論文の内容の要旨

論文題目 Establishment of new cell lines/mouse xenograft tumor models of human malignant mesothelioma and an analysis of its cancer stem cell properties

和訳　ヒト悪性胸膜中皮腫における新規細胞株・マウス移植腫瘍モデルの樹立と癌幹細胞特性の解析

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Research background

Malignant mesothelioma

Malignant mesotheloma (MM) is an aggressive neoplasm arising from mesothelial surface of the pleura, peritoneum and some other organ. 80% of MM cases accounts for pleural origin and the predominant cause of MM is inhalational exposure to asbestos. Latent period of MM is 20-60 years. The incidence of MM is increasing day by day and

this disease is responsible for approximately 15,000-20,000 deaths annually worldwide. A steady rise in cases is reported in North America, Europe, Australia and Asia.

At first, it was thought that MM began in the lymphatic system and spread to the lungs or abdomen. However, the realization that tumor developed from the mesoderm and the term mesothelioma came to be accepted in 1921.

Histologically, MM can be subdivided into three types, i.e., epithelioid, (50-70% of cases), sarcomatoid (10-20%), and mixed (20-35%).

MM is almost always associated with asbestos exposure. In tissue culture, asbestos fibers can cause mutagenic events, including DNA strand breaks and deletion mutations. Other causes of mesothelioma are simian virus-40, non-asbestos mineral fibers like erionite, taconite, and radiation.

Oncogenesis of MM is involved with chromosomal aberration, gene mutation, DNA methylation and activation of telomerase. Chromosomal damage may occur due to the direct interaction of asbestos fibers with mitotic spindle apparatus. A particularly high frequency of homo deletion is seen in the 9p21 region in MM, thus causing a high frequency of deletions of the P16 and P14 genes that are located on 9p21 and a loss of expression of their proteins. Neurofibromatosis 2 (NF2) gene is found to be mutated in MM.

Methylation of Tumor suppressor genes (TSG), including methylation of RASSF1A gene, has been observed in MM as well, strongly suggesting that methylation of the promoter region of TSGs contributes to the neoplastic transformation and progression of MM.

Three types of therapy are most frequently used for MM. They are surgery, chemotherapy and radiotherapy. Surgery can only be performed during MM stages I and II. Unfortunately, MM is not usually diagnosed until it reaches stage III or IV, when surgery is not an option. The combination of cisplatin and pemetrxed, a multitargeted antifolate agent, is the approved "standard of care" for patients with unresectable MM. Radiation therapy is most often used in conjunction with surgery. Radiation therapy may have a role in disease palliation but has no real impact on survival.

Cancer stem cell model

The cancer stem cell (CSC) model has been proposed as a unique subpopulation in tumor that possesses the ability to initiate and sustain tumor growth. These cells are

thought to be maintained by self-renewal and proliferated by asymmetric cell division. CSCs persist in tissue as a distinct population, express drug resistant and DNA repair phenotypes, and cause drug resistance, relapse and metastasis by giving rise to new tumors.

It has been found that pathways such as Notch, Sonic Hedgehog, Wnt that are associated with cancer may also regulate normal stem cell development. Origin of CSC is still unclear but it has been observed that stem cells, progenitor cells and in some cases differentiated cells might be the source of CSC.

Identification of CSC is important because CSCs are thought to be therapy resistant and cause of metastasis and relapse. Drugs could be developed for cancer without any relapse, once these cells are properly identified.

CSC was first identified in 1997 by John Dick in AML and subsequently verified in breast and brain tumor. After that, CSCs have been identified in colon, pancreatic, hepatocellular, overian and several other cancers.

The apparent similarities between normal stem cells and CSCs lead to the hypothesis that CSC may be relatively resistant to common chemotherapeutic agents compared to their more differentiated counterparts. Expression of multiple drug-resistance transporters, enhanced DNA repair capacity are other probable mechanism of resistance of CSCs to cytotoxic agent.

There are two types of targets for the design of CSC depleting agents. Eliminating CSC themselves by either killing or differentiating them and disrupting CSC niche. Another group of target derives from pathways that are crucial in regulating CSC self-renewal mechanisms.

A number of cell surface markers have been found to be useful for the identification of subsets enriched for CSC. In my present study, I tried to identify CSC signature from MM cells and found that CD9, CD24 and CD26 molecules have some stem cell like characters *in vitro* and *in vivo*.

