

CHEMORESISTANCE RELATED TO HYPOXIA ADAPTATION IN MESOTHELIOMA CELLS FROM TUMOR SPHEROIDS

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Background: Hypoxia has been noted as a key factor for induction and maintenance of cancer stemness thereby leading to therapy resistance. Three-dimensional (3D) spheroid models demonstrate a heterogeneity of hypoxic regions replicating the *in vivo* situation within tumors. Utilizing an established 3D spheroid model, we investigated whether extrinsic hypoxia reinforced chemoresistance in malignant pleural mesothelioma (MPM) spheroids. **Materials and Methods:** Tumor spheres were generated from Meso-1 (a typical human MPM cell line) cells having high spheroid-forming ability. To induce hypoxia condition, we utilized a hypoxia chamber with regulation of O₂ and CO₂ levels. Cell viability was estimated by a WST-8 assay. Real-time polymerase chain reaction and Western blot were performed to evaluate the expression at mRNA and protein levels. **Results:** Compared with cells cultured in the two-dimensional monolayer model, tumor sphere cells showed elevated mRNA levels of cancer stemness markers (CD26, CD44 and ABCG2) and protein levels of the stemness and hypoxia adaptation markers (ABCG2, ALDH1A1 and HIFs). Correlating with this, 3D spheroid cells were more resistant to permethrexed and topotecan than the two-dimensional cells, indicative of their potential for hypoxic adaptation. Furthermore, significantly stronger resistance to both chemotherapeutic agents was observed in spheroid cells upon hypoxic challenge compared to spheroid cells under normoxia. **Conclusion:** From the present data, it is concluded that hypoxia adaptation of MPM cells from tumor spheres could enhance their chemoresistance.

Key Words: chemoresistance, hypoxia, tumor spheroid, mesothelioma, stemness.

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Malignant pleural mesothelioma (MPM) is a highly aggressive type of tumor typically caused by exposure to asbestos and has limited treatment options. MPM develops slowly with an average latency period of ~40 years and is often diagnosed in its later stages, preventing many patients from being treated with curative intent during the most critical point of their disease [1]. MPM is highly chemoresistant and subsequently has a poor prognosis, with median survival ranging from 8 to 14 months after diagnosis [2]. With this unmet clinical need, understanding the mechanism of chemotherapy resistance could lead to better optimization of chemotherapy regimens for MPM and an improvement in the present treatment approach against MPM.

Chemoresistance results in cancer relapse and a poor response to therapy, with the resistance associated with a small sub-population of cells within tumors known as cancer stem cells (CSC) [3]. CSC typically have properties to allow unlimited self-renewal and to initiate the growth of heterogeneous cancer cell populations [4]. CSC are considered mediators of cancer metastasis, drug resistance and cancer relapse, and were first identified in hematopoietic malignancies and later on in a wide variety of solid tumors and

cultured cancer cell lines [4]. Studies have shown that the various environmental niches found in tumors play a central role in the development of CSC, and dictating their cellular properties [4]. Out of the niches, CSC rely most on hypoxic adaptation for survival and upkeep of their chemoresistance [5].

Identification of CSC is a complex process and it relies on different strategies. Currently, two-dimensional (2D) culture systems, in which flat monolayer cells are cultured, are most frequently used for the research of cell-based assays. The key limitation of the traditional 2D culture system is the loss of the *in vivo* architecture and resultant microenvironments [6]. On the other hand, three-dimensional (3D) spheroid cell cultures are recognized as a more accurate representation of *in vivo* tumors and their microenvironments, whilst retaining the biological characteristics of original tumors better than conventional 2D monolayer cultures. Spheroidal 3D cell culture systems are presently established as an effective method to easily concentrate CSC from heterogeneous parental cancer cells [7]. Recent studies have shown that the spherical 3D culture system is a highly efficient method of separating CSC from cancer cell lines or many solid tumors [7]. However, there is currently a paucity of published data that comprehensively demonstrate the CSC properties of spheroid derived MPM cells.

In this context, the present study was undertaken to investigate the changes in CSC characteristics, specifically chemoresistance related to hypoxia adaptation in a human MPM cell line (Meso-1) grown in 3D vs 2D cell culture conditions. Meso-1 is widely

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Abbreviations used: 2D – two-dimensional; 3D – three-dimensional; ABC – ATP-binding cassette; ALDH – aldehyde dehydrogenase; CSC – cancer stem cell; FBS – fetal bovine serum; HIF – hypoxia-inducible factor; MPM – malignant pleural mesothelioma.

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used in pre-clinical investigations and is known to have high spheroid-forming ability [8].

