Antiangiogenic therapies for malignant pleural mesothelioma

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## 1. ABSTRACT

Malignant pleural mesothelioma (MPM), arises from the mesothelial cells, is difficult to be diagnosed at an early stage, and is refractory to conventional chemotherapy and radiotherapy. Therefore, the establishment of novel effective therapies is necessary to improve the prognosis for many patients with this disease. Recent studies have demonstrated that angiogenesis plays a significant role in MPM progression, suggesting the importance of tumor vessels as therapeutic targets. To explore molecular pathogenesis and evaluate the efficacy of vascular targeting therapy in MPM, we developed orthotopic implantation SCID mouse models of MPM. We found that selective VEGF inhibitors were effective only in the treatment of high-VEGF-producing MPM models. On the other hand, multiple kinase inhibitor E7080, with inhibitory activity against various angiogenic cytokine receptors, suppressed the progression and prolonged survival of both high-VEGF-producing and low-VEGF-producing MPM models. Further understanding of the functional characteristics of tumor angiogenesis may be essential to improve targeting therapies in MPM. In this review, we introduce current status of clinical strategies and novel therapeutic approaches against angiogenesis in MPM.

## 2. INTRODUCTION

Malignant pleural mesothelioma (MPM) arises from the mesothelial cells that line the thoracic cavity. MPM grows aggressively with dissemination in the thoracic cavity and frequently produces malignant pleural effusions (1). Various respiratory symptoms such as dyspnea, shortness of breath, and chest pain affect the quality of life of patients with this disease.

Several etiological factors, including asbestos (2, 3), iron (4), and simian virus 40 (SV40) (1), are associated with the pathogenesis of MPM. Among these factors, asbestos is most closely related to the development of MPM, and individuals with a history of exposure to asbestos have a much greater risk of developing MPM. Even after bans on asbestos were initiated in the 1970s, the incidence of MPM is increasing worldwide because of its long latency period (30-40 years), and very high mortality rate in MPM is recognized as a social problem. In the United States, 2,000 to 3,000 patients die of MPM every year. Deaths from this disease are expected to peak in 2020-2025, and more than 250,000 MPM patients in Western Europe and Japan will die over the next 40 years (3).

**Table 1.** Target molecules involved in pathogenesis of MPM

| Process | Event | Molecules |
|---|---|---|
| Carcinogenesis | Environmental factor | Asbestos |
| | | SV40? |
| | Reactive oxigen species | Polymorphism of GSTM1 and Mn SOD |
| | | Ferritin heavy chain |
| | Loss of tumor suppressor gene | p16, p14, NF2, CDKN2A/ARF, MYO18B |
| Growth | Proliferation | TGF-β, PDGF, IGF-1, EGF, VEGF, IL-8, HGF |
| | Anti-apoptosis | Bcl-x1, telomerase, TNF-α, NF-kB |
| | Angiogenesis | VEGF, FGF-2, HGF, heparanase, IL-8, Cox-2 |
| Thoracic dissemination | Motility/Invasion | EphA2, EphB4,, NF2/merlin, MMP-2, MT1-MMP, |
| | | PDGF-BB, TGF-β, HGF, IGF, MYO18B |
| Pleural effusion | Permeability | VEGF |

While it remains to be clarified how asbestos fibers confer genetic/epigenetic alterations and induce cellular transformation in normal mesothelial cells, recent studies reported the involvement of several genes in the carcinogenesis of MPM (5). MPM is reported to have genetic inactivation of several tumor suppressor genes, such as $p16^{INK4a}/p14^{ARF}$ and neurofibromatosis type 2 (*NF2*), and epigenetic inactivation of RAS association domain family 1 isoform A (*RASSF1A)*. By contrast, no frequent mutations of major oncogenes such as *K-RAS* and *PIK3CA* have been identified. Receptor tyrosine kinases including the epidermal growth factor receptor (EGFR) family and MET, are frequently and downstream pathway, such as mitogen-activated protein kinase (MAPK) and phosphatidylinositol-3-kinase (PI3K)–AKT, are frequently activated in MPM cells (5). Further comprehensive analyses are warranted to identify key addiction pathways for cell survival and proliferation of MPM cells.

