VEGF levels depending on the environmental oxygen/redox conditions.

Effects of catecholamine (CA) metabolites on β-adrenoceptor (β-AR)-mediated relaxation evaluated in mouse/guinea pig (GP) trachea and rat thoracic aorta (TA)

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Effects of CA metabolites on β-AR-mediated relaxation were investigated in mouse (β_1)/GP (β_2) trachea and rat TA (β_3). Among tested seven CA metabolites, metadrenaline (MA) relaxed GP trachea even in the presence of clorgiline (CLG, MAO_A inhibitor). In mouse trachea, only in the presence of CLG, normetadrenaline (NMA) and MA significantly inhibited isoprenaline (ISO)-induced relaxation, which was also inhibited by 3,4-dihydroxyphenylglycol (DHPG) in the presence of 3,5-dinitrocatechol (3,5-DNC, COMT inhibitor). In GP trachea, NMA, MA, 3,4-dihydroxymandelic acid (DOMA), and DHPG significantly augmented ISO-induced relaxation, which was inhibited by NMA, and MA in the presence of 3,5-DNC or CLG plus 3,5-DNC, and by DHPG in the presence of 3,5-DNC. In rat TA, DHPG significantly inhibited the relaxation to CGP-12177A (β_3-AR partial agonist) in the presence of 3,5-DNC. Our findings indicate that 1) MA may have β_2-AR agonistic action; 2) NMA/MA have β_1-/β_2-AR antagonistic action although they enhance β_2-AR-mediated tracheal relaxation in the absence of CA metabolic inhibitors; 3) DHPG shows β_1-/β_2-/β_3-AR antagonistic action, and this is particularly remarkable for β_3-AR. Our observations may partly explain some of the pathologies associated with pheochromocytoma, which is characterized by elevated CA metabolites levels.

Splicing polymorphism and its function in TRPA1 channel

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The TRPA1 channel is Ca^{2+}-permeable non-selective cation channel that shows exquisite sensitivity to reactive oxygen species (ROS). While accumulating evidence has indicated that TRPA1 mediates various physiological functions, such as pain sensing, in mice, the function of TRPA1 is not fully understood in human. Here, we identified and characterized novel splice variants of TRPA1 from human brain cDNA library, one of which excludes the 5′ part of exon 2. Our electrophysiological and intercellular Ca^{2+} measurements revealed that this TRPA1 variant has higher redox sensitivity than intact TRPA1 despite no difference in the sensitivity to the TRPA1 agonist, allyl isothiocyanate. Interestingly, overexpression of the variant increases mitochondrial ROS levels. Altogether, these results suggest that the novel human TRPA1 splice variant exhibits hyper sensitivity to ROS by changing the cellular redox status.

Structural insights into the subtype-selective antagonist binding to the M2 muscarinic receptor

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Human muscarinic receptor, M_2 is one of the five subtypes of muscarinic receptors belonging to the family of G protein-coupled receptors. Muscarinic receptors are targets for multiple neurodegenerative diseases. The challenge has been designing subtype selective ligands against one of the five muscarinic receptors. We report high resolution structures of a thermostabilized mutant M_2 receptor bound to a subtype selective antagonist AF-DX 384 and a non-selective antagonist NMS. The thermostabilizing mutation S110R in M_2 was predicted using a theoretical strategy previously developed in our group. Comparison of the crystal structures and pharmacological properties of the M_2 receptor shows that the Arg in the S110R mutant mimics the stabilizing role of the sodium cation, that is known to allosterically stabilize inactive state(s) of class A GPCRs. Molecular Dynamics simulations reveal that tightening of the ligand-residue contacts in M_2 receptor compared to M_3 receptor leads to subtype selectivity of AF-DX 384.

Structural analysis of sphingosine 1-phosphate receptor

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The bioactive lipid sphingosine 1-phosphate (S1P) binds to five known G protein-coupled receptors, $S1P_{1-5}$, and acts as a second messenger during cell signaling. Among them FTY720 targeting $S1P_1$ is used for immunosuppressive agent for the treatment of autoimmune disease. However, FTY720 acts through multiple S1P receptors, the mechanism of action through one or more of these receptors may account for its side effects. In 2011, the X-ray crystal structure of antagonist-bound inactive state $S1P_1$ was solved, but FTY720 is an agonist. Solving the structure of agonist-bound active state $S1P_1$ is expected not only to elucidate the mechanism of $S1P_1$, but to design of a more selective and effective drug.

First, we attempted purification of $S1P_1$R and G protein complex for structural analysis. However, the expression level of wild-type $S1P_1$ is very low. To improve this problem, co-expressing dominant negative Gi and G$\beta\gamma$ with $S1P_1$ increased the yield and enhance the stability of $S1P_1$-G protein heterotrimer complexes. Negative stain electron microscope (EM) and 2D class averages revealed uniformity and stable complex particles suitable for cryo-EM.

