
PDPN Central
STRUCTURE, FUNCTION, FUTURE
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Podoplanin
Meeting 2019
February 12 - 13, 2019
Westin Maui Resort & Spa,
Ka'anapali, Maui, Hawaii
Topics Include:
PDPN Structure & Function
Role in Development
Activity in Disease
Opportunities for Biomarkers & Therapy
Website: www.PDPN.info

Pequod Room - Westin Maui Lahaina

Tuesday Feb 12

8 pm – 9 pm: Welcome committee and roundtable to define agenda.

Wednesday Feb 13

9:00 am – 9:30 am: Setup and introduction
Gary Goldberg (Rowan University)

9:30am - 10:00 am: Fibroblasts-dependent invasion of podoplanin-positive cancer stem cells in squamous cell carcinoma
*Genichiro Ishii, Tomoyuki Miyashita (*University of Tokyo and National Cancer Center Chiba)

10:00 am – 10:30 am: Targeting podoplanin-platelet interaction for suppressing tumor progression
Ai Takemoto (Japanese Foundation for Cancer Research)

10:30 am – 11:00 am: PDPN Promotes Destructive Pathways in Cancer and Arthritis
Harini Krishnan (Stony Brook University)

11:00 am – 11:30 am: Efficient detection of circulating tumor cells (CTCs) in malignant pleural mesothelioma (MPM) with "universal" CTC-chip and anti-podoplanin antibody NZ-1.2.
Kazue Yoneda (University of Occupational and Environmental Health)

11:30 am – 12:00 pm: Podoplanin targeting chimeric antigen receptor T (CAR-T) cells based on cancer specific monoclonal antibody show tumor-specific cytotoxicity against glioblastoma.
Lushun Chalise (Nagoya University)

12:00 pm – 1:30 pm: Lunch

1:30 pm – 2:00 pm: Platelet CLEC-2 upregulates podoplanin expression on macrophages and promotes their migration
Julie Rayes (University of Birmingham)

2:00 pm – 2:30 pm: The role of PDPN/CLEC-2 signaling in cutaneous allergic hypersensitivity
Miho Tsutsumi (Kyoto Prefectural University of Medicine)

2:30 pm – 3:00 pm: Regulation of T cell mediated tissue inflammation by podoplanin
Patrick Burkett (Harvard Medical School and Brigham and Women's Hospital)

3:00 pm – 3:20 pm: Fibroblast cadherins affect podoplanin expression, Src kinase induced cell motility, and transformed cell morphology
Stephanie Sheehan (Rowan University)

3:20 pm - 3:40 pm: Novel treatment modalities inhibit Src and PDPN activity in oral cancer
Edward Retzbach (Rowan University)

3:40 pm - 4:00 pm: Combination therapy targeting podoplanin to treat oral squamous cell carcinoma
Clinton A. Timmerman (Rowan University)

4:00 pm - 5:00 pm: Roundtable to define opportunities and future planning

6:00 pm: Dinner excursion

Fibroblasts-dependent invasion of podoplanin-positive cancer stem cells in squamous cell carcinoma

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Background: During the local invasion process, cancer cells often infiltrate into stroma with the aid of fibroblasts (fibroblasts-dependent invasion). Podoplanin is reportedly a cancer stem cell marker in squamous cell carcinoma. In this study, we examined whether podoplanin (+) cancer stem cells in squamous cell carcinoma have higher invasion activity during fibroblasts-dependent invasion.

Methods: We performed a collagen gel invasion assay using Fluorescent Ubiquitination-based Cell Cycle indicator (Fucci)-labeled A431 human squamous cell carcinoma cells. We counted the total number and number of invading A431 cells in S/G2/M phase using time-lapse imaging of sorted podoplanin (+) and podoplanin (-) A431 cells, co-cultured with fibroblasts. RNA interference was used to confirm that podoplanin is a functional molecule in increased invasion activity.

