Hypoxia affects in vitro growth of newly established cell lines from patients with malignant pleural mesothelioma

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(AReceived 20 November 2012; and accepted 26 November 2012)

ABSTRACT

Human malignant pleural mesothelioma (MPM) is highly aggressive, and its prognosis is very poor. For an early diagnosis of MPM and developing new therapeutic strategies against the malignancy, it is necessary to better understand biological characteristics of MPM. In this study, we established two cell lines from pleural effusions derived from patients with MPM. Both cell lines expressed tumor markers of mesothelioma such as mesothelin, podoplanin, WT1, calretinin and keratin 5/6 whereas they did not express either CEA or TTF-1 which are often used as markers of lung adenocarcinoma. The cell lines harboured wild-type TP53, produced hyaluronic acid, and were not infected with SV40. When these two cell lines were cultured under hypoxia (1% O₂), they showed particular responses to the hypoxic condition, distinct from those to normoxic condition (21% O₂). Namely, the ability to form a colony originating from a single cell (plating efficiency and cloning efficiency) was stimulated under hypoxia in both cell lines. On the other hand, when the assays of cell growth were started at a relatively high cell density, the growth of both cell lines, regardless of anchorage-dependent or -independent, decreased under hypoxia. The differences of their growth between under hypoxia and under normoxia, and those depending on the cell density, may provide useful hints for developing a new strategy for diagnosis or therapy of MPM.

Human malignant pleural mesothelioma (MPM) is a tumor arising from mesothelial cells lining the visceral or parietal pleura. It is now widely known that MPM is an asbestos-induced cancer with the long latency period of up to 40 years (12). And it is predicted that patients with MPM will increase in Japan till the next 20–30 years since legal controls were not imposed on usages of asbestos in the past (23). Generally it is difficult to diagnose MPM at its early stage and in many cases the patients have poor prognosis when they are diagnosed as MPM. Clinically MPM is characterized by dyspnea, chest wall pain and anorexia, and it often shows resistance to treatment intervention and short survival (8). For an early diagnosis of MPM and developing new therapeutic strategies for the disease, it is necessary to better understand biological characteristics of MPM.

In solid tumors, hypoxia often occurs during the expansion of a tumor mass because of structural and functional abnormality of the tumor blood vessels (27). It is easy to speculate that hypoxic regions exist in mesothelioma tissues as well as in other tumor tissues. It is well known that tumor hypoxia has the Janus face. In some cases, hypoxia inhibits cell proliferation, and induces differentiation, apoptosis or
necrosis (3, 28). In other cases, it promotes malignant progression such as invasion, metastasis and acquiring resistance to chemo- and radio-therapies (5, 7, 26).

So far there are few reports on the influence of hypoxia on the malignancy of MPM cells (6, 14). In this study, we established new cell lines from pleural effusion of patients with malignant pleural mesothelioma, and examined the influence of hypoxia on their in vitro growth.

MATERIALS AND METHODS

Establishment of MPM cell lines. Novel MPM cell lines were established from pleural effusions of two patients (male, 57-year-old, and male, 64-year-old) who underwent surgery for MPM at Hokkaido University Hospital in 2006 and 2007. The patients were histopathologically diagnosed as having malignant mesothelioma after the surgery. They agreed to the aim and contents of this study and provided a written informed consent, and the study protocol was approved by the institutional review board of Hokkaido University Hospital. Pleural effusions were collected from the patients at surgical operation. The collected effusions were centrifuged at 240 × g for 10 min, and cell pellets were resuspended in Dulbecco’s modified Eagle’s minimum essential medium and Ham’s F12 medium (DME/F12) and centrifuged again. The cell pellets were resuspended again in DME/F12 supplemented with 10% fetal bovine serum (FBS) and antibiotics (100 units/mL of penicillin and 100 μg/mL of streptomycin), and the cell suspensions were transferred into tissue culture dishes. The cells were successively subcultured for 6 to 8 months and thereafter defined as MPM cell lines. The cell lines established from the patient (57-year-old) and the other patient (64-year-old) were designated as HMM-1 and HMM-3, respectively. They were routinely checked for no infection with Mycoplasma by using Nested-PCR analysis according to JCRB Cell bank (Tsukuba, Japan) procedure.