General Introduction

MM is an aggressive neoplasm and is rarely suitable for radical surgical resection and usually resistant to radiotherapy and chemotherapy. Despite the advance of new therapy,

most patients experience tumor progression or relapses. On the other hand, according to CSC theory, biological characters of CSCs are often similar to those of normal stem cells and CSCs are believed to be potential reasons for chemoresistance, metastasis and recurrence. Therefore, my present study has been undertaken to identify CSCs from human MM cells.

In this study, I first tried to establish a serial transplantation model of MM in NOD/SCID mouse to obtain unlimited MM tissue supply which is keeping the hierarchical system of CSC. Simultaneously, I tried to establish new MM cell lines. Finally, I searched CSC signatures of MM using my new cell line and the existing cell lines.

Among the CSC signatures, SP phenotype was observed in the majority of MM cell lines. Extensive cell surface antigen analysis revealed that CD9 and CD24 were also expressed in heterogeneous pattern. In some of the cell lines, asymmetric cell division-like proliferation was observed when the cells were divided into SP or CD24-expressing cells. In the transplantation assay of NOD/SCID mouse, the expressions of CD9, CD24, and CD26 were significantly correlated with tumor formation capacity. Moreover, these markers were also remarkably associated with several *in vitro* signatures including cell cycle, drug-resistance, invasion capacity, expressions of stem cell genes and sphere formation capacity. These results suggest that CD9, CD24 and CD26 correlate with stem cell signatures of MM and may be important targets for effective therapy.

Result

Establishment of new mouse lines and cell lines of MM by transplanting patient samples into NOD/SCID mice

I obtained total 9 MM tissues from patient surgical samples. Using these primary tumors, I performed serial transplantation assay by subcutaneous injection of minced tumor tissue or enzymatically dissociated cells into immune-deficient NOD/SCID mice. After 2-4 months later, secondary tumors were observed in all samples. Initial transplantation tumors were formed in thorax or subcutaneous region. I collected minced tumor tissue and injected the secondary tumors into new mice and continued injecting by the same way up to 3rd or 4th generation. However, formation of tumor was accelerated generation by generation.

Next, I tried to establish new cell lines from primary surgical samples or

generated mouse tumors by culturing dissociated tumor cells. 5 cell lines could be established from secondary or tertiary tumors of the NOD/SCID mice.

Identification of SP cells in the MM cell lines

MM is often highly chemoresistant, therefore, I attempted to determine whether MM cells contain CSC characteristics such as SP cells. I performed SP analysis of a total of 9 cell lines and found that 6 cell lines (JMN, H226, H2452, MSTO, Meso-1, and TUM1) contained a population of SP cells ranging from 0.6% to 21.9% total cells. I did not detect SP cell populations in H28, H2052 and Meso-4, suggesting that not all MM cell lines contain SP cells.

Next, I performed cell sorting and culture assays of SP cells. Following the cell sorting by flow cytometry of 6 SP-positive cell lines, I evaluated both SP and non-SP cells (main population, MP) for the appearance of SP cells at 7 days after the separation. In the case of H226 and H2452, only SP cells could regenerate both SP and MP cells, whereas MP cells could not regenerate SP cells. These results suggest that some of the MM cell lines proliferate by asymmetric cell division-like manner.

Identification of cell surface markers in the MM cell lines

Next, I attempted to determine cell surface markers that correlate with stem cell signatures. For this purpose, 9 cell lines with 106 types of antibodies were examined by FACS. Heterogeneous expressions of several markers were observed within some of the cell lines. The observed markers were CD24/CD54/CD56 in JMN, CD24 in H226, CD9/CD24 in TUM1, and CD56/CD90 in H2452.

Intriguingly, small subpopulation of CD24-positive cells (1.0-5.1%) was observed in JMN, H226, and TUM1. Expression of CD24 was also examined in other cell lines and found that CD24 was expressed in 7 out of 9 cell lines.

In the case of CD9, TUM1 cells expressed CD9 heterogeneously but not as a small subpopulation. All of the cell lines except JMN expressed CD9 significantly high and quite homogeneously. Tissue sample of the transplanted mouse tumor of patient 2 (the same origin as of TUM1) also expressed CD9 heterogeneously. These results suggest that some of the MM cell lines are composed of heterogeneous cell populations, and a hierarchy-like system exists even in the established cell lines.