Results: When A431 cells were seeded without fibroblasts, there was no significant difference between the number of invading podoplanin (+) and podoplanin (-) A431 cells. On the contrary, the number of invading podoplanin (+) A431 cells was significantly higher than that of podoplanin (-) A431 cells when they were co-cultured with fibroblasts. The frequency of cells in S/G2/M phase among the invading podoplanin (+) A431 cells was similar as that of podoplanin (-) A431 cells. Knockdown of podoplanin decreased the number of invaded A431 cells significantly when they were co-cultured with fibroblasts.

Conclusion; Our current study clearly shows that podoplanin (+) A431 cells display higher invasion activity during fibroblasts-dependent invasion process, suggesting that some biological functions of cancer stem cells might become evident only within the fibrous tumor microenvironment.

Targeting podoplanin-platelet interaction for suppressing tumor progression

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Podoplanin (PDPN), an O-glycosylated transmembrane protein, has been identified as a tumor metastasis-promoting factor through its platelet aggregation-inducing ability. The expression of PDPN brings a platelet aggregation-inducing ability to tumor cells via the interaction with CLEC-2, a receptor on platelets, which increases tumor embolization leading to metastasis. In addition, growth factors released from aggregated platelets enhance tumor growth and metastasis property. Therefore, PDPN-CLEC-2 interaction is the possible target for suppressing tumor progression.

Many anti-PDPN monoclonal antibodies (mAbs) have been established and reported. Our PG4D2 mAb recognizing PLAG4 domain of human PDPN which is most crucial for the CLEC-2 binding shows the neutralizing activity against human PDPN-CLEC-2 interaction, which results in suppression of PDPN-dependent platelet aggregation. To evaluate the therapeutic availability of PG4D2, we examined the toxicity induced by suppressing the interaction of PDPN expressed in normal tissues and CLEC-2, and its tumor suppressive effects in vivo using osteosarcoma xenograft model.

PLAG4 domain is highly conserved in mammal species. However, its perimeter structure recognized by PG4D2 contains various amino acid residues dependently on species. Thus, PG4D2 showed weaker binding and neutralizing activity against monkey PDPN than human PDPN. Therefore, we constructed the surrogate antibody using homologus region of PG4D2 epitope in monkey PDPN as an antigen. One clone, 2F7 mAb could bind and neutralize monkey PDPN and suppress the monkey PDPN-dependent platelet aggregation and hematogenous metastasis likely as PG4D2 showed against human PDPN. And, we found the high-dose treatment of 2F7 to cynomolgus monkey did not induce the acute toxicity.

We also tested the suppression of metastasis and tumor growth by PG4D2 treatment using human osteosarcoma xenograft model. Osteosarcoma has been reported to express PDPN at high frequency. We searched PDPN-positive osteosarcoma cell lines and tested availability for xenograft and metastasis model. Seven out of 11 (64%) osteosarcoma cell lines were PDPN-positive. Highly PDPN-expressing (PDPN<U />high</U>) cell lines (at least 3 out of 7) exhibited high and PDPN-dependent platelet aggregation ability, while low-leveled PDPN-expressing (PDPN<U />low</U>) cell lines showed weak ability. Analyses using the cell lines available for xenograft model, PDPN<U />high</U> xenograft tumors were significantly reduced in growth by PG4D2 treatment, however, PDPN<U />low</U> tumors were not. Furthermore, hematogenous metastasis using PDPN<U />high</U> cells was also suppressed by PG4D2 treatment. As SCID-Beige mice lacking T, B cells and NK activity were used for the models, PG4D2 probably suppressed the tumor progression by the neutralization activity of PDPN-CLEC-2 interaction.

From these results, we propose PG4D2 targeting PDPN-CLEC-2 interaction would be a promising strategy for suppression of PDPN<U />high</U> tumors. Screening strategy of PDPN<U />high</U> patients suitable for this treatment could be also important.