Cell culture. The cells were grown on tissue culture dishes in DME/F12 medium supplemented with 10% FBS. The usual subculture and the culture under a normoxic condition were done in a CO₂ incubator (5% CO₂ balanced with air, which included 21% O₂) whereas the culture under a hypoxic condition was done in a hypoxia chamber (5% CO₂ and 1% O₂ balanced with N₂).

Examination of MPM-related markers. To confirm that the established two cell lines were mesothelioma, we examined the expressions of tumor markers of mesothelioma. The cells (5 × 10⁶/mL of DME/F12 supplemented with 10% FBS) were seeded on 100-mm tissue culture dishes and cultured for 24 h. The cultures were washed twice with DME/F12 and then replaced with 10 mL of DME/F12. After 24 h-incubation, the media were collected into 15 mL-tubes and centrifuged at 240 × g for 10 min, and then the supernatants were centrifuged at 20,000 × g for 10 min. The supernatants were used for measuring the concentration of hyaluronic acid by latex agglutination turbidimetric immunoassay at SRL (Tachikawa, Japan). After removing the media, the cells were harvested into two 15 mL-tubes per cell line and centrifuged for making cell pellets. One cell pellet was used for protein extraction and another one was subjected to extraction of total RNAs.

RNA extraction and RT-PCR. Total RNA was extracted from monolayer cultures of MPM cells with TRIZOL (Invitrogen, Carlsbad, CA) according to the manufacturer’s instruction. For preparation of cDNA, 1 μg of total RNAs was subjected to reverse transcription with Oligo(dT) primers and Superscript III reverse transcriptase (Invitrogen). PCR amplification of cDNA was performed in 25 μL reaction mixture containing 1 μL cDNA, 5 μL of 5× Green GoTaq Buffer (Promega), 3 μL of 2 mM dNTPs mix, 1 μL of 5 μM of each specific primers and 0.125 μL GoTaq DNA polymerase (Promega). PCR products were electrophoresed in a 2% agarose gel containing ethidium bromide and intensity of the bands was observed under an ultraviolet transilluminator. The sense/antisense primers for RT-PCR were designed as follows; (a) keratin 5/6 (KRT5/6), 5′-GGTTGATGCACTGATGGATG-3′/5′-AGATTGGCGCACTGTTTCTT-3′; (b) podoplanin (PDPN), 5′-GAAGAAGATGACCCGTGGAAC-3′/5′-TGGCCTACTTGGGAGAAACA-3′; (c) Wilms’ tumor 1 (WT1), 5′-ATACCGGTGCTTCTGGAAACTA-3′/5′-GA CCTCGGGAATGTTAGACAAG-3′; (d) calretinin (CALB2), 5′-ACATTTGACGACATGACTCTGCT-3′/5′-CACAGCAGCACCTTTCCATT-3′; (e) mesothelin (MSLN), 5′-CAAGAAGTGGAGCTGGAAAC-3′/5′-GTCCTCCAGGACGTCAACATT-3′; (f) carcinoembryonic antigen (CEA), 5′-CATGATTGGAGTGCTGGTTG-3′/5′-TGCTTGATCTTGGTCAG-3′; (g) thyroid transcription factor 1 (TTF-1), 5′-AAACGCACACGATCCGTT-3′/5′-GTGCTCCAGCTAAC-3′.
**TP53 status.** The yeast functional assay was performed according to our previous report (32). Colorimetric evaluation of yeast colonies (red or white colonies) was done after 48 h culture.

**p16 expression.** Expression of p16 protein was examined by Western blot analysis. It was performed in the same manner described in our previous report (32) by using mouse monoclonal antibody to p16 (clone G175-405; PharMingen, San Diego, CA, USA) and Saos-2 cell lysates as a positive control.