Annexin A2 is involved in activation of ERK upon endothelin-1 stimulation

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Endothelin receptors (ETRs) is one of G protein coupled receptors, and consist of ET type A receptor (ET_AR) and ET type B receptor (ET_BR). The overexpression of endothelin (ET)-1 or ETRs is related to malignancy of human cancer, although ET-1 was originally identified as an endothelium-derived vasocontractile peptide. In cancer cells, ET-1 activates various signaling pathways, including mitogen-activated protein kinase, phosphatidylinositol 3-kinase, protein kinase C through ETRs, although the mechanisms by which ET-1 activates these signaling pathways remain uncertain. Here, we found that ETRs interacted with annexin A2, which is overexpressed in various cancers. Annexin A2 bound to ET_AR and ET_BR. Upon ET-1 stimulation, serine phosphorylation of annexin A2 increased, while there is no change in tyrosine phosphorylation of annexin A2. Furthermore, we found that annexin A2 silencing suppressed activation of ERK upon ET-1 stimulation. These results suggested that interaction of ETRs and annexin A2 may play important roles in activation of extracellular signal-regulated kinase upon ET-1 stimulation.

Inhibition of aquaporin-3 in macrophages by a monoclonal antibody as potential therapy for liver injury

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Aquaporin 3 (AQP3) is a water and hydrogen peroxide (H_2O_2)-transporter expressed in various epithelial cells and in macrophages. Here, we developed an anti-AQP3 monoclonal antibody (mAb) that inhibited AQP3-facilitated H_2O_2 transport and prevented liver injury in an experimental animal model. Using AQP3 knockout ($AQP3^{-/-}$) mice in a CCl_4-induced model of liver injury and fibrosis, we found that AQP3-facilitated H_2O_2 uptake into macrophages was responsible for nuclear factor-κB (NF-κB) cell signaling and macrophage activation during acute liver inflammation. The hepatic inflammation, oxidative stress, and stellate cell activation caused by activated macrophages was dependent on macrophage AQP3 expression. Administration of an anti-AQP3 mAb, which targeted an extracellular epitope on AQP3, prevented liver injury by a mechanism involving inhibition of AQP3-mediated H_2O_2 transport and macrophage activation. These findings implicate the involvement of macrophage AQP3 in liver injury, and provide evidence for mAb inhibition of AQP3-mediated H_2O_2 transport as therapy for macrophage-dependent liver injury.

Amino acid starvation induces glycine transporter 1 gene expression.

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Glycine is a co-agonist at NMDA receptors and an agonist at glycine receptors. Glycine transporters reuptake glycine from the synaptic cleft and regulate glycine concentration. Glycine transporter subtype 1 (GlyT1) is abundant in astrocytes and GlyT1 inhibitors have analgesic effect. We found that GlyT1 mRNA expression in C6 glioma cells was increased during amino acid starvation. To investigate the mechanisms underlying GlyT1 mRNA upregulation, we focused on ATF4 (activating transcription factor 4) that is activated during amino acid starvation and mTOR (mammalian target of rapamycin) that is inactivated during amino acid starvation. Tunicamycin, an ER stress inducer that upregulates ATF4 expression, led to increase of GlyT1. Next, we examined the involvement of mTOR in GlyT1 expression. The mTOR inhibitor rapamycin increased GlyT1 mRNA expression in the culture medium with amino acids. Moreover, ATF4 mRNA expression was also increased by rapamycin. These results indicate that ATF4 increases GlyT1 and mTOR downregulates GlyT1 gene expression.

SIRT1, a protein deacetylase, contributes to mitophagy by promoting autophagosome-lysosome fusion in the cardiomyocyte.

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Background: Damaged mitochondria is removed by autophagy. This process includes engulf of mitochondria by autophagosomes and degradation of autophagosomes by lysosomes. We recently reported that activation of SIRT1, a protein deacetylase, promotes autophagosome degradation and reduces damaged mitochondria in the heart of a mouse model of muscular dystrophy. Here, we examined how SIRT1 participates in mitochondrial autophagy (mitophagy) in the cardiomyocyte.

Methods and Results: Mitophagy was induced by CCCP (20 μM), a mitochondrial uncoupler, in H9c2 cardiomyocytes. Western blotting showed that levels of succinate dehydrogenase B and cyclophilin F, mitochondrial proteins, were reduced by CCCP. These reductions in mitochondrial proteins were significantly blocked by siRNA-mediated SIRT1 knockdown (KD). CCCP increased level of LC3-II, an autophagosome marker; however, LC3-II level was rather increased in SIRT1 KD cells, suggesting a role of SIRT1 in autophagosome degradation. Mitophagosomes defined as autophagosomes (LC3 dots) including fragmented mitochondria (Tomm20) in immunostaining were increased by CCCP. In contrast, SIRT1 KD promoted accumulation of mitophagosomes compared with control cells, suggesting disturbance of mitophagosome clearance. Finally, CCCP-induced autophagosome-lysosome fusion analyzed by colocalization of LC3 dot and LAMP1, a lysosome marker, was significantly suppressed by SIRT1 KD.

Conclusion: These findings suggest that SIRT1 plays a role in mitophagy at autophagosome-lysosome fusion in cardiomyocytes.