Next, I separated the $CD24^+$ and $CD24^-$ populations by FACS and cultured them for additional days. I found that asymmetric cell division-like proliferation occurred only in the $CD24^+$ cells of JMN and H226. $CD24^+$ cells could subsequently give rise to both $CD24^+$ and $CD24^-$ cells to repopulate the original pattern, whereas $CD24^-$ cells repopulated almost $CD24^-$ cells. These results suggest that CD24-positive cells undergo an asymmetric cell division in some of the MM cell lines.

Expression of CD26 correlates with that of CD24 in the MM cells

CD26 has been found to be a promising target for monoclonal antibody therapy, therefore, I sought to examine the expression of CD26 in the MM cell lines and found that all the cell lines except MSTO express CD26 in significantly high level. Next, I examined the correlation between CD24 and CD26 expressions in each cell line. I found that the expressions of both markers were significantly correlated with each other, especially in the sarcomatoid type cell lines JMN and H28. These results suggest that CD26 might be an additional CSC marker, and targeting CD26 by monoclonal antibody might eliminate CD24$^+$ cells simultaneously.

Correlation of tumor growth potential with cell surface marker expression

Next, I performed transplantation assay of the cell lines to evaluate tumor development in NOD/SCID mice. My preliminary examination of transplantation potentials in MM cell lines showed that JMN, H226, and TUM1 cells could generate tumor in the mice. I also transplanted dissociated cells from mouse tumor tissue of patient 2. The cells were then separated into CD9^{hig}/CD9^{low}, CD24$^+$/CD24$^-$, CD26$^{+/high}$/CD26$^{-/low}$ or SP/MP and transplanted into the mice and monitored everyday up to 8-19 weeks. Although statistically insufficient in some cases, CD marker-positive cells or SP cells generated relatively larger tumors in general. In the case of CD24$^+$/CD26$^+$ cells of JMN and CD9^{high} cells of tumor tissue cells (secondary mouse tumor derived from patient 2), they generated significantly larger tumors than negative cells (*P < 0.05). These results suggest that the expressions of these markers correlate closely with the tumorigenic potential.

Identification of cellular characteristics of stem cell in the isolated MM cells

As MM cells often exhibit drug-resistance, therefore, next I examined the relationships between these marker expressions and drug-resistance. 12 anti-cancer drugs in JMN, H226, and TUM1 cells were examined and found that these cell lines actually showed drug-resistance against some of the drugs. Among them, daunomycin (JMN and TUM1) and etoposide (H226) significantly affected the proportion of CD24-expressing cells. Although the expression of CD49e (Integrin α5) was not affected by drug treatment, CD24 (all cell lines) and CD26 (JMN) -positive cells survived and consequently the proportion of them were significantly increased, suggesting that CD24$^+$ and CD26$^+$ cells are relatively drug-resistant.

Next, I performed cell cycle analysis of these cell lines. In the case of CD9, CD9^{high} cells of TUM1 contained G2/M-phase cells in significantly higher population than their low counterparts. CD24$^+$ and CD26$^+$ cells of JMN, CD24$^+$ cells of H226 and TUM1 also show significantly higher G2/M proportion than negative cells, indicating the higher proliferating potential of marker-positive cells.

Finally, I performed the matrigel invasion assay of the sorted cells. The

sorted cells were incubated in matrigel invasion chamber. I found that the cells with relatively higher expression of CD9, CD24 and CD26 exhibited higher invasive activity than the cells with lower expression of these cell surface markers. Taken together, these results suggest that expression of CD9, CD24, and CD26 are highly correlated with several cellular signatures of CSCs, and CD26 also could be a new cellular marker of CSC of MM in addition to CD9 and CD24.

Spheroid colony formation and induction of stem cell specific gene expressions in the MM cells

Spheroid colony formation in the stem cell medium containing human epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF) is observed in various solid tumors and this potential is one of the stem cell signatures. To determine whether MM cells have similar properties, I cultured each of the above cell line in the stem cell medium for 6 days. In the case of TUM1, most cells lost attachment to the plate and formed many floating irregular colonies. Further culture for 3-4 weeks, these cells formed typical spheroid colonies in the stem cell medium.