**PCR analysis for Simian virus 40 infection.** To determine whether the cell lines were infected with Simian virus 40 (SV40), we performed genomic PCR analysis to detect any viral gene encoding SV40 large T antigen. Genomic DNA was extracted from the MPM cells, and HEK293T cells (Riken Bioresource Center, Cell Bank, Tsukuba, Japan) as positive control, with GenElute Mammalian Genomic DNA Miniprep Kit (Sigma) according to the manufacturer’s instruction. PCR amplification and detection of PCR products were carried out in the same manner as RT-PCR, described in the above session of RNA extraction and RT-PCR in MATERIALS AND METHODS. The following sets of sense/antisense primers were used for amplification: 5'-AGC AGTGGTGGAATGCCCTTTAATGAGG-3'/5'-TTAGCAATTCTGAAGGAAAGTCCTTGG-3' (10).

**Morphological observation.** Cells growing at low and high cell densities on tissue culture dishes were observed under a phase-contrast microscope (ECLIPSE TE300; Nikon, Tokyo, Japan).

For scanning electron microscopy, HMM-1 and HMM-3 cells were seeded on 60-mm tissue culture dishes in DME/F-12 supplemented with 10% FBS. After 3 days incubation, the cells were fixed with 2.5% glutaraldehyde for 10 min, post-fixed with 1% OsO4 for 5 min and dehydrated with ethanol. After replacing ethanol with N-buthyl alcohol and drying in a freeze dryer (VFD-21S t-butanol freeze dryer; Shinkuu, Mito, Japan), the cells were coated with platinum and palladium. Surface morphology of the cells was observed with SEM (S-4500; Hitachi, Tokyo, Japan).

**Cell growth assays.** Four kinds of cell growth assays were carried out. First, an assay for anchorage-dependent growth as cell population was performed by a colorimetric crystal violet-staining method. The cells (1 × 10³, 2 × 10³ or 4 × 10²/0.1 mL; DME/F-12 supplemented with 10% FBS) were plated on 96-well plates. Two, 4 and 6 days after the cell seeding, crystal violet-staining was done by the same procedure as described in our previous report (32). Each data was represented as the mean ± standard deviation (SD) of triplicate wells. Absorbance values obtained by crystal violet assay were linearly correlated with the cell numbers within a range from 100 cells/well to 3 × 10⁴ cells/well in both cell lines.

The second was an assay for plating efficiency which measured colony-forming ability of a single cell in an anchorage-dependent manner. For the assay, the cells (5 × 10² or 1 × 10⁴/mL; DME/F-12 supplemented with 10% FBS) were seeded on 6-well plates in triplicate wells. Two weeks after the cell seeding, the cells were fixed with 10% formalin and stained with 50 mM 3-Cyclohexylamino-1-propanesulfonic acid buffered solution containing 0.1% crystal violet for 30 min. After removing the staining solution, colonies consisting of more than 50 cells were macroscopically counted. Plating efficiency was evaluated as (number of colonies/number of cells seeded) × 100 (%) and represented as the mean ± SD of triplicate wells.

The third was spheroid formation assay which was performed to evaluate anchorage-independent growth originated from multiple cells. For spheroid formation, the cells (2 × 10³/0.1 mL; DME/F-12 supplemented with 10% FBS) were seeded in ultra-low attachment 96-well round-bottomed plate (Celltight; Sumitomo Bakelite, Tokyo, Japan). After 12 days incubation, spheroids were observed under a phase-contrast microscope. The size of a spheroid was evaluated as mean diameter calculated from the major and the minor axes and represented as the mean ± SD of triplicate wells.