Hyaluronan synthase inhibitor induces apoptosis in canine mammary tumor cells through inhibition of spheroid formation

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Hyaluronan (HA) is one of the main components of the extracellular matrix. HA synthase (HAS) and hyaluronidase (HYAL) isoforms have been shown to influence malignant potential in cancer cells. Formation of a cohesive multicellular group has been reported to facilitate cancer progression, leading to distant metastasis without apoptosis. Previously, we demonstrated that the HAS inhibitor 4-methylumbelliferone (4-MU) inhibits cell proliferation and mobility. In this study, we used the canine mammary tumor cell line AZACB to investigate whether inhibiting HA production reduces cancer spheroid formation and induces apoptosis. In AZACB cells cultured under standard conditions, 4-MU decreased HA production, cell proliferation, and mobility, and increased Bim expression as an apoptosis marker. In addition, 4-MU inhibited the expression of HAS2 and the HA receptor RHAMM. The plastic ware was coated with poly (2-hydroxyethyl methacrylate) (poly-HEMA) to obtain a low adhesive scaffold. Cells cultured on the poly-HEMA-coated plastic ware exhibited spheroid formation without altering Bim expression. Addition of 4-MU decreased cell viability and increased Bim expression and the number of the annexin V/PI-positive (AP) cells. Moreover, exogenously applied HYAL decreased the number of spheroids and increased the number of AP cells. HA is likely necessary for spheroid formation and thus apoptosis evasion in cancer cells, suggesting that HA production could be a possible pharmacological target for tumors.

Azulene derivatives act on mitochondria and induce apoptosis of cancer cells.

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Cancer cells obtain anaerobic glycolysis and metabolic abnormalities due to reduced mitochondrial function. We have found that guaiazulene affects the metabolism of the nematode *C. elegans* and cultured mammalian cells. We investigated whether azulene derivatives synthesized from guaiazulene as a precursor have more effective interference with metabolism.

Adding azulene derivatives to cultured mammalian cells, cell growth was suppressed in immortalized cells and cancer cells, but not normal fibroblast. This result suggests that azulene derivatives affect cells with increased metabolic activity. Next, we investigated whether azulene derivative-treated cells did not proliferate due to cell cycle arrest or apoptosis. Whereas the treatment of azulene derivatives did not significantly change the cell cycle in flow cytometry, cleaved PARP was increased in HeLa cells in a time-dependent manner. These results indicate that the azulene derivative induces apoptosis instead of cell cycle arrest. Moreover, we observed the mitochondrial status using MitoProbe™ JC-1. HeLa cells treated with azulene derivative were observed to undergo mitochondrial depolarization. Our findings indicate that azulene derivatives reduce mitochondrial function to induce apoptosis. We are analyzing where azulene derivatives act in the metabolic pathway.

Protective effects of Rab1a protein against cytotoxicity

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The prenylated Rab acceptor 1 (PRA1) superfamily member PRAF3 plays crucial roles in membrane traffic as a GDI displacement factor *via* physical interaction with a variety of Rab proteins, as well as in the modulation of antioxidant glutathione through its interaction with EAAC1. It is known that the toxicity of the host cell is induced by the overexpression of PRAF3, however, the factors capable of cancelling the cytotoxicity remained unknown. Our findings demonstrate that Rab1a can protect from the toxicity of PRAF3-overexpressed cells. Protective effects of Rab1a protein against the cytotoxicity could further suggest that PRAF3 and Rab1a are closely related to each other physiologically and genetically.

Expression profile of xenobiotic efflux transporters in mouse neural stem cells

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Neural stem cells (NSCs) play important roles in neurogenesis since they have self-renewal ability and pluripotentiality to differentiate into neurons and glial cells. Recently, it has been reported that differentiation of NSCs is regulated by endogenous metabolites. Therefore, we hypothesized that efflux transporters such as ATP-binding cassettes (ABCs) may regulate function of NSCs via excretion of metabolites. We first examined mRNA expression of ABCs in primary cultured NSCs derived from murine embryonic cortex: Quantitative PCR was performed at 3, 6, and 9 days after the primary culture. Abcb1b, Abcg2, Abcc1, Abcc4, and Abcc5 were expressed in the NSCs. Among them, expression of Abcc5 was the highest and increased in a culture period-dependent manner, whereas that of other ABCs was decreased. We also characterized the NSCs by evaluating mRNA expression of basic helix-loop-helix (bHLH) transcription factors which regulate neuronal differentiation. Especially, expression of Math1 and Mash1, activators of neuronal differentiation were increased, whereas that of Hes1 and Hes5, suppressors of neuronal differentiation were decreased during the culture period. Thus, some of the xenobiotic efflux transporters may be associated with differentiation of NSCs, and further studies are required to understand detailed regulatory mechanisms.