Currently, the only curative therapeutic modality for MPM is surgical resection at early stages. However, the majority of MPM patients are found at the advanced stage, and it is difficult to eradicate MPM by an operative approach alone (1). In addition, MPM is refractory to conventional chemotherapy and radiotherapy. These problems are associated with poor prognosis in MPM, and the median survival time of MPM patients from onset is approximately one year. Novel effective therapies are necessary to improve the prognosis of this disease.

## 3. MALIGNANT PLEURAL METHOTHELIOMA AND ANGIOGENESIS

Progression of MPM consists of multiple processes, including carcinogenesis, growth supported by angiogenesis, dissemination in the thoracic cavity, and production of pleural effusion. Each event is regulated by the balance of various molecules (Table 1). Angiogenesis, the formation of new blood vessels to deliver oxygen and nutrients to the expanding tumor mass, is crucial for growth and progression of solid tumors (6). The notion is supported by the fact that in many types of cancer, including MPM, there is an inverse correlation between microvessel density in tumors and patient survival (7-9). The positive regulators of angiogenesis in MPM include vascular endothelial growth factor (VEGF), fibroblast growth factor-2 (FGF-2), platelet-derived growth factor (PDGF), hepatocyte growth factor (HGF), and interleukin-8 (IL-8) (10). Therefore, these proangiogenic molecules are expected as effective therapeutic targets of MPM. Among these proangiogenic molecules, VEGF is the most potent endothelial cell-specific mitogen associated with tumor neovascularization. VEGF also significantly increases vascular permeability, thus mediating the development of pleural effusion and ascites (11, 12).

## 4. ESTABLISHMENT OF ORTHOTOPIC IMPLANTATION MODLE FOR MALIGNANT METHOTHELIOMA

To clarify the cellular and molecular pathogenesis of MPM, a suitable animal model that shows "patient-like" tumor progression is required. It is widely accepted that orthotopically-growing tumors progress to a greater extent and at a higher rate than ectopically- growing tumors. As it has become clear that local milieu of the host tissue is critical for the development of transplanted tumors in nude or SCID mice (13), we established an orthotopic implantation model for MPM using SCID mice (14) (Figure 1).

We investigated more than ten human MPM cell lines, and found that five cell lines developed tumors in the thoracic cavity of SCID mice after orthotopic implantation (EHMES-10, Y-Meso-14, NCI-H290, MSTO-211H, and NCI-H226) (14 – 16) (Table 2). Among these five cell lines, MSTO-211H and NCI-H226 produced thoracic tumors without fluid. On the other hand, EHMES-10, Y-Meso-14, and NCI-H290 cells developed thoracic tumors with large amounts of bloody pleural effusion. It is known that a subgroup of MPM patients develop large amounts of bloody effusion whereas others do not. Therefore, our orthotopic implantation models are suitable for investigating progression courses of human MPM in terms of underlying pathophysiology of effusion-producing and effusion-nonproducing subgroups.

