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All-*trans*-retinoic acid inhibits tumor growth of malignant pleural mesothelioma in mice

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Short running head: ATRA inhibits mesothelioma growth

ABSTRACT

Malignant pleural mesothelioma (MPM) is an aggressive malignant tumor of mesothelial origin associated with asbestos exposure. Because MPM has limited response to conventional chemotherapy and radiotherapy, the prognosis is very poor. Several researchers have reported that cytokines such as interleukin-6 (IL-6) play an important role in the growth of MPM. Previously we reported that all-*trans*-retinoic acid (ATRA) inhibited the production and function of interleukin-6 (IL-6) and transforming growth factor (TGF)-β_1 in the experiments using lung fibroblasts.

We investigated whether ATRA had an inhibitory effect on the cell growth of MPM, the origin of which was mesenchymal cells similar to lung fibroblasts, using a subcutaneous xenograft mouse model. We estimated the tumor growth and performed quantitative measurements of IL-6, TGF-β_1 and platelet-derived growth factor (PDGF) receptor beta (PDGFR-β) mRNA levels both of cultured MPM cells and grown cells in mice with or without the administration of ATRA.

ATRA significantly inhibited MPM tumor growth. In vitro studies disclosed that the administration of ATRA reduced 1) mRNA levels of TGF-β_1, TGF-β_1 receptors, and PDGFR-β, and 2) TGF-β_1-dependent proliferation and PDGF-BB-dependent migration of MPM cells.

These data may provide a rationale to explore the clinical use of ATRA for the treatment for MPM.

Key words: cytokines, mesothelioma,

INTRODUCTION

Malignant pleural mesothelioma (MPM) is an aggressive malignant tumor of mesothelial origin associated with asbestos exposure (1). Although recently, asbestos usage has decreased throughout the world, the incidence of MPM is expected to markedly increase over the next few decades because there is the long latency period (20–40 years) between asbestos exposure and tumor development (2). MPM has limited response to conventional chemotherapy and radiotherapy, so the prognosis is very poor with median survival durations of 8–18 months (3). Despite many researches into MPM treatment, there has been little progress in effective therapeutic and preventive strategies against MPM, and the development of novel treatment is urgently needed (4).

According to recent researches investigating cytokines in relation to malignant pleural mesothelioma, several cytokines, such as interleukin-6 (IL-6) (5) and hepatocyte growth factor/scatter factor (HGF) (6) play an important role in the growth of malignant pleural mesothelioma.

All-*trans*-retinoic acid (ATRA), a physiological metabolite of Vitamin A, is known to affect cell differentiation, proliferation and development, and has been widely used in differentiating therapy for acute promyelocytic leukemia (APL) with the ability to overcome PML/RAR fusion protein. There have been several reports about the effects of ATRA on cytokine production (7–9). ATRA induced the growth inhibition of myeloma cells, which proliferated in IL-6 autocrine and paracrine manners, with the reduction of both IL-6

production and its receptor expression (10, 11). In the previous report, we demonstrated that ATRA reduced irradiation-induced proliferation of lung fibroblasts by inhibiting both IL-6 production and its receptor expression (12). Moreover, we recently reported that ATRA prevented both irradiation- and bleomycin-induced pulmonary fibrosis in mice via an inhibitory effect on both IL-6-dependent fibroblast proliferation and transforming growth factor (TGF-β_1) dependent-transdifferentiation of fibroblasts into myofibroblasts (13).

Here we investigated whether ATRA had an inhibitory effect on the cell growth of malignant pleural mesothelioma, the origin of which was mesenchymal cells similar to lung fibroblasts and associated with several cytokines, including IL-6.

METHODS

Cell culture

Human malignant pleural mesothelioma (MPM) cell lines H28 (epithelioid), H2052 (sarcomatoid), H2452 (biphasic), MSTO-211H (biphasic), and human mesothelial cell line MeT-5A were obtained from the American Type Culture Collection (Rockville, MD). These cells were cultured in RPMI 1640 (Sigma Chemical Co., St Louis, MO) supplemented with 10% heat-inactivated fetal calf serum. ATRA (Sigma) was added to the growth medium to yield the final DMSO solvent concentration < 0.05 % (v/v). In some experiments, the cells were preincubated with proteasome inhibitor MG-132 (5 μM, Calbiochem, San Diego, CA) (14) JNK inhibitor SP600125 (10 μM, Calbiochem) (15), p38MAPK inhibitor SB203580 (10 μM, Calbiochem) (16), or ERK1/2 inhibitor PD98059 (25 μM, Calbiochem) (13) for 60 minutes.