MATERIALS AND METHODS

All cultures and reagents were purchased from Nacalai Tesque (Japan), Sigma-Aldrich (USA), Thermo Fisher Scientific (USA) and PeproTech (USA), unless otherwise indicated. Anti-hypoxia-inducible factor (HIF)-1 α and anti-HIF-2 α antibodies were purchased from Novus Biologicals (USA) and Acris (Germany), respectively, and anti-CD26 antibody was obtained from Abcam (UK). All other primary antibodies were purchased from Cell Signaling Technology (USA). As secondary antibodies, anti-mouse IgG-peroxidase and rabbit IgG-peroxidase antibodies were obtained from MBL (Japan).

Meso-1 human MPM cell line (Riken BioResource Center, Japan) was used in this study. The cells were cultured in RPMI-1640 medium containing 10% fetal bovine serum (FBS) and 0.5% penicillin/streptomycin in humidified 21% O₂, 5% CO₂ at 37 °C, as a 2D culture method. As a mild hypoxia 2D culture condition, humidified 1% O₂, 5% CO₂, at 37 °C was utilized in a hypoxia chamber with regulation of O₂ and CO₂ levels (Biospherix Ltd. USA). To form tumor spheres from Meso-1 cells, after 2D culture, the cells were suspended in stem cell medium, which consisted of DMEM/F12 medium (Wako Co., Japan), 20 ng/mL epidermal growth factor, 20 ng/mL basic fibroblast growth factor, 2% B27 supplement and 0.5% penicillin/streptomycin [9]. The cells were suspended in the stem medium at a density of 5000 cells/ml, and each cell suspension was transferred into each 100 mm Ultra-Low Attachment Culture Dish (Corning Inc, Japan). The cells were cultured for 7–21 days, and subsequently, the spheres were filtered using a spheroid catcher with a 77 μ m diameter filter (Watson Co., Japan). The residues on the filters were used as tumor sphere samples.

Cell viability was routinely evaluated using the WST-8 method. In the case of determining cell viability in tumor spheres, the MTT method was utilized. Protein level was estimated by Western blot analysis. Each mRNA level was analyzed by real-time polymerase chain reaction using SYBR protocol, and the expression was quantified using 2^{- Δ Ct} method with normalization to mRNA expression of RPL32. The sequences of each primer set are shown as below.

Aldehyde dehydrogenase (ALDH) 1A1 primers:

Forward: 5'-AGTGCCCTTTGGTGGATTC-3'

Reverse: 5'-AAGAGCTTCTCTCCACTCTTG-3'

ATP-binding cassette (ABC) G2 primers:

Forward: 5'-ATCCAAGGCCTCCTGAGCAG-3'

Reverse: 5'-ACTGGCTTAGACTCAAGCACAGCA-3'

CD26 primers:

Forward: 5'-AATCACATGGACGGGGAAAG-3'

Reverse: 5'-AGACCACCACAGAGCAGAGTAGG-3'

CD44 primers:

Forward: 5'-AATGGCCCAGATGGAGAAAG-3'

Reverse: 5'-GGGAGGTGTTGGATGTGAGG-3'

RPL32 primers:

Forward: 5'-AACCTGTGTCAATGCCTC-3'