## 5. CORRELATION BETWEEN PROGRESSION PATTERN AND PROANGIOGENIC CYTOKINE PRODUCTION IN ORTHOTOPIC MODELS

We previously reported that pleural effusion from MPM patients contained high levels of VEGF (17, 18), and that the production of experimental malignant pleural effusion was dependent on the VEGF levels in the thoracic cavity (11). Intrathoracic (i.t.) injection of low-VEGF-producing human MPM NCI-H226 cells developed tumors in the thoracic cavity but did not produce pleural effusion. In contrast, i.t. injection of VEGF gene-transfected NCI-H226 cells developed not only thoracic tumors but also a large volume of pleural effusion (11). As VEGF was originally identified as the vascular
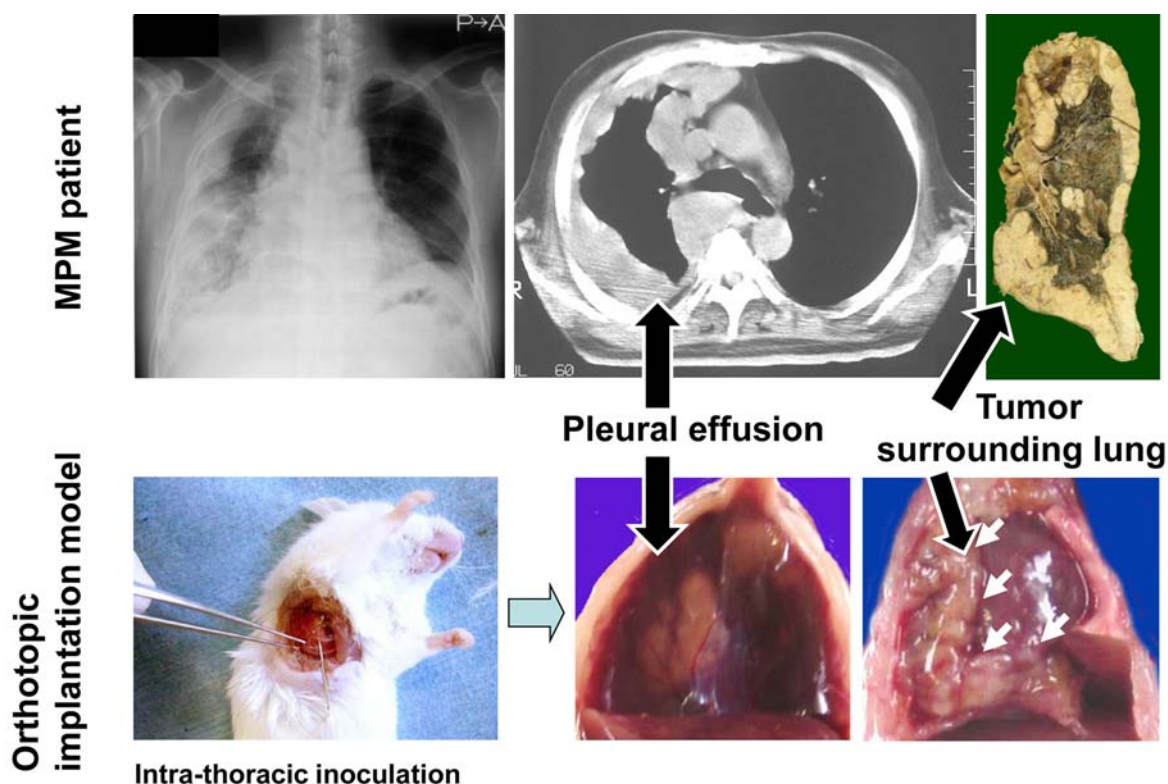
**Figure 1.** Clinically relevant orthotopic implantation model of MPM. Upper panel. The clinical feature of advanced MPM. MPM produces tumor surrounding the lung and frequently develops malignant pleural effusion. Lower panel. Mouse model of MPM. For orthotopic implantation, SCID mice were anesthetized with ether and the right chest wall was shaved. After sterilization of the chest wall with 70% ethanol, the right chest skin and subcutaneous tissue were cut and the parietal pleura was exposed. EHMES-10 cells $(1 - 3 \times 10^6$ cells/100μL) were injected into the thoracic cavity. Four weeks after inoculation, the SCID mice developed thoracic tumors surrounding the lung as well as a large amount of bloody pleural effusion, resembling the clinical behavior of MPM in patients. Figure reproduced with permission from (14).

permeability factor (19), the mechanism was explained at least in part that high amount of VEGF in the thoracic cavity increased local vascular permeability, leading to bloody pleural effusion (11). Consistent with these previous results, there was a correlation between progression patterns in the orthotopic models and proangiogenic cytokine production by MPM cells (Table 2). Among five cell lines that developed thoracic tumors, high-VEGF-producing cell lines EHMES-10 and Y-Meso-14 induced large amounts of bloody pleural effusion. In contrast, low-VEGF-producing MSTO-211H and NCI-H226 led to the development of thoracic tumors without pleural effusion. The latter group was found to produce high levels of FGF-2 instead of VEGF. It is suggested that overexpressed FGF-2 may compensate for VEGF deficiency and that FGF receptor (FGFR)-mediated signaling may play an important role in the progression of these MPM models including angiogenesis without producing fluid. Another cell line NCI-H290 developed thoracic tumors with bloody effusion. However, this cell line did not produce high levels of VEGF or FGF-2 (16). It is likely that some other factors than VEGF play important roles in producing fluid in certain groups of MPM patients. Further studies are required to determine the molecule responsible for the production of pleural effusion by this cell line to understand the VEGF-independent pathogenesis of pleural effusion.