Development of Alveolar Epithelial Type II Cells from Human Induced Pluripotent Stem Cells

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It is well known that some drugs cause pulmonary toxicities, such as interstitial pneumonia and it is important to minimize the risk of the drug-induced respiratory diseases for patient safety. However, it is difficult to obtain human lung cells and culture the cells for a long-term period to explore the mechanistic approach for the adverse effects. In this study, we tried to induce alveolar epithelial type II (AT2) cells from human induced pluripotent stem cells (iPSCs) using two-dimensional culture method. Differentiation was performed by mainly two steps; the first step was to generate lung progenitor cells and the second step was to induce AT2 cells from lung progenitor cells. The differentiated cells were collected, extracted RNA, and characterized by quantitative real-time PCR. We found that the differentiated cells from human iPSCs expressed AT2 cell markers, such as surfactant protein C, surfactant protein B, ATP binding cassette subfamily A member 3, and solute carrier family 34 member 2, suggesting the cells exhibit AT2-like properties. We are currently working on drug-induced pulmonary toxicities using AT2 cells.

Development of human intestinal organoids from iPS cell technology

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Intestinal analysis has been usually performed using established cell lines or primary cells in 2D culture. However, these culture systems can not satisfy the complexity of 3D structure and diversity of composed cell types in the intestinal epithelial tissue.

Here, we report the generation of intestinal organoids using human iPS cells (iPSCs) by sequential treatment with various cytokines and compounds. We observed that almost cells were double positive for the definitive endoderm markers SOX17 and FOXA2 at day 3 of differentiation. The expression of *CDX2*, a marker of the mid/hindgut, was upregulated at day 7 of differentiation, and floating and semi-adherent spheroids were positive for CDX2. Within several days after floating spheroids were embedded in Matrigel and incubated in intestinal growth medium, round organoids were observed at day 21. Immunocytochemical analysis revealed that these organoids consisted of monolayer cells, which were positive for intestinal markers E-cadherin (ECAD) and KLF5. In addition, RT-qPCR analysis revealed that multiple epithelial cell markers, *LGR5* (intestinal stem cells), *VIL1* (enterocytes), *MUC2* (goblet cells) and *LYZ* (paneth cells) were upregulated on day 21.

These data suggest that human iPSCs are successfully differentiated into intestinal organoids consisting of epithelial monolayers.

Role of a mechanosensitive ion channel PIEZO1 in muscle satellite cells

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Muscle-resident stem cells called muscle satellite cells (MuSC) play an essential role in muscle regeneration. Mechanosensation is presumed to be critical for activation of MuSCs, but the molecular entity that determines the cell fate in MuSCs through converting the mechanical stimuli into biochemical signals remains to be elucidated. Here we identify PIEZO1, a mechanosensitive ion channel that is activated by membrane tension, as a critical determinant for activation of MuSCs. *In silico* analysis demonstrates that PIEZO1 is predominantly expressed in MuSCs but not in mature myofibers. By utilizing *Piezo1-tdTomato* mice where endogenous PIEZO1 is fused with a fluorescent protein tdTomato, our immunofluorescent analysis reveals that PIEZO1 is accumulated to the cleavage furrow during cell division of MuSCs. Moreover, a conditional deletion of *Piezo1* leads to delayed myofibers regeneration after cardiotoxin-induced myofiber injury, at least in part due to the cell division delay in MuSCs. Thus, our results indicate that PIEZO1 is a bona fide mechanosensor whose ion channel activity is required for completion of cell division in MuSCs.

LPA-induced increase in triple-negative breast cancer stem cells via IL-8 production.

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Triple-negative breast cancer (TNBC) is a highly aggressive cancer with fewer effective targeted therapy. Since growing evidence suggests that TNBC is originated from breast cancer stem cells (BCSCs), it is required to elucidate the molecular mechanism of BCSC proliferation for new drug development. We have previously reported that a lipid mediator lysophosphatidic acid (LPA) increased BCSCs via Ca^{2+} signaling pathway. In this study, we examined whether calcineurin/NFAT pathway is involved in the LPA-induced increase in BCSCs. We found that LPA stimulation increased the transcriptional activation of NFAT. The calcineurin inhibitor cyclosporine A inhibited both LPA-increased NFAT activation and increase in BCSCs. We next examined the downstream signaling pathway. To identify NFAT target gene which is involved in the LPA-induced increase in BCSCs, we performed RNA-sequencing using MDA-MB-231 cells. We identified that 428 transcripts were upregulated by LPA by two fold or more. Among them, we focused on proinflammatory cytokine IL-8 which promoter contains NFAT consensus sequence. We found that LPA increased IL-8 production in MDA-MB-231 cells. In addition, a selective IL-8 receptor antagonist inhibited the LPA-induced increase in BCSCs. These results suggested that LPA increases BCSC through the NFAT-mediated IL-8 production.

Assessment of drug-induced contractility by simultaneous recording of cell motion imaging and electrical impedance

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Drug-induced cardiotoxicity is critical in the non-clinical testing. Applications of iPS-derived cardiomyocytes (iPS-CMs) hold great promise as a human cell-based platform. To date, multielectrode array (MEA) system has been widely used as a standardized assay to detect proarrhythmia risk with iPS-CM. In addition, evaluation of inotropic effects in vivo is recognized as a safety pharmacology in drug development. Given its impact on drug development, it should be useful to detect the drug-induced effects on contractility in vitro. In the present study, we used the cell motion imaging system (CMI) and the measurement of cell-induced electrical impedance (IMP) for the contractility assessment. We confirmed the effects of isoprenaline and verapamil using these systems. Simultaneous recording of CMI and IMP showed clear correlation between CMI and IMP. Our results suggest that both CMI and IMP can monitor the contraction movement of iPS-CMs.