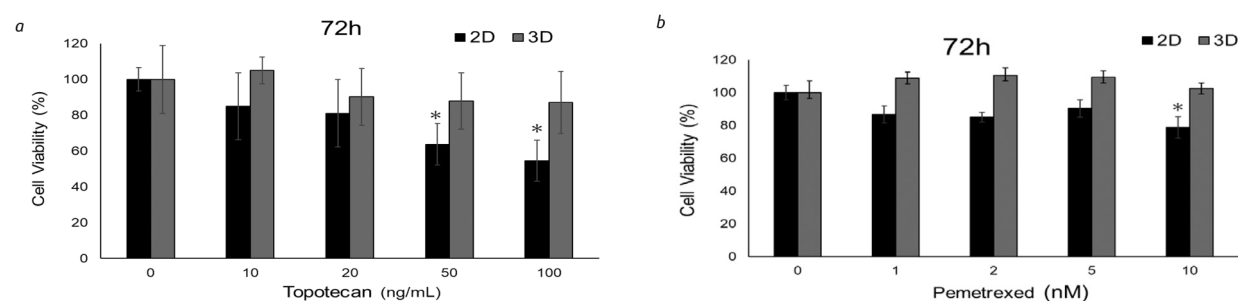
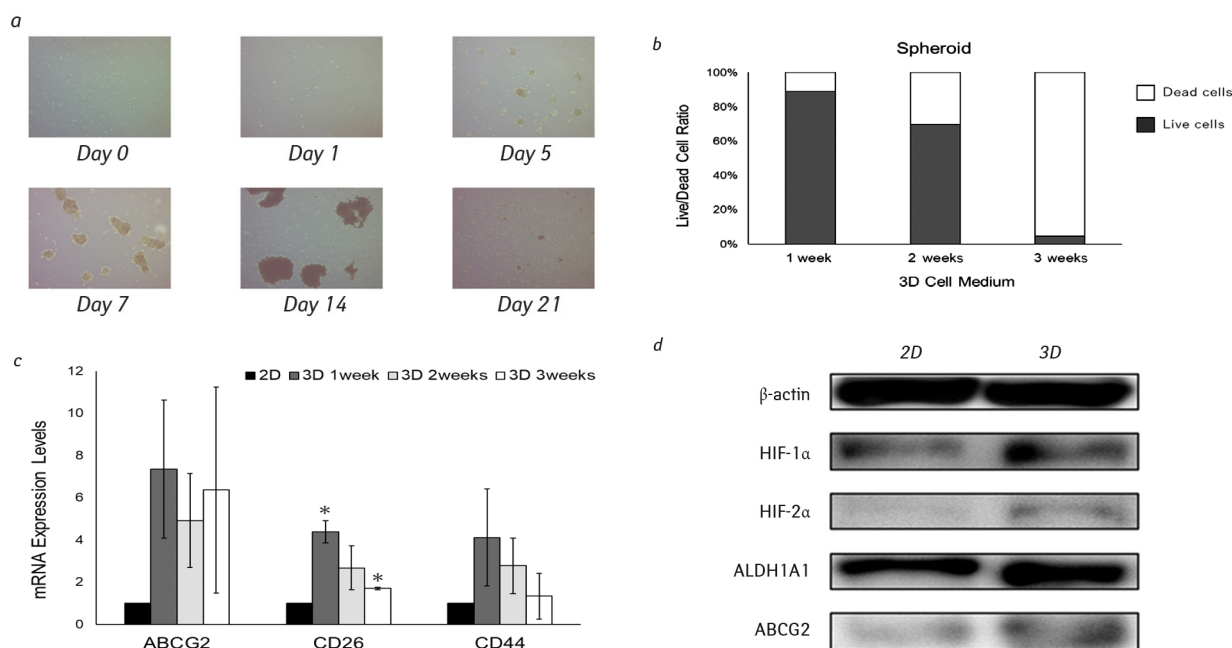
Reverse: 5'-CATCTCCTCTCGGCATCA-3'

Each experiment was repeated independently at least three times under the same conditions. Differences between control and each dose group were analyzed using Student's *t*-test. Differences among groups were analyzed by one-way ANOVA followed by Tukey — Kramer's multiple comparison test. All data were represented as mean and standard deviation (mean $\pm$ SD). For all statistical tests, *p* < 0.05 was considered as a significant difference.

RESULTS AND DISCUSSION

Several methods to concentrate CSC from the parental cell population have been reported [10]. In this study, concentration of MPM stem cells was based on their ability to form tumor spheroids in a 3D culture system using a stem cell medium without FBS [9]. As shown in Fig. 1, a, on day 5 after the start of 3D culture, small tumor sphere formation was observed, and on days 7 and 14, the size of each sphere became bigger, and the contour of the shape was clear. However, most of the spheres were diminished on day 21. Linked with the observation, viability in cells from tumor spheres on day 21 was much lower than that in the cells on days 7 and 14 (Fig. 1, b). To check the identity of the concentrated cells, mRNA levels of a panel of established MPM stem cell markers [11–13] were compared between cells from 2D culture and cells from tumor spheres formed by 3D culture (Fig. 1, c). Each mRNA level in MPM stem markers (*ABCG2*, *CD26* and *CD44*) was higher in 3D culture groups than in 2D culture group, and, of the 3D culture groups, mRNA levels of *ABCG2*, *CD26* and *CD44* in the group on day 7 showed highest level among three different groups. Additionally, we confirmed that protein levels of HIF-1 α and HIF-2 α related to hypoxia adaptation, and *ALDH1A1* and *ABCG2* related to chemoresistance [9, 14, 15] in 3D culture group on day 7 were higher than those in 2D culture group on day 7 (Fig 1, d). As these results indicated that the 7-day culture period using the present 3D culture condition was sufficient to concentrate MPM stem cells from parental MPM cells, this 3D culture condition was utilized in this study.