**6. THERAPEUTIC STRATEGY FOR HIGH-VEGF-PRODUCING MPM**

VEGF, also known as VEGF-A, is a member of the VEGF/PDGF gene family and is present in at least six isoforms in human (VEGF121, VEGF145, VEGF165, VEGF183, VEGF189, VEGF206), which are regulated by splicing at the mRNA level. VEGF165 is the most abundant and biologically potent isoform. VEGF binds with high affinity to two tyrosine kinase receptors, VEGFR-1 (Flt-1) and VEGFR-2 (Flk-1/KDR), on the endothelial cells, and the VEGF-VEGFR interaction induces receptor dimerization, autophosphorylation, and signal transduction. Many studies have shown VEGF to be overexpressed in most human tumors (20). Antiangiogenic strategies targeting the VEGF pathway, such as monoclonal antibodies directed against VEGF or its receptors VEGFR-1 and VEGFR-2, and the small molecule tyrosine kinase inhibitors, have been developed, and clinical trials have shown that these targeting drugs have promising antitumor effects.
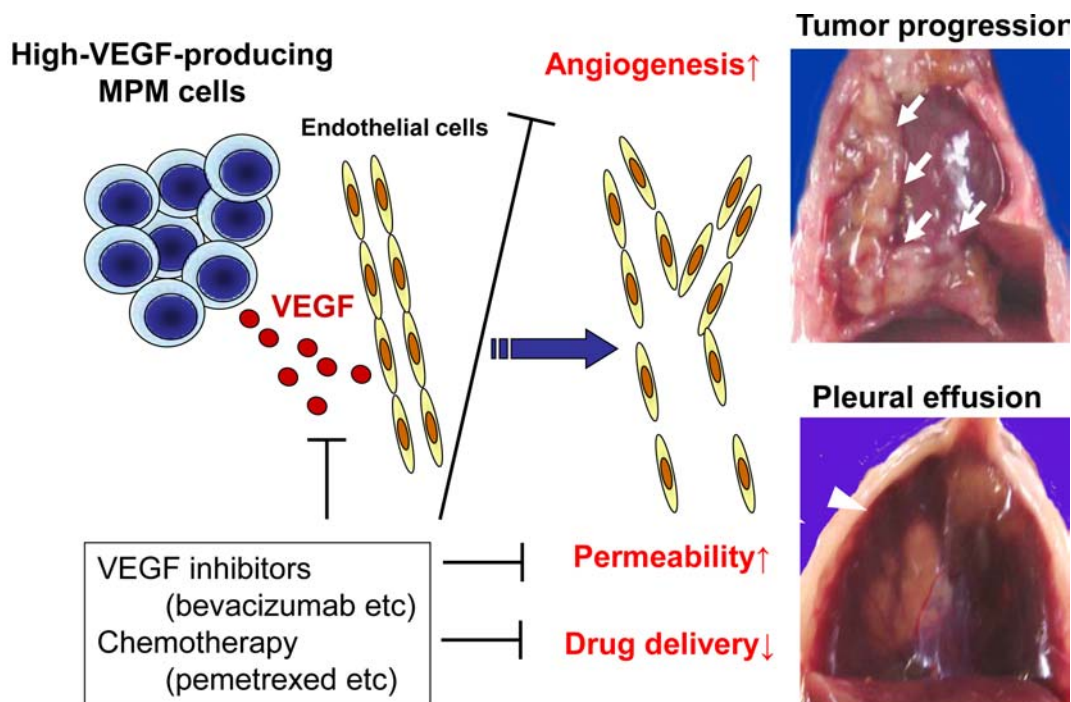
**Figure 2.** Therapeutic strategy against high-VEGF-producing MPM. VEGF produced by MPM cells promotes angiogenesis and thereby tumor growth in the thoracic cavity. VEGF also induces vascular hyperpermeability and causes bloody pleural effusion. Furthermore, VEGF increases interstitial pressure and decreases drug penetration into tumors. Combined use of VEGF inhibitors and chemotherapeutic agents may be ideal, because inhibition of VEGF suppressed tumor growth promoted by angiogenesis and pleural effusion promoted by hyperpermeability, and recovers drug penetration into tumors by normalizing interstitial pressure. Figure reproduced with permission from (14).

**6.1. Anti-VEGF antibody, bevacizumab**

Bevacizumab (Avastin™), a humanized anti-VEGF monoclonal neutralizing antibody, blocks binding of VEGF to its receptor, and neutralizes all of the isoforms of human VEGF (21). Several clinical trials have demonstrated the survival benefit of bevacizumab when combined with chemotherapy in various types of malignancies, including lung, colorectal, and breast cancers (22 – 25). Bevacizumab has been approved for the treatment of colorectal cancer and non-small cell lung cancer (NSCLC) in many countries, including Japan.