The fourth was cloning efficiency assay which was performed to evaluate colony-forming ability from a single cell in an anchorage-independent manner. The cells (1 × 10⁴ or 2 × 10⁵) were suspended in 0.3% agarose in DME/F-12 supplemented with 10% FBS, and seeded in 6-well plates over 0.6% agarose base layer. After 2-week incubation, colonies were counted under a microscope. Cloning efficiency was evaluated by the calculation of (number of colonies/number of cells seeded) × 100 (%) and represented as the mean ± SD of triplicate wells.

**Statistical analysis.** Statistical significance was determined by one-way analysis of variance followed by Fisher’s probable least-squares difference analysis as a *post hoc* test. In all statistical comparisons, *P* < 0.01 was used to indicate statistically significant difference.
RESULTS AND DISCUSSION

Characterization of newly established MPM cell lines

The results of RT-PCR analysis of mesothelioma markers in HMM-1 and HMM-3 are shown in Table 1. Both cell lines expressed keratin 5/6, podoplanin, WT1, calretinin and mesothelin which are often used as mesothelioma markers for pathological diagnosis (11). On the other hand, neither cell line expressed TTF-1 or CEA; they are not generally expressed in MPM, and are often used as markers of lung adenocarcinoma (11). The expressions of those marker genes of mesothelioma and origin of HMM-1 and HMM-3 cell lines suggested that the two cell lines were mesothelioma cells. Yet, since most of MPM cell lines are known to produce hyaluronic acid, to have wild-type *TP53* gene but not mutant one, and not to express p16 protein (13, 16–18, 22, 30), we examined these properties to further confirm the above (Table 1). Both cell lines produced hyaluronic acid in the conditioned media, harboured wild-type *TP53* and did not express p16 proteins. Since there are some reports on association of Simian virus 40 (SV40) with MPM (4), we also examined whether the two cell lines were infected with SV40. There was no evidence of SV40 infection. This coincided with the report that the frequency of SV40 infection in Japanese mesothelioma cases is extremely low (1).

Morphological characterization of the mesothelioma cell lines

When HMM-1 cells were sparsely cultured on tissue culture dishes, their shapes were round or oval; when they were cultured in confluency, they became polygonal and some of them piled (Fig. 1A and B). On the other hand, HMM-3 cells showed fibroblastic shape, especially when they were seeded sparsely on tissue culture dishes, compared to HMM-1 cells (Fig. 1D). When they were cultured in confluency, their cytoplasms tended to overlap partially (Fig. 1E). Observation with a scanning electron microscope revealed that HMM-1 cells exhibited a cauliflower-like bumpy surface whereas HMM-3 cells had thick and short cytoplasmic protrusions on their surface (Fig. 1C and F). Yanagihara et al. classified MPM cell lines in 4 types based on the cell surface ultrastructure by SEM observation: the cells with (i) numerous, long thin microvilli; (ii) short microvilli; (iii) thick and short cytoplasmic projections and (iv) a cauliflower-like bumpy surface (30). According to their classification, the cell surface structures of HMM-1 and HMM-3 cells agreed to the type (iv) and (iii), respectively. The two cell lines were thus recognized as those of mesothelioma morphologically, as well as by RT-PCR analysis on mesothelioma markers.

Comparison of in vitro growth of MPM cells between under normoxia and under hypoxia

HMM-1 and HMM-3 cells were seeded on 96-well tissue culture plates at cell densities of 1,000, 2,000 or 4,000 cells/well, and the cell number per well was chronologically counted by a colorimetric crystal violet-staining method (Fig. 2). The cell growth tended to reduce under hypoxia when the cell densi-
ty became high: more than $1 \times 10^4$/well in HMM-1 cells and more than $1.5 \times 10^4$/well in HMM-3 cells. Especially 6 days after seeding 4,000 cells, the cell number of HMM-1 under hypoxia was approximately 65% of that under normoxia and that of HMM-3, approximately 50%.

Assay for plating efficiency (colony formation in an anchorage-dependent manner), which evaluates the ability of single cells to form 2-dimensional colonies (one colony from one cell), showed more colonies under hypoxia than under normoxia, in both cell lines (Fig. 3). HMM-1 cells formed compact colonies with tight cell-cell adhesion whereas HMM-3 cells formed colonies consisting of sparse cells beside compact ones.