Since it is well known that chemoresistance as well as hypoxia are representative properties of CSC [5], the two properties in cell population from tumor spheres were evaluated to clarify cancer stemness in the population. In this study, two anticancer agents, topotecan and pemetrexed, were used to check the potential of chemoresistance in MPM cells from the tumor spheres, because the former is a typical substrate for *ABCG2* related to chemoresistance [14] and the latter is clinically utilized as a chemotherapeutic agent in MPM therapy [15]. As shown in Fig. 2, a and 2, b, chemoresistance against the two anticancer agents in 3D-cultured cells was more persistent than that in 2D-cultured cells, and throughout the treatment doses of the two anticancer agents, 3D-culture cells showed almost unchanged cell viabilities. On the



contrary, 2D-cultured cells showed dose-dependent decreases on their cell viabilities, and topotecan treatment and pemetrexed treatment at maximum doses caused significant 46 and 32% decreases, respectively, compared with non-treatment.

It has been reported that hypoxia condition could enhance chemoresistance in CSC [5], so we investigated if the hypoxia condition could contribute to the reinforcement of the chemoresistance in MPM stem cells. As shown in Fig. 3, *a* and 3, *b*, both of topotecan- and pemetrexed-treated group in hypoxia had no changes on cell viability throughout the treatment doses, on the other hand, the two anticancer agents-treated groups in normoxia showed a dose-dependent decrease on the viability. Topotecan treatment and pemetrexed treatment at each maximum dose in nor-

moxia had about 23% decreases together on cell viability in comparison with that in hypoxia.

While many cancer cell lines and solid tumors have been optimized for 3D spheroid growth [16–18], there have been limited studies utilizing MPM cell lines in this model. In this study, we demonstrate how an established MPM cell line, Meso-1, can be utilized in this culturing model to identify stemness characteristics and differences in hypoxia response that are more apparent in the 3D culture derived cells. We first confirmed that CSC-enriched spheroid could be formed by Meso-1 cells. Our present data indicate that Meso-1 cells grown in 3D culturing model exhibit enhanced expression of genes involved in CSC properties. 3D spheroids generated from Meso-1 seem to be able to shape their own microenvironments by adapting to hypoxic con-

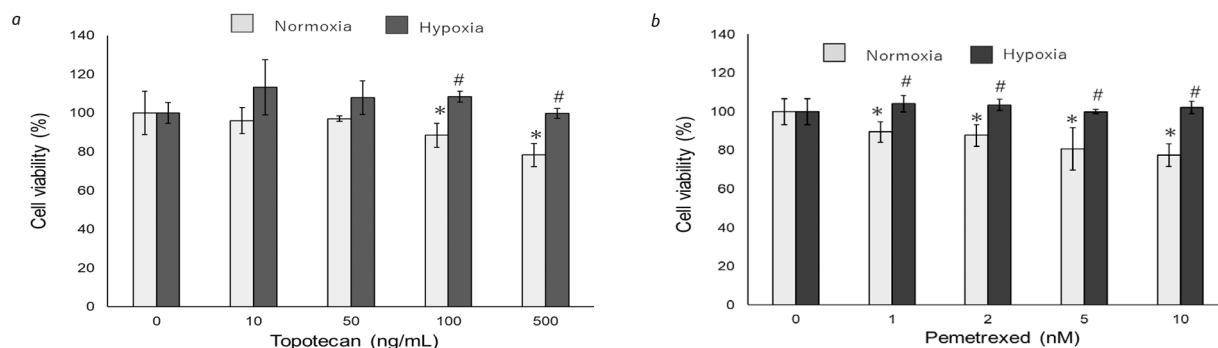


Fig. 3. Hypoxia condition can reinforce chemoresistance in MPM cells from tumor spheres. The cells from tumor spheres formed by a 3D culture for one week were cultured under hypoxia condition using hypoxia chamber with regulation of O₂ and CO₂ levels (1% O₂, 5% CO₂, 74% N₂) or normoxia condition (21% O₂) for 48 h, and further treatment of topotecan (a) or pemetrexed (b) was carried out for 24 h under the same hypoxia or normoxia condition. After the treatment, cell viability was determined using a WST-8 method. All values are means ± SEM (n = 5). *Significant difference from each non-treatment group and #significant difference from each normoxia group

ditions. Whilst under the high cellular stress induced by hypoxia, Meso-1 derived 3D spheroids developed a drug-resistant phenotype differentiating them from the 2D monolayer culturing methods.