Pemetrexed (Alimta™) is a new anticancer drug which interferes with the replication of cancer cells by preventing the conversion of folic acid to folinic acid. Pemetrexed undergoes intracellular activation by poly-gamma-glutamylation that is essential for its antiproliferative activity. It inhibits at least three key enzymes of intracellular folate pathway, i.e., thymidylates synthase, dihydrofolate reductase, and glycinamide ribonucleotide formyltransferase (26). This drug has recently been approved for the treatment of MPM. At present, combined use of pemetrexed and cisplatin is considered the standard chemotherapy for advanced MPM. However, the median survival period with pemetrexed plus cisplatin is only 2.8 months longer than with cisplatin alone (27). In our orthotopic implantation MPM models, bevacizumab showed a potent inhibitory effect against the production of thoracic tumors and prevented pleural effusion, thus prolonging the survival of mice bearing high-VEGF-producing EHMES-10 cells (15). Moreover, these effects were augmented by combined use of pemetrexed (15). The mechanism by which bevacizumab potentiates the effects of chemotherapy can be explained by the hypothesis proposed by Jain *et al*; successful inhibition of VEGF normalizes tumor vascularization, and improves interstitial pressure and drug delivery to tumors (28). Normal vasculatures are composed of mature vessels and they are maintained by fine tuning of pro- and anti-angiogenic signaling balances. In contrast, abnormal tumor vasculatures are thought to be composed largely of immature vessels with increased permeability and interstitial fluid pressure, and disorganized structure in terms of vessel diameter, tortuosity, density, and so on. These functionally and morphologically abnormal vessels in tumor tissues compromise the delivery of therapeutics. "Normalization of tumor vasculature" by antiangiogenic molecules is expected to prune immature vessels and reconstruct them so that they more closely resemble normal-looking vasculature and more efficiently deliver therapeutics and oxygen (28). Therefore, inhibition of VEGF seems to be a potent strategy to control pleural effusion in MPM patients with high-VEGF-producing tumors (Figure 2).

**6.2. Triple tyrosine kinase inhibitor, vendetanib**

Several drugs that target key molecules have been tested in clinical trials for MPM patients. However,

**Table 2.** Characteristics of MPM cell lines

| | | | Cell line | | |
|---|---|---|---|---|---|
| | EHMES-10 | Y-Meso-14 | NCI-H290 | MSTO-211H | NCI-H226 |
| In vitro production | | | | | |
| VEGF | ++++ | +++ | + | + | + |
| FGF-2 | - | - | - | +++ | +++ |
| HGF | - | - | - | - | ND |
| In vivo behavior | | | | | |
| Thoracic tumor | +++ | ++ | ++ | +++ | ++ |
| Pleural effusion | ++++ | +++ | ++ | - | - |
| Survival of mice | 4W[1] | 4W | 4W | 3W | >8W |
| Therapeutic effect | | | | | |
| Bevacizumab | Yes | ND | ND | No | ND |
| E7080 | ND[2] | Tes | Yes | Yes | ND |

Abbreviations: weeks[1], not determined[2]

early studies on imatinib, an inhibitor of multiple receptor tyrosine kinases such as PDGF receptor (PDGFR), c-Kit, and Bcr/Abl, and gefitinib, a selective inhibitor of EGFR tyrosine kinase, have shown limited or no activity in MPM patients (29 – 31). These disappointing results may be due to the poor expression/activation of these target molecules in MPM tumor cells. Alternative therapeutic approaches are currently being investigated, including inhibitors of histone deacetylase, proteasome and angiogenesis (32).

Vandetanib (ZD6474, Zactima™) is a novel, orally active agent that, in isolated enzyme assays, inhibits the tyrosine kinase activities of VEGFR-2 ($IC_{50}$ = 40 nM), RET ($IC_{50}$ = 100 nM), and EGFR ($IC_{50}$ = 500 nM) (33– 35). Vandetanib inhibits VEGF signaling and angiogenesis *in vivo* and shows broad-spectrum antitumor activity in a range of histologically diverse tumor xenograft models (33). We also reported that vandetanib inhibited metastatic spread of human small cell lung cancer (SCLC) cells (36), and the production of malignant pleural effusion by NSCLC cells in a mouse model (37). Phase II trials of vandetanib in NSCLC showed efficacy as monotherapy compared with gefitinib (38). Moreover, vandetanib was reported to be beneficial in combination with docetaxel compared with docetaxel alone (39), and in combination with the carboplatin/paclitaxel regimen (40). Based on these encouraging results, vandetanib is currently being investigated in phase III trials in advanced NSCLC.