Next we examined anchorage-independent growth of the two cell lines. When the cells were seeded in ultra-low attachment plates, they formed one cell aggregate (spheroid) per well (Fig. 4A). Compared to HMM-1 cells, HMM-3 cells made so tight spheroids that it was difficult to recognize the border between the cells. After incubation for 12 days, sizes of the spheroids were compared between hypoxia and normoxia. As shown in Figure 4B, those formed under hypoxia were significantly smaller than the ones under normoxia.

Finally we analyzed cloning efficiency (colony formation in an anchorage-independent manner), namely the ability of single cells to form 3-dimensional colonies (one colony from one cell), by cul-

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Fig. 1  *In vitro* morphology of HMM-1 and HMM-3 cell lines. Phase-contrast microphotographs of *in vitro* morphology (*A, B, D, E*), and scanning electron-microscopic photographs (*C, F*) of HMM-1 and HMM-3 cell lines. *A, B*: HMM-1, cultured at a low cell density and in confluency, respectively. A small nucleus was localized at marginal region of cytoplasm. *D, E*: HMM-3, cultured at a low cell density and in confluency, respectively. Bar, 100 μm. *C*: a single cell of HMM-1 cell line; *F*: a single cell of HMM-3 cell line. Bar, 4.3 μm.
many other cell lines consisting of fibroblastic-type cells or a mixture of fibroblastic-type cells and polygonal cells (15, 24, 30). On the other hand, HMM-1 cells are all in polygonal shape and forming a pavement-like structure (Fig. 1B). This morphology bears a striking resemblance to structure of primary cultured mesothelial cell monolayers (9, 25). From these, we assume that HMM-1 is less malignant and that the cell line may be useful for analysis of molecular mechanisms for malignant progression of MPM cells.

In summary of the results of the 4 types of cell growth assays, the ability of single cells to form colonies (one colony from one cell) was enhanced in both cell lines under hypoxia, regardless of anchorage-dependent or -independent growth (Figs. 3 and 5). We also observed that, when the growth assays were started at a relatively high cell density, growth abilities of both cell lines reduced under hypoxia (Figs. 2 and 4). And the saturation density was especially low in both cell lines in hypoxic conditions compared to that in normoxic conditions. We speculate that the hypoxia-enhanced colony formation was due to cellular heterogeneity in the cell lines: A cell line contains cell populations with various responses to hypoxia, and it may contain a small cell population which is driven to proliferate by hypoxia but not by normoxia. In other words, hypoxia may convert a small population of dormant (non-proliferative) cells into active (proliferative) cells. Several recent studies based on cancer stem cell (CSC) theory support this interpretation. It is explained in the theory that CSCs are a small population existing in a tumor tissue or a cell line, and are capable of self-renewing, differentiating and tu-

![Fig. 2 Anchorage-dependent cell growth of HMM-1 and HMM-3 cell lines. Indicated number of cells were seeded on 96-well tissue culture plates. Indicated days after the cell seeding, cell numbers were counted by a colorimetric crystal violet-staining method. Data are presented as the mean ± SD (n = 4). *P < 0.01 vs normoxia.](image)

![Fig. 3 Anchorage-dependent colony formation of HMM-1 and HMM-3 cell lines. Indicated number of cells were seeded on 6-well tissue culture plates. Two weeks after the cell seeding, number of colonies formed was counted macroscopically. Data are presented as the mean ± SD (n = 3). *P < 0.01 vs normoxia.](image)

turing the cells in semi-solid media containing 0.3% agarose. Colony-forming ability of HMM-1 cells was considerably low compared to that of HMM-3 cells. Nevertheless hypoxia promoted colony-forming abilities of both cell lines (Fig. 5).