In general, normal stem cells characteristics (stemness) are maintained by hypoxia (usually defined as 1% oxygen), with higher atmospheric oxygen levels (21% oxygen) inducing the loss of the stemness *in vitro* [19]. Similarly, CSC usually localize within hypoxic tumor niches; the hypoxia maintains the CSCs in an undifferentiated state with increased stem-like characteristics [20]. Additionally, like normal stem cells, physiologically normal oxygen levels induce differentiation in CSC *in vitro* [21]. Thus, it is considered that hypoxic conditions could be essential for the development of the stemness in CSC such as chemoresistance. In this study, we showed that under usual hypoxic conditions (1% oxygen), MPM stem cells from tumor spheres had enhanced chemoresistance against typical chemotherapeutic agents (pemetrexed and topotecan) compared to MPM stem cells under normal atmospheric oxygen levels (21% oxygen). The relationship between hypoxia and stemness is coordinated by HIFs, primarily the two major isoforms of the α -subunits, HIF-1 α and HIF-2 α [22]. HIFs act as transcription factors under hypoxic conditions of less than 5% oxygen, inducing several genes required for hypoxic adaptation [23]. Of the genes regulated by HIFs, there are three main ABC transporters, that is, ABCB1, ABCC1 and ABCG2, that efflux several anticancer drugs, contributing to chemoresistance in CSC [24]. In this study, we observed that of the ABC transporter isoforms, especially ABCG2 levels were enhanced by the hypoxia, corresponding with the chemoresistance against topotecan; a known substrate for the ABC transporter isoform [14]. In addition to the efflux of anticancer agents via ABC transporters, quiescence (a transient dormant state where cell cycle progression is arrested) induced by hypoxia, is one of the main factors governing chemoresistance against anticancer agents targeting rapidly dividing cancer cells [20]. Additionally, pemetrexed, a multitarget antifolate, is an effective agent in treating MPM, with its mechanism of action resulting in inhibition of enzymes involved in pyrimidine and purine

synthesis (e.g., thymidylate synthase), causing reduced DNA repair and synthesis [15]. Thus, hypoxic induced quiescence of MPM stem cells under hypoxia may contribute to the chemoresistance against pemetrexed. Overall, it is considered that hypoxia adaptation of MPM stem cells is closely related to reinforcement of the chemoresistance against anticancer agents, mainly via the stabilization of HIFs. Thus, it seems possible that the effective inhibition of HIFs-dependent function under hypoxia could lead to attenuation of chemoresistance in MPM.

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ХІМІОРЕЗИСТЕНТНІСТЬ, ПОВ'ЯЗАНА З АДАПТАЦІЄЮ ДО ГІПОКСІЇ КЛІТИН МЕЗОТЕЛІОМИ В ПУХЛИННИХ СФЕРОЇДАХ

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Стан питання: Гіпоксія є ключовим фактором, який впливає на індукцію та підтримку пулу пухлинних стовбурових клітин (ПСК), що в результаті забезпечує резистентність до терапії. Тривимірні (3D) сфероїдні моделі демонструють неоднорідність гіпоксичних ділянок, що відтворюють ситуацію *in vivo* в пухлинах. Використовуючи отримані 3D-сфероїд-моделі, нами проведено дослідження впливу гіпоксії на чутливість сфероїдів злоякісної мезотеліоми плеври до хімотерапевтичних препаратів. **Матеріали та методи:** Пухлинні сфероїди, одержані з клітин Meso-1 (типова клітинна лінія ЗМП людини), які мають високу здатність до утворення сфероїдів. Для індукування стану гіпоксії було використано гіпоксичну камеру з регулюванням рівнів O₂ і CO₂. Життєздатність клітин оцінювали тестуванням з WST-8. Експресію досліджуваних маркерів на рівні мРНК і білка проводили із застосуванням полімеразної ланцюгової реакції в реальному часі та Western-блот-аналізу. **Результати:** Встановлено достовірно вищі показники маркерів ПСК (CD26, CD44 і ABCG2) на рівні мРНК та маркерів адаптації до гіпоксії (ABCG2, ALDH1A1 і HIFs) у клітинах сфероїдів порівняно з аналогічними показниками у моношаровій моделі (2D). Як наслідок, клітини сфероїдів є менш чутливими до дії перметрекседу і топотекану порівняно з клітинами в моношарі, що вказує на вищий потенціал для адаптації до гіпоксії. Продемонстровано підвищення резистентності до обох хімотерапевтичних агентів у клітинах сфероїдів в умовах гіпоксії порівняно з клітинами, які культивувалися за умов нормоксії. **Висновки:** Отримані результати свідчать, що адаптація до гіпоксії клітин ЗМП в сфероїдах може посилити їх хіміо-резистентність.

Ключові слова: хіміорезистентність, гіпоксія, пухлинні сфероїди, мезотеліома, стовбурність.