We have recently found that high-VEGF-producing cell line EHMES-10 has an oncogenic gene rearrangement of RET. Vandetanib markedly induced apoptosis and inhibited RET phosphorylation of EHMES-10 *in vitro* (41). In addition, vandetanib inhibited the progression of EHMES-10 tumors *in vivo* at least in part through suppression of angiogenesis (41). Therefore, dual inhibitory effects of vandetanib on tumor proliferation through RET and on angiogenesis through VEGFRs may be a promising therapeutic strategy for high-VEGF-producing MPM cells like EHMES-10 To our knowledge, this is the first report of the identification of rearranged-RET in MPM cells; however, the incidence of RET mutations in MPM patients remains unknown and a clinical study on this issue is a subject for future study.

## 7. THERAPEUTIC STRATEGY FOR LOW-VEGF-PRODUCING MALIGNANT PLEURAL MESOTHELIOMA

As described above, while bevacizumab effectively inhibited thoracic tumors and pleural effusion induced by the high-VEGF-producing MPM cell line EHMES-10, it had no effect on thoracic tumors developed by the low-VEGF-producing MPM cell line MSTO-211H (15). It is strongly suggested that MPMs are heterogeneous in the context of VEGF production and that novel therapeutic modalities are necessary to improve the management of low-VEGF-producing MPMs.

One strategy for controlling low-VEGF-producing MPMs is to use multi-tyrosine kinase inhibitors. E7080 is an orally active inhibitor of VEGFR-2, with additional activity against other receptor tyrosine kinases, including FGFRs, PDGFRs, and c-Kit (42). E7080 shows potent antitumor effects in xenograft models of various tumor types by inhibiting angiogenesis, especially through VEGFR suppression (43). Moreover, E7080 can cause regression of tumors induced by human SCLC H146 cells which produce stem cell factor, due to its antiangiogenic activity mediated by inhibition of both c-Kit and VEGFR signaling (42). These findings suggest that E7080 may inhibit VEGF-dependent and VEGF-independent angiogenesis and have antitumor activity against a broad spectrum of human solid tumors by suppressing not only VEGFR-2 but also other receptor tyrosine kinases. Therefore, we postulated that E7080 may be therapeutically useful for both high-VEGF-producing and low-VEGF-producing MPMs.