PDPN Promotes Destructive Pathways in Cancer and Arthritis

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Cancer and arthritis present an enormous challenge to society. They share pathogenic pathways that involve extracellular matrix degradation, tissue invasion, and inflammation. Most cancer and arthritis treatments affect normal cell function to cause significant adverse effects in patients. Specific pathways that promote cancer and arthritis progression must be elucidated to design more targeted and effective therapeutics. Podoplanin (PDPN) is upregulated in cancer cells, cancer-associated fibroblasts, immune cells, and synoviocytes that increase tissue invasion and inflammation to promote both cancer and arthritis. PDPN expression is particularly strong at the invasive front of tumors and hyperplastic regions in arthritis, where it activates matrix metalloproteases that destroy tissue architecture. In addition, PDPN and inflammatory cytokines stimulate the expression of each other by unknown mechanisms to promote inflammation in cancer and arthritis. Accordingly, targeting PDPN inhibits tissue invasion and metastasis in cancer and suppresses inflammation in arthritis. Taken together, PDPN can serve as a target for therapeutics in both cancer and arthritis. While there is clear evidence that PDPN promotes cancer and arthritis progression, the specific mechanisms by which PDPN activates matrix degradation and inflammation pathways are largely unknown. PDPN has a very short intracellular domain that binds ERM proteins and is phosphorylated by PKA and CDK5 kinases. PDPN extracellular domain is known to bind ligands such as CLEC2, CD44, galectin 8, CD9, and CCL21. These interactions need to be further characterized to understand PDPN behavior during pathological conditions. We outline some future studies that use novel biochemical tools to gain more insight into PDPN structure and function.

Efficient detection of circulating tumor cells (CTCs) in malignant pleural mesothelioma (MPM) with "universal" CTC-chip and anti-podoplanin antibody NZ-1.2.

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Background: Circulating tumor cells (CTCs) are shed from primary tumor and circulate in the peripheral blood. CTCs, as a surrogate of distant metastasis, can be potentially useful in diagnosis and monitoring therapeutic effects in malignant tumors. Among a variety of systems for detection of CTCs, the "CellSearch" is the only approved system for clinical use. However, EpCAM-negative tumor cells, such as those originating from non-epithelial cells and those undergoing epithelial-mesenchymal transition (EMT) cannot be captured with the "CellSearch" that is an EpCAM-based isolation system. Therefore, we have developed a novel polymeric microfluidic device ("universal" CTC-chip) in which any antibody to capture CTCs is conjugated (*Oncol Rep. 2017*). The CTC-chip conjugated with an anti-podoplanin antibody (clone E1) achieved a higher efficiency in capturing of mesothelioma cells than the CellSearch, and provided a significant diagnostic and prognostic information in MPM (*Cancer Sci. in press*). In the present study, we evaluated another anti-podoplanin antibody (clone NZ-1.2) as a capture antibody for the CTC-chip.

Methods: The CTC-chip was used after two-step coating with an antibody to capture CTCs (*Oncol Rep. 2017*). For E1-conjugated CTC-chip, a goat anti-mouse IgG antibody was coated as a base antibody, and then the anti-mouse podoplanin antibody clone E1 (Santa Cruz Biotech) was conjugated at the concentration of 20 μg/mL (E1-chip). For NZ-1.2-conjugated CTC-chip, a goat anti-rat IgG antibody was coated, and then NZ-1.2 was conjugated at several concentrations ranging from 200 μg/mL to 10 mg/mL. Cell capture efficiencies (the number of captured cells/the number of cells sent into the chip) were examined in experimental models in which CFSE-labeled mesothelioma cells (ACC-MESO-4) were added in blood sampled from a healthy volunteer. Then, CTC-detection performances were evaluated in 42 peripheral blood samples drawn from patients with the diagnosis of MPM. Captured cells were immuno-stained with cytokeratin, CD45 and Hoechst to distinguish CTCs from contaminated leukocytes.

Results: In experimental models, the capture efficiency with the NZ-1.2-chip was 97.9 %, which was significantly higher than that with the E1-chip (74.5%, p=0.032). In clinical samples, one or more CTCs were detected in 59.5% (25/42) with the NZ-1.2-chip and in 40.5% (17/42) with the E1-chip.