Animals

Six-week-old C.B-17/Icr-*scid* Jcl (*scid* /*scid*) (SCID) female mice were purchased from Clea Japan (Tokyo, Japan) and maintained in our specific pathogen-free animal facility. All animals were kept according to the Animal Protection Guidelines of Hyogo College of Medicine. All protocols for animal use and euthanasia were reviewed and approved by the Institute of Laboratory Animals, Graduate School of Medicine, Hyogo College of Medicine, Japan.

Ectopic (subcutaneous) xenograft model

To produce subcutaneous (s.c.) tumors, a single-cell suspension of 10^7 MSTO-211H cells was implanted s.c. into the back of SCID mice. In some experiments, mice were injected intraperitoneally with 0.5 mg of ATRA dissolved in 0.1 ml cottonseed oil or 0.1 ml cottonseed oil alone (controls). Injections were repeated three times weekly 1) throughout the course, or 2) in the latter half of the period from inoculation to the end of the observation period. In our previous study (12,13), 0.5 mg of ATRA administration 3 times per week for 6 months did not produce noticeable morbidity and mortality. The tumors were measured every 7 days with calipers, and their volumes were calculated using the formula: $a\ (b^2) / 2$, in which a and b represent the longest and shortest diameters, respectively.

Quantitative real-time RT-PCR

Total RNA was isolated by RNeasy Mini kit (QIAGEN, Valencia, CA), and reverse-transcribed by High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA). Quantitative real-time RT-PCR was performed as previously described (18, 19), using TaqMan Gene expression products for human IL-6, IL-6 receptor, TGF-β_1, TGF-β_1 type 1 receptor, TGF-β_1 type 2 receptor, platelet-derived growth factor (PDGF) beta (β), PDGF receptor beta (PDGFR-β), and CYP26A1. 18SrRNA served as an endogenous control (Applied Biosystems).

Measurement of NF-κB p65, p38MAP kinase, and JNK

Nuclear extracts were prepared and protein concentration in nuclear extracts was measured as previously described (12). Nuclear NF-κB p65, cytoplasmic phospho-p38MAPK

($pThr^{180}$/$pThr^{182}$), and cytoplasmic JNK were detected by ELISA Kit (BioSource, Sigma, and Active Motif, respectively).

Cell proliferation assay

Cell proliferation assay was performed as previously described (12). Cells were cultured in 96-well flat-bottomed culture plates for 2 days with or without TGF-β_1 (1–10000 pg/ml), and/or ATRA (10^{-5} M).

Cell Migration Assay

In vitro migration assays were performed by CytoSelect 24-well Cell Migration Assay (8μm, Colorimetric Format) (CELL BIOLABS, Huissen, Netherlands), according to the manufacturer's instructions. Briefly, MSTO-211H cells were pre-cultured overnight with or without ATRA (10^{-5} M) and were then suspended at a density of 1 x 10^6 cells/mL in RPMI1640 and placed in the upper half of the Boyden chamber. The lower half of the Boyden chamber was filled with RPIM1640 containing 10 ng/mL human recombinant PDGF-BB (PeproTech, London, UK) or RPMI1640 alone.

Analysis of Apoptosis

MPM cells undergoing apoptosis were detected in tissue sections by the terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate-biotin nick-end labeling (TUNEL) method, using the *in situ* apoptosis detection TUNEL kit (Takara, Shiga, Japan) according to the manufacturer's instructions.

Statistical analysis

The results are given as the mean ± SD of three experiments performed in triplicate. Statistical analysis was performed using the Bonferroni/Dunn multiple comparisons test. In all tests, a p-value < 0.05 was considered significant.

RESULTS

Inhibitory effect of ATRA on proliferation of MPM cells

We first investigated the effect of ATRA on the growth of both MPM (H28, H2052, H2452, MSTO-211H) and mesothelial (MeT-5A) cells. Cells were cultured with or without various concentrations of ATRA for 48 hours. The addition of ATRA had a suppressive effect on the proliferation of all of these MPM cells in a dose-dependent manner. The maximum inhibitory effect was observed at the concentration of 10^{-5} M ATRA (H28, 59% decrease [$p<0.0001$]; H2042, 33% decrease [$p<0.0001$]; H2452, 45% decrease [$p<0.0001$], and MSTO-211H, 29% decrease [$p<0.0001$], Fig. 1A–D), whereas the lower concentration (10^{-7} M or 10^{-6} M) of ATRA showed a minor effect compared to 10^{-5} M. On the other hand, ATRA had no effect on the proliferation of MeT-5A (Fig. 1E). The final concentration of DMSO (0.05 % (v/v)) had no gross effect on any cells (data not shown). The concentration of 10^{-5} M ATRA had no effect on cell viabilities in any cell lines (data not shown).