Using these cells, I performed SP analysis. In each cell line, proportion of SP cells was significantly reduced in the stem cell medium, suggesting that EGF and FGF signaling pathways play certain roles in SP phenotype and differentiation of CSCs.

I also examined the expression of stem cell related genes (c-Myc, Oct3/4, Klf4, Sox2, and Nanog) between the stem cell and standard medium. Interestingly, treatment of the stem cell medium leads to the enhanced expression of Nanog in each cell line. Expression of Sox2 was significantly elevated in JMN and TUM1. Klf4 was also up-regulated in H226. These results indicate that MM cell lines express several stem cell genes and these genes are significantly activated in the stem cell medium.

Finally, I examined the potential of spheroid colony formation in the FACS-separated TUM1 cells. I incubated the isolated CD9$^{high/low}$ or CD24$^{+/-}$ cells for 3 weeks in the stem cell medium and counted the number of spheroid colonies. In each experiment, CD9^{high} or CD24^{+} cells formed more spheroid colonies than CD9^{low} or CD24^{-} cells, indicating that expression levels of these markers correlate with the potential of spheroid colony formation.

Discussion and future direction

One of the difficulties of CSC research is that there is limited supply of human tumor samples. In this study, I could establish the mouse xenograft model of human MM while keeping hierarchy system of CSC by serial transplantation of MM tissues. This *in vivo* model may retain CSCs within the generated tumor tissue compared to the

tumor generated from established cell lines. Therefore, my strategy may allow me to obtain fresh tumor cells with unlimited CSCs for CSC studies. Moreover, using this novel approach, I also established 5 new MM cell lines TUM1-5.

By SP anlysis, I found that 6 of 9 cell lines contained SP cells, some of which exhibited the asymmetric cell division-like proliferation. SP is originally the phenotype of multi-drug resistant gene (ABC transporter) activity, but this phenotype was reduced in the stem cell medium. Transplantation assay of SP was performed by using cell lines. SP cells formed larger tumor than MP cells but that was not statistically significant. Therefore, SP cells did not always correlate with CSC properties *in vivo* in my experiment. Further investigation, using the patient sample may clarify more about the CSC properties of SP cells in MM.

In the extensive analysis of CD markers, I found that CD24 and CD9 express heterogeneously in some cell lines. These cells also correlated with several stem cell signatures such as asymmetric proliferation, drug resistance, invasiveness *in vitro* and *in vivo* transplantation analysis.

CD24 is known as a heat-stable antigen, and previous studies have reported that CD24 may be an important marker for CSCs of pancreatic cancer and ovarian cancer. The expression of CD24 correlated significantly with CD26 in some MM cell lines, especially in sarcomatoid type. Moreover, in transplantation studies $CD24^{+}CD26^{+/high}$ cells generated significantly larger tumors than their negative counterpart. CD26 is widely expressed on human tissues and certain types of cancers, and known to be involved in cancer metastasis and progression. More recent study showed that a subpopulation of CD26-positive cells correlated with tumorigenic and metastatic capacities within human colorectal CSCs.

Although CD24 and CD26 markers were widely expressed in both normal and cancer cells, they can be new CSC markers in MM when they are expressed in significantly high levels. Such case is CD44, and targeting this molecule, could eradicate AML stem cells.

Another intriguing marker is CD9, which belongs to the tetraspanin super family. In my experiment, CD 9 positive cells of patient sample formed significantly larger tumors than their negative counterpart and also showed higher invasion and sphere formation potential. My research group has already reported that CD9 correlates with CSC potentials in B-ALL and anti-human CD9 monoclonal antibody (5H9) has been shown to inhibit the proliferation of stimulated T cells and induce

apoptosis of Jurkat T cells. Therefore, future studies will examine the effectiveness of the anti-human CD9 monoclonal antibody in the MM.

I also found that several stemness genes were significantly up-regulated in the stem cell medium. As the stemness genes have an essential role in self-renewal and fate determination basal expressions of these genes suggest that the MM cells of the cell lines inherently have stem cell potentials or contain subpopulation of CSCs.

In conclusion, my study identified CD9, CD24, and CD26 as important stem cell signatures in MM. Since total eradication of cancer cells is very difficult without elimination of CSCs, targeted therapy against these markers and stem cell signatures will be a novel therapeutic approach in the near future.