Assessment of developmental neurotoxicity using human iPS cells

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Evaluation of developmental toxicology has been an integral part of safety assessment issue for new compounds. One of the basic ideas behind developmental toxicity tests is that children differ significantly from adults in some aspects of their basic biology and responses to compound exposures.

Because current developmental neurotoxicity (DNT) guideline (OECD TG426) requires a lot of animals and costs, it is necessary to establish more predictable approach using human iPS cells (hiPSCs). Here, we tried to search suitable structural and functional endpoints by evaluating antifouling agent, such as tributyltin (TBT), as a positive compound using hiPSCs, which was expected to provide novel human cell-based applications for alternative in vitro testing approaches.

We focused on neural differentiation process (Structure) using hiPSCs. TBT reduced the expression of several genes, including *OTX2*, a marker of neurogenesis. We further focused on electrophysiological properties (Function) using hiPSC-derived neurons. TBT reduced the number of spikes and network burst neurons using microelectrode array (MEA) recordings. These data suggest that TBT inhibited neural differentiation from iPSCs and spontaneous firing of neurons. Our data indicate that integrated analyses using iPSCs and iPSC-derived neurons are useful for DNT assessment.

Deep learning for the prediction of seizure liability and MoA of drugs based on the electrophysiological activities in hiPS cell-derived neurons

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Human iPSC-derived neurons are expected to be applied to toxicity evaluations in nonclinical studies and drug screening. Microelectrode array (MEA) measurement system is suitable to evaluate the neuronal electrophysiological responses to drugs. We have previously reported the electrophysiological responses to convulsants using MEA in cultured hiPSC-derived neurons. In this study, we aimed to develop an analytical method enabling the evaluation of toxicity and the classification of MoA of convulsants using multivariate analysis and deep learning. hiPSC-derived cerebral cortical neurons were cultured on Micro-electrode array (MEA) plate, and the pharmacological responses over 10 drugs in spontaneous firings were obtained. We firstly constructed the raster plots of spontaneous firing and the divided image data. The 4096 feature vectors of the divvied image data in raster plots were extracted by pre-trained model. Next, CNN model was trained with feature vectors each drug name. Using this trained CNN model, we have succeeded in separating the responses between non-convulsive drugs and convulsants, and classifying the MoA of convulsants. This deep learning methods are useful for the prediction of seizure liability and the classification of MoA of new drugs.

Bisphosphonate induces the mitophagy of osteoblastic cells by forming the chelate with intracellular metal ions

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Extracting teeth of patients treated with bisphosphonate (BP) occasionally induces the necrosis of jaw, but the cause of disease is still unclear. I have proved that the BPs taken into osteoblastic cells were gradually accumulated in lysosomes. In the present study, I investigated the mechanism of BP-induced cytotoxicity in osteoblast, focusing on mitochondria. MC3T3-E1 cells were used as osteoblastic cells. The uptake of BP into cells was observed by fluorescent BP. The intracellular reactive oxygenspecies (ROS) were evaluated by CM-H_2-DCFDA. Detection of autophagy and mitophagy was used DALGreen and mtphagy dye, respectively. The intracellular Ca^{2+} and mitochondrial Fe^{2+} were measured by Fluo 4-AM and Mito-FerroGreen, respectively. Zoledronate (ZD) impaired cells dose-dependently. BP taken into cells was accumulated into lysosomes. MC3T3-E1 cells were always occurred autophagy flux, but bafilomycin A1(BM), a lysosome inhibitor induced cell death, by inhibiting autophagy flux. ZD slightly suppressed the autophagy flux, however the combination of BM and ZD strongly enhanced cell death. ZD decreased intracellular Ca^{2+} and mitochondrial Fe^{2+}, and inhibited the response of intracellular ROS generation by oxidative stress, resulting in promotion of mitophagy. These results suggest that BP may form the chelate with Ca^{2+} and Fe^{2+}, and promote mitophagy of damaged mitochondria. Furthermore, the accumulation of BP into lysosomes indicates to induce cell death by inhibiting the autophagy flux.