Conclusions: The anti-podoplanin antibody NZ-1.2 showed a higher performance of capturing and detecting CTCs in MPM.

Podoplanin targeting chimeric antigen receptor T (CAR-T) cells based on cancer specific monoclonal antibody show tumor-specific cytotoxicity against glioblastoma.

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Chimeric antigen receptors (CAR) are recombinant T-cell receptors genetically constructed from a single-chain variable fragment (scFv) from a monoclonal antibody (mAb) connected by a transmembrane hinge region, CD3ζ, and costimulatory signaling domains such as CD28, 4-1BB, OX-40 and inducible co-stimulator. CAR transduced T (CAR-T) cells can recognize specific tumor surface antigens independent of major histocompatibility complex (MHC) restriction, which is often down-regulated in tumors including Glioblastomas (GBM). CAR-T cells can migrate through the microvascular walls and penetrate into the tumors making it a potential therapeutic choice against tumors of the central nervous system. Podoplanin (PDPN) is overexpressed in several solid tumors including brain tumors and it is positively correlated with tumor malignancy in glial tumors and is primarily expressed in the worst prognosis mesenchymal subtype of GBM. We recognized the potential of PDPN as a therapeutic target to treat brain tumors and previously generated a third generation of CAR that targeted PDPN, by using anti-PDPN antibody NZ-1-based scFv. NZ-1-CAR T cells showed specific efficacy against PDPN-positive GBM cells in vitro and inhibited the growth of intracranial glioma xenografts in vivo. However, NZ-1 reacts with podoplanin-expressing normal cells such as lymphatic endothelial cells. To overcome this issue, we produced a new CAR (Lp2-CAR) based on cancer specific monoclonal antibody, LpMab-2 against human PDPN. LpMab-2 is a cancer-specific mAb (CasMab) that reacts with podoplanin-expressing cancer cells but not with normal cells. This novel third generation CAR from LpMab-2 recombinant scFv specifically targeted PDPN expressing glioma cells but spared PDPN expressing normal cells. Lp2-CAR transduced T cells (Lp2-CAR-T) also showed significant cytolysis against multiple patient derived glioma stem cells, demonstrating their clinical potential against GBM. Lp2-CAR-T cells could also be used in combination with other PDPN targeting agents and novel anti-cancer therapies like oncolytic viruses to generate a higher therapeutic efficacy against GBM.

Platelet CLEC-2 upregulates podoplanin expression on macrophages and promotes their migration

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Platelet interaction with macrophage modulates platelet and macrophage functions in thrombo-inflammation, infectious diseases and cancer. Platelets interact with inflammatory macrophages and dampen the secretion of pro-inflammatory cytokines while promoting their migration. We have recently shown that the protective role of platelets is mediated by the interaction of CLEC-2 with podoplanin on inflammatory macrophages. Furthermore, we show that the interaction of CLEC-2 with podoplanin induces the upregulation of podoplanin on macrophages in a stimuli-dependent manner. Wild-type but not CLEC-2 deficient platelets bind to podoplanin-positive macrophages and induce the upregulation of podoplanin. From the other hand the interaction of CLEC-2 with podoplanin increases the spreading and migration of macrophages while reducing the secretion of inflammatory cytokines. These results show that during infection and inflammation, platelet CLEC-2 induces the upregulation of podoplanin on macrophages promoting their migration to the site of infection.

The role of PDPN/CLEC-2 signaling in cutaneous allergic hypersensitivity

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Podoplanin (PDPN; also known as gp38) is a transmembrane glycoprotein with high expression by fibroblastic reticular cells (FRCs), lymphatic endothelial cells (LECs) and various other cell types outside of lymph nodes (LNs). PDPN is the endogenous ligand for the C-type lectin receptor CLEC-2 (CLEC1b), which is expressed by platelets, dendritic cells (DCs), Macrophages, neutrophils, and a part of lymphocytes. Some reports indicated that PDPN/ CLEC-2 signaling contributes to DCs migration to lymph nodes during inflammation, and LN enlargement in part via relaxation and expansion of the FRC network. However, particular kinds of CLEC-2 expressing lymphocytes, function in inflammation, interaction with PDPN positive LECs and FRCs are almost unknown.