Effect of ATRA on TGF-β_1/ TGF-β_1 receptor mRNA expression of MPM cells

As shown in Figure 2A, the TGF-β_1 mRNA as a ratio of 18SrRNA expression was decreased following 7 hours of culture with 10^{-5} M ATRA by 29% in H28 ($p<0.0001$), 47% in H2052 ($p=0.0002$), 38% in H2452 ($p=0.0002$) and 56% in MASTO-211H cells ($p<0.0001$) compared to cells incubated with DMSO alone. On the other hand, ATRA had no effect on TGF-β_1 mRNA expression in mesothelial cells, MeT-5A. Both TGF-β_1 type 1 and type 2 receptor mRNA/ 18SrRNA ratios were also decreased with ATRA by (type 1: 19% in H28 [$p=0.0023$], 22% in H2052 [$p<0.0001$], 29% in H2452[$p<0.0001$], 42% in MSTO-211H

[p<0.0001], type 2: 48%[p<0.0001], 27%[p=0.0003], 43%[p<0.0001], and 57%[p<0.0001], respectively) compared to cells incubated with DMSO alone. On the other hand, ATRA had no effect on TGF-β_1 type 1 and type 2 receptor mRNA expression in MeT-5A (Fig. 2B, C).

Involvement of NF-κB in the suppressive effect of ATRA on TGF-β_1 mRNA expression

Proteasome inhibitor MG-132 is also known to have inhibitory effect on NF-κB activity. Pretreatment of MPM cells with MG-132 led to decreased TGF-β_1 mRNA levels by 47% in H28 (p<0.0001), 68% in H2052 (p<0.0001), 60% in H2452 (p<0.0001), and 75% in MSTO-211H (p<0.0001) compared to cells incubated with DMSO alone (Fig. 3A). Next we demonstrated that the levels of nuclear NF-κB p65 in these cells were suppressed in the presence of ATRA by 11% in H28 (p=0.0162), 13% in H2052 (p=0.018), 10% in H2452 (p=0.0302), and 14% in MSTO-211H (p=0.0012) compared to cells incubated with DMSO alone (Fig. 3B). Although pretreatment with inhibitors for both p38MAPK and JNK led to decreased TGF-β_1 mRNA levels in these cells, the level of phospho-p38MAPK and activity of JNK were not affected by ATRA (data not shown). Inhibitors of ERK1/2 had no effect on TGF-β_1 mRNA levels (data not shown)

Effect of ATRA on TGF-β_1 mediated proliferation of MPM cells

To clarify the involvement of TGF-β_1 in the development of MPM tumor growth, we studied the effect of TGF-β_1 on the proliferation of MPM cells and mesothelial cells, MeT-5A. As shown in Fig. 4A, the addition of TGF-β_1 stimulated all MPM cell growth in a

dose-dependent manner and reached a plateau at the concentration of 1000 pg/ ml (H28, 18% increase [p=0.0012]; H2042, 19% increase [p=0.0015]; H2452, 30% increase [p<0.0001], and MSTO-211H, 46% increase [p=0.0069], Fig. 4A). On the other hand, TGF-β_1 had no effect on the proliferation of mesothelial cells, MeT-5A (Fig. 4A).We then evaluated the effect of ATRA on TGF-β_1-mediated proliferation of MPM cells, and showed that the TGF-β_1-mediated proliferation was decreased with ATRA by 39% in H28 (p<0.0001), 48% in H2052 (p=0.0013), 80% in H2452 (p<0.0001), and 73% in MSTO-211H (p<0.0001) compared to cells incubated with DMSO alone (Fig. 4B).

Antitumor efficacy of ATRA in a subcutaneous xenograft model

We next examined the effect of ATRA on a subcutaneous xenograft mouse model of MPM cells. A single cell suspension of 10^7 H28, H2052, H2452, and MSTO-211H cells with a viability of >95% was implanted subcutaneously into the back of SCID mice. Only MSTO-211H cells could grow on the back of SCID mice. To study the "preventive" and "therapeutic" effects of ATRA on MSTO-211H cell growth, intraperitoneal injections of ATRA were repeated three times weekly (1) throughout the course, or (2) for the latter half period from inoculation to the end of the observation period. As shown in Fig. 5A, intraperitoneal administration of ATRA three times per week throughout the course greatly inhibited tumor growth at 28 days after inoculation (68% decrease, p<0.0001). Moreover, treatment with ATRA three times per week for the latter half period from inoculation to the end

of the observation period ameliorated tumor growth better than the vehicle control group (36% decrease, p=0.0011). The injection intraperitoneally with 0.5 mg of ATRA three times a week for 28days had no effect on the health of the mice.