Clioquinol changes the expression profiles and redox states of proteins involved in copper/zinc homeostasis

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Clioquinol, extensively used as an amebicide to treat indigestion and diarrhea in the mid-1900s, was withdrawn from the market due to an increase in the incidence of subacute myelo-optic neuropathy (SMON). Yet, the pathogenesis of SMON has not been fully elucidated. Since clioquinol is known as a chelator and ionophore of copper and zinc ions, we focused on proteins involved in homeostasis of these metal ions. A global analysis on human neuroblastoma cells demonstrated that among 4 isoforms of metallothionein (MT), a family of metal-binding proteins, 7 subclasses of MT -1 and MT-2A were remarkably up-regulated by clioquinol. Clioquinol-induced up-regulation of SLC30A1 (zinc exporter ZNT1) was further verified by quantitative PCR. Up-regulation of these proteins suggested that clioquinol activated metal regulatory transcription factor 1 (MTF1)-dependent transcription. We alsoexamined antioxidant 1 (ATOX1), a copper chaperone which has a redox-sensitive metal binding motif and is known to promote neuronal survival. Monitoring the redox state of ATOX1 showed clioquinol-induced thiol oxidization, possibly resulting in the inactivation of ATOX1. Collectively, dyshomeostasis of copper and zinc may be involved in the neurotoxicity of clioquinol.

Molecular mechanisms for cigarette smoke tar phase-induced cell death

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Cigarette smoke is divided in tar phase containing nicotine and gas phase. The gas phase of cigarette smoke is prepared by passing cigarette smoke through Cambridge filter. The tar phase is extracted from Cambridge filter by 2-propanol. We have previously elucidated that the gas phase induce cell death by intracellular Ca^{2+}- and protein kinase C (PKC)-dependent manner, and identified acrolein and methyl vinyl ketone as the major cytotoxic compounds in the gas phase (Mai et al., 2012; Noya et al., 2013; Higashi et al., 2014). In this study, we examined molecular mechanism (s) for cigarette smoke tar phase-induced cell death in lung cancer cells. Lung adenocarcinoma, small cell carcinoma, and non-small cell carcinoma cell lines are all sensitive to cigarette smoke tar phase. Tar phase-induced cell death is intracellular Ca^{2+}- and PKC-independent, whereas intracellular Ca^{2+} chelator and PKC inhibitor effectively suppressed gas phase-induced cell death. These results indicate that the molecular mechanisms for cell death induction by cigarette tar phase is different from that of cigarette smoke gas phase.

High-throughput immunocytochemical assay to detect adverse effects of substances using cultured hippocampal neurons

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To detect adverse effects of toxic substances on neurons, we quantitated neuron number, dendrite length and synaptic status of cultured neurons. An actin-binding protein, drebrin accumulated in the postsynaptic sites of glutamatergic synapses and a tubulin-binding protein, MAP2 were used as markers to detect synaptic changes and to visualize neuronal cell body and dendrites, respectively. We have applied this method for high-throughput analysis and showed that glutamate treatment for 10 min significantly reduced drebrin cluster density of 21-days-in-vitro (DIV) neurons in a dose-dependent manner. In this study, we examined the effects of other toxic substances. Treatment of 0.5-50 μM latrunculin A, which sequesters monomeric actin, for 5 min significantly reduced drebrin cluster density of 21-DIV neurons in a dose-dependent manner. We also confirmed that exposure of 1 Gy X-irradiation to 1-DIV neurons reduces neuron number, dendrite length and drebrin cluster density in the neurons at 21-DIV. In addition, our analysis could efficiently detect staurosporine-induced neuronal cell death in mature neurons. 24 hours exposure of 0.3 and 1.0 μM staurosporine to 21-DIV neurons significantly reduced neuron number. These results suggest that our high-content imaging analysis is useful for analyzing the effects of various toxic substances.

Assessment of Developmental Neurotoxicity during Neuronal Differentiation using a Triple-Transgenic Zebrafish Line

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The developing brain is extremely sensitive to many chemicals. Various screening methods have been used to assess the developmental neurotoxicity (DNT) of chemicals. However, assessment of toxicity during progenitor cell differentiation into neurons, astrocytes, and oligodendrocytes often requires immunohistochemistry, which is a reliable but labor-intensive and time-consuming assay. Here, we report the development of a triple-transgenic zebrafish line that expresses distinct fluorescent proteins in neurons (Cerulean), astrocytes (mCherry), and oligodendrocytes (mCitrine), which can be used to detect DNT during neuronal differentiation. Using in vivo fluorescence microscopy, we could detect DNT by 6 of the 10 neurotoxicants tested after exposure to zebrafish from 12 h to 5 days' post-fertilization. Moreover, the chemicals could be clustered into three main DNT groups based on the fluorescence pattern: (i) inhibition of neuron and oligodendrocyte differentiation and stimulation of astrocyte differentiation; (ii) inhibition of neuron and oligodendrocyte differentiation; and (iii) inhibition of neuron and astrocyte differentiation, which suggests that reporter expression reflects the toxicodynamics of the chemicals. Thus, the triple-transgenic zebrafish line developed here may be a useful tool to assess DNT during neuronal differentiation.

Development of drug assessment in central and peripheral neuronal networks using oriented nanofiber devices.

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In vitro Microelectrode array (MEA) assay systems using human iPSC-derived neurons and rodent primary cultured cells are expected to be useful for drug discovery and pre-clinical studies for toxicity and efficacy. However, as experimental problems, there are problems that the sample is likely to aggregate, it takes time until functional maturation, and dispersion culture has random structure and does not reflect the tissue structure. This is particularly remarkable for iPSC-derived neurons. In addition, since the actual state of nerve function is not yet elucidated, the fact that evaluation parameters are not established also contributes to difficulty.