With regard to proangiogenic cytokine production profiles of MPM cell lines, MSTO-211H and Y-MESO-14 cells produce high levels of FGF-2 and VEGF, respectively (Table 2). In our study, E7080 successfully suppressed the phosphorylation of VEGFR-2 and FGFR-1, and thus inhibited the proliferation of human microvascular endothelial cells (HMVEC) *in vitro* (16). Administration of E7080 to low-VEGF-producing MSTO-211H and high-VEGF-producing Y-MESO-14 MPM models potently inhibited the progression of both tumors *in vivo*, and markedly prolonged survival of these mice with different profiles of proangiogenic cytokines production (Figure 3). The therapeutic efficacy was associated with decreased numbers of tumor-associated vessels and proliferating
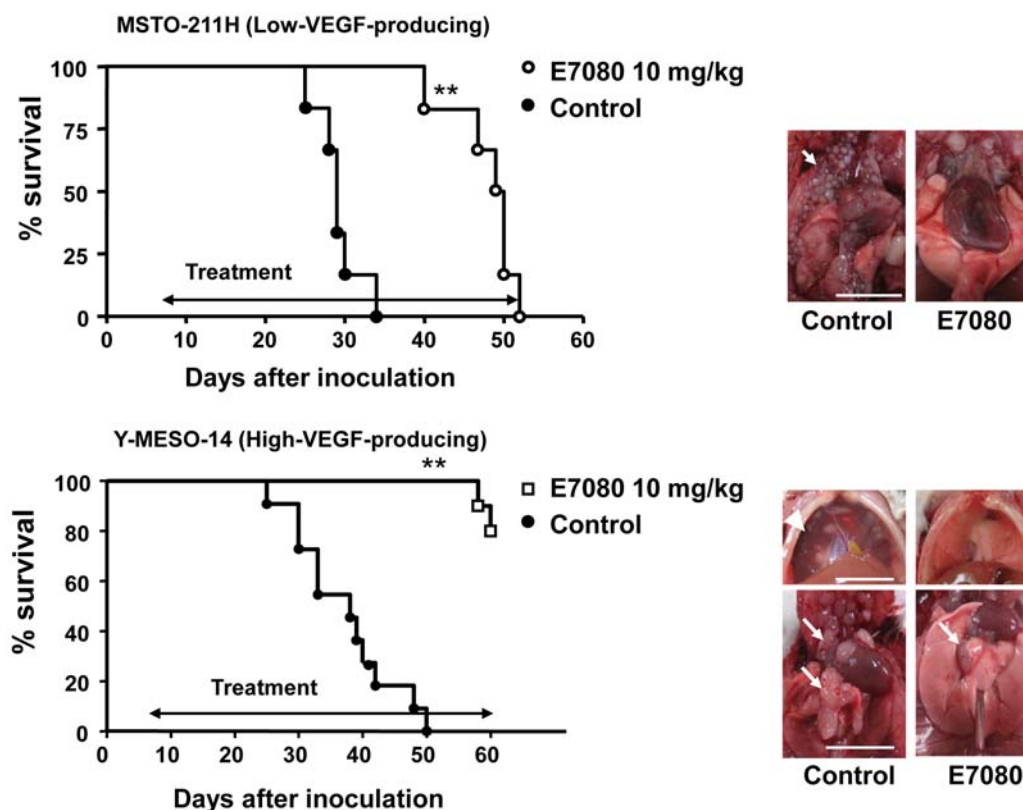
**Figure 3.** Prolongation of survival by multi-tyrosine kinase inhibitor, E7080, in SCID mice inoculated with MPM with different proangiogenic cytokine production profiles. SCID mice were inoculated orthotopically with MSTO-211H or Y-Meso-14, and treated orally with E7080 (10 mg/day) from day 14. E7080 significantly prolonged survival of the SCID mice. Bar: 10 mm. Figure reproduced with permission from (16).

MPM cells in the tumors (16). At clinically available concentrations (< 1000 nM) (44), however, E7080 did not directly affect the proliferation of MPM cells *in vitro*. Therefore, the therapeutic potential of E7080 against orthotopic MPM models may be due to inhibition of angiogenesis. These results strongly suggest broad-spectrum therapeutic effects of E7080 in MPM patients with different proangiogenic cytokine production profiles. In addition, our findings further suggest that novel strategies using E7080 in combination with chemotherapeutic agents, such as pemetrexed and gemcitabine, may be highly effective in treating for both effusion-producing type MPMs and non-effusion type MPMs. Preclinical experiments on these combinations are currently underway in our laboratory.

E7080 is currently being evaluated in clinical trials against a wide variety of solid tumors. In a phase I clinical studies, 27 patients with advanced solid tumors were treated with E7080 (0.5 – 20 mg, twice daily)(44). The maximum tolerated dose was determined to be 13 mg twice daily, and $C_{max}$ was 302 ng/mL (577 nM). Preliminary results showed that E7080 had durable disease control activity in various types of tumor, including one colorectal cancer case with a confirmed partial response. In another phase I trial, 52 patients with various tumor types were treated with 0.2 to 32 mg/once daily E7080, which was safe and well tolerated at doses up to 25 mg daily (45). Although the number of patients was limited, tumor

regression was observed in cases of melanoma ($n = 2$), renal cell carcinoma ($n = 2$), and sarcoma ($n = 1$). Based on these promising results, phase II clinical trials are currently being planned.

## 8. FUTURE ASSIGNMENTS

Molecular targeted drugs with significant clinical impact have been developed for various malignant diseases. However, no such drugs have been successfully developed for MPM. This may be due to the biological heterogeneity and insufficient understanding of molecular pathogenesis of this disease. Recent studies have demonstrated the significant involvement of angiogenesis in progression of MPM, suggesting that angiogenic vessels are therapeutic targets for this disease. Further understanding of the functional characteristics of tumor angiogenesis may be essential to establish novel drugs with significant therapeutic efficacy against MPM.

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