Overall, HMM-1 cells showed much lower growth ability than HMM-3 cells in all the growth assays performed in this study. The doubling time 52 h of the HMM-1 cell line (unpublished data) may also characterize the cells, compared to those of other MPM cell lines (15, 30): TCC-MESO-1 (24.5 h), TCC-MESO-2 (26.2 h), TCC-MESO-3 (31.3 h), MPM1 (26 h), MPM2 (46 h) and MPM3 (43 h), showing the slowest growth of HMM-1 cells. Morphologically HMM-3 cells are not so distinct from many other cell lines consisting of fibroblastic-type cells or a mixture of fibroblastic-type cells and polygonal cells (15, 24, 30). On the other hand, HMM-1 cells are all in polygonal shape and forming a pavement-like structure (Fig. 1B). This morphology bears a striking resemblance to structure of primary cultured mesothelial cell monolayers (9, 25). From these, we assume that HMM-1 is less malignant and that the cell line may be useful for analysis of molecular mechanisms for malignant progression of MPM cells.
Mesothelioma cell growth in hypoxia

And CSCs are believed to be resistant to chemo- and radio-therapies because of their properties capable of maintaining their dormant statuses. Yeung et al. showed that hypoxia increased colony formation in Matrigel by colorectal cancer cells and that the colonies formed in hypoxic condition, but not those in normoxic condition, expressed CSC markers, suggesting that hypoxia maintained CSC-like property of colorectal-cancer-derived CSCs (31). Ma et al. showed that plating efficiency of two human prostate cancer cell lines was enhanced under hypoxia and that hypoxia increased the number of the cells with CSC-like properties such as CD44, ABCG2 and Oct3/4 (19). Further, it was demonstrated that colony-forming ability of glioblastoma cells cultured in methyl cellulose was higher in hypoxic condition than in normoxic condition and that hypoxia increased CSC-like cells (2). All these results support the idea that hypoxia enhances the proliferation of CSCs.

**Fig. 4** Phase-contrast microphotographs of anchorage-independent growth (spheroid formation) of HMM-1 and HMM-3 cell lines. A: Morphology of spheroids. Bar, 100 μm. B: The bar graph shows diameters of colonies (spheroids). Data are presented as the mean ± SD (n = 3). *P < 0.01 vs normoxia.

**Fig. 5** Anchorage-independent colony formation of HMM-1 and HMM-3 cell lines. Indicated numbers of cells were seeded in semi-solid media. Two weeks after the cell seeding, colonies were counted with an inverted microscope. Data are presented as the mean ± SD (n = 3). *P < 0.01 vs normoxia.
ports indicated that hypoxia provided CSC-like cells with favorable microenvironments for their survival and growth. Namely hypoxia may convert dormant CSCs into active ones in MPM cells although a possibility remains that growth or survival of cell populations other than CSCs may be stimulated by hypoxia.

We also found evidence that hypoxia reduced saturation density of MPM cells. Generally tumor cells tend to metabolize glucose by glycolysis even in aerobic condition (known as the Warburg effect), although it is less efficient for ATP production compared to oxidative phosphorylation (29); and in various kinds of tumor cells the glucose uptake is increased concomitantly (20). It is well known that hypoxia increases glucose influx by increasing expressions of glucose transporters such as GLUT1 and GLUT3 (20). Taken together, we may be able to explain that the tumor cells rapidly exhausted glucose contained in the media under hypoxia, which caused slow growth or growth arrest under hypoxia at high cell density in the present experiments.

Our findings suggest the complexity of tumor microenvironment where additive factors such as changes of cell density alter the responses of MPM cells to hypoxia. Presently we have no evidence to determine whether growth responses to hypoxia and to culture cell density arise in other MPM cell lines or other types of tumor cell lines besides those we used in this study. We are now investigating to determine generality of our findings by using a variety of human tumor cell lines. We expect that further studies using these two MPM cell lines would provide us with useful hints for developing a new therapeutic strategy targeting tumor microenvironments.

Acknowledgement

The authors thank Ms. Yanome for her help in preparing the manuscript. The authors have no financial relationship to disclose.

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