As one of the methods to solve these problems, we are working on the construction of an evaluation method in which neurons are cultured on an oriented nanofiber device (NFD). When human iPSC-derived central neurons were cultured on NFD, it was found that aggregation was suppressed and synchronous burst firing, which is an indicator of maturation, was detected early. Since this NFD forms a neural network along the fiber, it can give direction to activity propagation in the network. When excitatory drugs acting on synapses were administered, the propagation speed in the network changed. The change in propagation speed reflects synaptic function, suggesting that it is useful as a drug efficacy evaluation parameter. In addition, rat DRG neurons, which are peripheral nerves, were cultured on NFD and measured by CMOS-MEA. As a result, we succeeded in measuring the axonal conduction of one cell along the fiber in multiple points. A change in conduction speed due to drug administration was detected, suggesting that it is also effective in evaluating peripheral neurotoxicity such as axon disorder.

Clinical study of risk factors associated with magnesium oxide for treatment of constipation in different age groups

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[Background] Magnesium oxide is an osmotic laxative widely used for treatment of constipation in children and the elderly. However, it has been administered indiscriminately, and side effects have been reported. [Methods] Using medical records, the present study investigated children and the elderly who had been prescribed magnesium oxide, in order to clarify the associated risk factors and side effects. Children aged 0-14 years and elderly patients aged 65 years or older who received magnesium oxide were studied. Univariate analysis was carried out, and the risk factors of side effects were investigated. Fisher's exact test was performed to calculate the odds ratios. [Results] Children who developed side effects were significantly younger than those who did not. It was also clarified that lean children were 6.5 times more likely to develop side effects than normal to obese children (P<0.05). On the other hand, elderly individuals who developed side effects had significantly higher Cr and BUN levels than those who did not. Fisher's exact test also revealed that patients with low body weight, poor renal function, and a history of hyperuricemia had higher risks of developing hypermagnesemia than those who did not (P<0.05). [Conclusion] These results suggest that younger children have a higher risk of developing side effects when taking magnesium oxide preparations, whereas elderly individuals with a lower body weight and poor renal function are also more at risk.

Predication of the risk of anaphylaxis during the general anesthesia

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In present study, we aimed to investigate the feasibility of machine-learning-based classification using clinical features of patients for risk predication of anesthesia-related anaphylaxis.

After data pre-processing, the performance of four classification methods, which were integrated with four feature selection methods, were evaluated using two-layer cross-validation. Linear Discriminate Analysis in conjunction with Recursive Feature Elimination presented the best performance, with accuracy of 0.867 and Matthews correlation coefficient of 0.558 with 25 features used in the classification.

This study presents initial proof of the capability of a machine-learning-based strategy for forecasting low-prevalence anesthesia-related anaphylaxis. In future, we plan to utilize an extended database including preoperative information and vital-sign streams to define personalized risk status for anaphylaxis.

Retrospective survey on the approved antibody drugs in Japan for the perspective of antibody "microdose" dose.

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A microdose (MD) of antibody drugs for clinical study is still not defined by any guidance.
In this survey, we studied retrospectively on the starting dose in the first in human (FIH) clinical trials of antibody drugs. We survey 52approved antibody drugs that were listed in the National Institute of Health Sciences' website as of October, 2018. We used the database survey of Pharmaceuticals and Medical Devices Agancy (PMDA) web homepage and reviewed the information from package insert, interview form, and review report. We defined the lowest dose of a human-equivalent dose of no-observed-adverse-effect levels (NOAEL) calculated from nonclinical studies as NHD and the smallest start dose treated for clinical trial as FHD. NHD in 10 items of the antineoplastic drugs was unknown as a toxicity appeared at the minimum dose set in toxicity studies. FHD in 3 items was less than 100 micrograms. FHD is normally selected and assumed the lower than the dose expected not to exert any pharmacological actions. The MD is also expected to be lower than the effect level. When NHD is unknown, 100 micrograms seems to be low enough for MD dose of antibody.

Association between use of oral hypoglycemic agents in Japanese patients with type 2 diabetes mellitus and risk of depression: a retrospective cohort study

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Type 2 diabetes mellitus (T2DM) is a risk factor for depression. Since brain insulin resistance plays a potential role in depression, the future risk of depression in patients with T2DM may be altered depending on the class of oral hypoglycemic agent (OHA) used for T2DM therapy. The aim of the present study was to determine if specific classes of OHAs are associated with a risk for co-morbid depression in T2DM. Japanese adult patients with T2DM (n = 40,214) were divided into a case group (with depression; n = 1,979) and control group (without depression; n = 38,235). After adjustment for age [adjusted odds ratio (AOR) for 10 years: 1.03; 95% confidence interval (CI): 0.99 – 1.07; P = 0.1211], sex [AOR for female: 1.39; 95% CI: 1.26 – 1.53; P < 0.0001], hemoglobin A1c [AOR for 1.0%: 1.18; 95% CI: 1.11 – 1.26; P < 0.0001], duration of T2DM [AOR for 1 year: 1.00; 95% CI: 0.99 – 1.01; P = 0.4089], and history of seven medical conditions, the odds ratios for the development of depression was significantly lower for dipeptidyl peptidase-4 (DPP-4) inhibitors [AOR: 0.31; 95% CI: 0.24 – 0.42; P < 0.0001]. However, there was no significant association for the other classes of OHAs. Therefore, this study finds that there is less risk of depression associated with the use of DPP-4 inhibitors for the treatment of T2DM.