We examined the role of PDPN/CLEC-2 signaling in CLEC-2 positive lymphocytes using murine allergic contact dermatitis model. Mice (balb/c, 6w-9w, female) were sensitized by application on the shaved abdominal skin of 25ml of 1% (w/v) TNCB in acetone/olive oil (4:1) or 25ml of acetone/olive oil alone as a control. At 7 days after sensitization, we isolated abdominal skin and bilateral inguinal lymph nodes, then processed abdominal skin into dermis and epidermis. Cells were isolated from dermis, epidermis and lymph nodes by using MACs dissociator. Skin cells were stained with CD4, CD25, CLEC-2 and Lymph node cells were stained with CD4, CD25, CLEC-2 or CD62L, CD44, CLEC-2 and analyzed by flowcytometric analysis.

We researched whether CD4+CD25+cells, CD62L+CD44lo (T naïve, Tn), CD62L+CD44hi (central memory T cell, Tcm), CD62L-CD44hi (effector memory T cell, Tem) express CLEC-2 and difference between control and sensitization model.

In control, about 60 percent of CD4+cells in lymph nodes, and about 90 percent of the cells in epidermis and dermis was positive for CLEC-2.

CLEC-2 positive proportion of CD4+CD25+cells in draining lymph nodes and epidermis was decreased compared with control mouse, that of CLEC-2 positive Tn was increased and Tcm, CD4+Tcm was decreased in draining lymph nodes. Significant difference was not seen in dermal cells. Counts of CLEC-2 positive CD4+CD25+cells in draining lymph nodes was decreased compared with control mouse and that in epidermis was increased. Significant difference was not seen in dermis cells, either. Counts of CLEC-2 positive Tem, Tcm cells and CD4+Tem were increased.

In conclusion, our data suggest possibility that lymphocytes participate in inflammation reaction by controlling CLEC-2 expression, such as recruitment of CD4+CD25+ cells to inflammation cites and localize Tcm at regional lymph nodes.

Now, we investigate CLEC-2 positive lymphocyte migration ability and chemotaxis to recombinant PDPN protein or PDPN positive LECs using IVIS imaging and chemotaxis assay.

Regulation of T cell mediated tissue inflammation by podoplanin

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Pathways that regulate T cell responses in non-lymphoid tissues are critical for maintaining peripheral tolerance and preventing autoimmunity, but are also frequently co-opted by tumors to subvert anti-tumor immunity. We recently found that the cell surface glycoprotein podoplanin (PDPN) is expressed by both CD4 T helper (Th)-17 cells and regulatory T cells (Tregs), particularly those residing in certain non-lymphoid tissues, as well as by tumor infiltrating lymphocytes (TILs). Notably, PDPN was one of a module of co-inhibitory cell surface receptors that were coordinately regulated by interleukin (IL)-27, and expression of PDPN was positively correlated with expression of PD-1 and Tim-3 in both autoreactive Th17 cells and TILs. T cells which overexpress PDPN had impaired peripheral survival, while deletion of PDPN or its ligand, CLEC-2, resulted in spontaneous peripheral autoimmunity. Analysis of mice with T cell specific deletion of PDPN revealed exacerbated tissue inflammation in a model of multiple sclerosis, but also demonstrated more robust anti-tumor responses. Therefore, PDPN/CLEC-2 represents a novel pathway that regulates peripheral T cell tolerance in multiple contexts.