Clustering therapeutic drugs using FDA Adverse Event Reporting System

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Therapeutic drugs have been classified based on pharmacodynamics and disease indications. However, it has gradually been revealed that profiling of side effects can be used to classify therapeutic drugs and to find novel disease indications of drugs. In this study, we generated multidimensional vectors for each therapeutic drug based on the cosine similarity of indications or side effects in the US FDA Adverse Event Reporting System (FAERS). Using the spatial density, the multidimensional vectors were clustered based on the indications and side effects in FAERS. By comparing these clusters, we were able to identify several sets of therapeutic drugs that were common in the two clusterings, including a few sets comprising of therapeutic drugs with different pharmacodynamics and different disease indications. These findings suggest that clustering therapeutic drugs based on similarities of indications and side effects reported in public databases can be useful to find new functions of therapeutic drugs.

Introduction of Interprofessional education at St. Marianna University School of Medicine and Showa Pharmaceutical University

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In recent years, medical care by multidisciplinary team has become increasingly important in order to achieve higher quality medical care. Educational institutions those train for medical professionals are also introducing interprofessional education (IPE) to help students understand the need for team practice. In 2018, we started IPE in collaboration with St. Marianna University School of Medicine and Showa Pharmaceutical University, and we conducted a questionnaire survey on students and faculty members to clarify student responses and future challenges.
For the 4th year students from both universities, the IPE program held a small group discussion using simulated cases, followed by a presentation. At the end of the IPE, questionnaire surveys were conducted on students and faculty members in charge of IPE on the day, and responses were compiled.
This survey gave a positive response regarding the implementation and content of IPE and the addition of undergraduate students other than medicine and pharmacy. On the other hand, 33% answered "difficult" regarding the difficulty level of the task. It is not easy to conduct IPE, but 89% of respondents answered that they understood the importance of collaborate in multidisciplinary team. It is necessary to continue IPE and improve the program, though creating an educational program is challenging.

Survey and Promotion of Use of Radioisotopes in Molecular Imaging and Cancer Research

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Japan Radioisotope Association

1. Background

Japan Radioisotope Association is an organization promoting the beneficial use of radioisotopes to contribute to the development of science and technology in Japan. Facing a decreasing trend of the use of radioisotope (RI) reagents, we conducted customer surveys. The result shows there is a potential demand in the fields of molecular imaging and cancer research. It is also found that the services providing information and supplying short-lived RI are required to encourage the use in these fields. We carried out further investigation to know what information and which nuclides the researchers in these fields need.

2. Information service

We conducted a questioner survey to know what type of information is needed. Based on the result of the survey, we created the user's guides about 1) usage of RI in the field of molecular imaging, 2) fundamental method of life-science experiment using RI and 3) safety handling of RI reagents in cooperation with the experts.

3. Supply of short-lived RI

We carried out another survey about the needs for the short-lived RI. The result shows there is an increasing interest in short-lived RI such as Cu-67, Zr-89, I-124, At-211, Ra-223, and Ac-225. To date, we have established the supply routes of these short-lived RI products except for At-211.

4. Future development

As a future development, we are planning the service to introduce the news and reviews on molecular images and cancer therapy researches. Also, we will continue the survey on the customer's needs for the information and short-lived RI to meet the demand timely.

Pretest for the development of a novel medication adherence assessment tool focusing on the autonomy of the elderly (1st report) -Factors affecting the total score of adherence assessment tools

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Purpose

This study aims to obtain suggestions on factors affecting the total score of evaluation tools based on the results of pretests for the development of a medication adherence assessment tools focusing on the autonomy of the elderly.

Methods

A survey using an anonymous self-administered questionnaire was conducted on 30 nurses working at acute hospitals and home-visit nursing stations. Recalling a case of elderly patients aged 65–74 having difficulties in self-management of medications, and another case in which self-management was successful, the subjects were asked to answer the same questions. The survey comprised the personal attributes of the recalled patient and 91 questions regarding autonomous adherence. A t-test or one-way analysis of variance for total scores and background factors was performed.

Results

Based on the responses of 19 nurses (recovery rate, 63.3%), we obtained data for a total of 38 cases (19 in the difficult group and 19 in the appropriate group). The total medication adherence score significantly reduced with the cognitive function of the elderly. However, it was not associated with gender, age, number and types of medications and doses taken per day, medication management method, and the use of psychiatric and external medications.

Conclusion

Cognitive function was shown to influence medication adherence. Hence, its utility as a scale to measure management ability based on patient autonomy was shown. Further evaluation of reliability and validity as a medication adherence assessment tool through an expanded survey is needed.