Fibroblast cadherins affect podoplanin expression, Src kinase induced cell motility, and transformed cell morphology

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Cancer kills over 8 million people worldwide annually, and metastasis is responsible for the vast majority of these deaths. Cancer associated fibroblasts (CAFs) remodel the tumor microenvironment to promote tumor invasion and metastasis. Src tyrosine kinase activity is often increased in cancer cells and CAFs where it disrupts adherens junctions, increases podoplanin (PDPN) expression and augments cell motility. Adherens junctions are formed by cadherins which mediate homotypic adhesion between cells. We are investigating how junctions formed by E-cadherin (E-cdh) and N-cadherin (N-cdh) affect PDPN expression, migration, and tumor progression of cells expressing oncogenic Src kinase activity. We are utilizing temperature sensitive Src (tsSrc) and fluorescently tagged E-cdh and N-cdh constructs to study the effects of cadherins, PDPN, and Src activity in real time. We are also using homozygous null cadherin knockout cells as a clear background on which to examine the effects of cadherins on, PDPN expression, cell growth, migration, and morphology. We found that forced expression of E-cdh or N-cdh decreases the motility of Src transformed cells to levels comparable to nontransformed cells in both wound healing and invasion assays. However, levels of Src kinase activity in transformed cells are not affected by forced expression of either of these cadherins. In addition, temporal regulation by temperature shift reveals that consistent Src activity is required to maintain transformed cell morphology and motility. Moreover, transformed and nontransformed cells form cadherin junctions with each other, and these appear to modulate transformed cell growth, PDPN expression, and motility within the microenvironment. These dynamic interactions are applicable to a variety of cancers including oral squamous cell carcinoma (OSCC) which can be modeled in the system. Taken together, these results indicate that cadherins act downstream of Src to regulate malignant tumor progression and may mediate tumor-stromal interactions in the cancer field.

Novel treatment modalities inhibit Src and PDPN activity in oral cancer

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Oral cancer is diagnosed in over 300,000 people worldwide every year, killing over 11 people every hour. Oral cancers derive from precancerous lesions such as oral leukoplakias (OL). However, mechanisms underlying this transformation are poorly understood. Src tyrosine kinase is overactive in oral cancer and increases PDPN expression. We can combine natural and synthetic products to inhibit Src activity. We have also developed a system that uses temperature sensitive Src (tsSrc) to induce PDPN expression. This system will help us understand the mechanisms behind PDPN expression. Podoplanin (PDPN), a transmembrane receptor, has been detected in OL and oral cancer. PDPN increases cancer cell motility and metastasis. We and others have developed potential chemotherapies to target PDPN. For instance, Maackia Amurensis seed lectin (MASL) and antibodies can be used to inhibit OSCC motility and viability. PDPN also appears to undergo cancer specific changes that can be further exploited to treat patients. Taken together, these data indicate that Src and PDPN can serve as a functionally relevant target to prevent and combat oral cancer.

Combination therapy targeting podoplanin to treat oral squamous cell carcinoma

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Oral cancer is the 11th most common malignancy in the world. The vast majority of these cancers are caused by oral squamous cell carcinoma (OSCC). These cells are resistant to current chemotherapeutic reagents and require surgical excision as the main form of treatment. This approach results in a five-year survival rate of approximately 50% depending on stage and other factors. In addition to this mortality, current treatment options cause disfigurement and permanent sequelae that decrease the quality of life for survivors. New methods are clearly needed to combat oral cancer. Extracellular receptors are very effective chemotherapeutic targets. This approach has been demonstrated by a number of agents that target cognate receptors to treat a variety of cancers. Podoplanin (PDPN) has been identified as a transmembrane receptor that promotes the progression of a number of cancers including OSCC. *Maackia amurensis* seed lectin (MASL) can target PDPN with surprising efficiency to decrease OSCC cell motility and growth. Here, we investigate the ability of MASL to work with a variety of chemotherapeutic agents to inhibit OSCC cell viability. Metabolic and Western blot data from these experiments indicate that MASL decreases mitogen activated protein kinase (MAPK) activity in OSCC cells, and displays an additive killing effect when combined with doxorubicin, but not other reagents including cisplatin, 5-fluorouracil, paclitaxel, etoposide, or methotrexate. Taken together, these data suggest that MASL may be considered as a monotherapy or combined with other chemotherapeutic agents to inhibit oral cancer progression. This opens the possibility of alternative therapies to combat oral cancer as well as further characterizing PDPN and its role in tumor development.