The mRNA levels of TGF-β_1 and PDGF receptor beta in implanted grown tumors on SCID mice

TGF-β_1 mRNA levels of implanted grown MPM tumors on SCID mice at 28 days after inoculation with or without an intraperitoneal injection of ATRA were analyzed by real-time RT-PCR. We demonstrate in Fig. 5B that TGF-β_1 mRNA expression was significantly suppressed by the administration of ATRA (32% decrease, p=0.0008). To study whether another mechanism was involved in the preventive effect of ATRA on MPM tumor progression, we focused on previous reports demonstrating that human mesothelioma cell lines expressed PDGFR-β (22). We then analyzed PDGFR-β expression in implanted grown MPM tumors and found that in mice treated with ATRA, the levels of PDGFR-β expression were markedly decreased as compared to in mice without ATRA (31% decrease, p=0.0004, Fig. 5C).

Inhibitory effect of ATRA on PDGF receptor beta expression and PDGF-BB-induced migration of MPM cells

We next examined the impact of ATRA on PDGFR-β mRNA expression in MPM cells. As shown in Fig. 6A, ATRA decreased the expression of PDGFR-β mRNA by 32% in

MSTO-211H cells ($p<0.0001$); however, ATRA had no effect on the production of PDGF-BB (data not shown). It is well known that cell migration plays an important role in tumor cell invasion, especially in the wide spread of MPM tumors. We therefore performed an *in vitro* migration assay to study the effect of PDGF, which is a potent mitogen and chemotactic factor for several mesenchymal cells (18), on MPM progression, and revealed that MSTO-211H cell migration was induced (1.3 fold increase, $p=0.0004$) by PDGF-BB, which was inhibited in the presence of ATRA (19% decrease, $p=0.0011$, Fig. 6B).

DISCUSSION

MPM is an aggressive malignant tumor of mesothelial origin associated with asbestos exposure that has limited response to conventional chemotherapy and radiotherapy; the prognosis is very poor. Recently, the multi-targeted anti-folate pemetrexed has been approved as a first-line agent in combination with cisplatin for the treatment of MPM; however, overall survival remains very poor (19).

We have previously reported that ATRA prevented both irradiation- and bleomycin-induced pulmonary fibrosis in mice by the inhibition of both IL-6-dependent proliferation and TGF-β_1-dependent transdifferentiation of lung fibroblasts. As MPM cells originate from mesenchymal cells similar to lung fibroblasts, here we examined the effect of ATRA on the progression of MPM tumor in SCID mice.

In the present study, we found that ATRA inhibited the proliferation of MPM cells, but not mesothelial cells. Several factors, including IL-6, TGF-β_1, and PDGF have been reported to be associated with MPM cells (5, 6, 17, 20). Here we first examined whether the IL-6/IL-6R system also plays an important role in ATRA-mediated inhibition of MPM cell proliferation; however, in this study, ATRA had no effect on IL-6/ IL-6 receptor mRNA expression in MPM cells.

We then investigated the effect of ATRA on the production of TGF-β_1, another key cytokine in ATRA-mediated inhibition of pulmonary fibrosis, as we have previously

demonstrated. We here showed that ATRA suppressed mRNA expressions of both TGF-β_1 and TGF-β_1 receptors in these cells, and moreover, inhibited TGF-β_1-dependent cell proliferation, suggesting that ATRA demonstrated the inhibitory effect on MPM cell proliferation via "TGF-β_1/TGF-β_1R autocrine mechanism". Generally, TGF-β_1 is produced by various normal cells observed surrounding MPM tissues, including fibroblasts, macrophages, neutrophils and lymphocytes (21, 22). In vivo therefore, in addition to the inhibitory effect of ATRA via the "TGF-β_1/TGF-β_1R autocrine mechanism", ATRA could decrease MPM tumor progression via the "TGF-β_1/TGF-β_1R paracrine loop" by MPM cells and these TGF-β_1 -producing surrounding cells. To determine the cellular mechanism in the regulation of TGF-β_1 production of MPM cells, we used some well-characterized pharmacologic inhibitors (23). There are at least three distinct MAPK signal transduction pathways in mammalian cells that lead to activation of the ERK, JNK, and p38MAPK pathways. As the induction of most cytokine genes requires the activation of NF-κB, we next examined whether changes in NF-κB activity were involved in the suppressive effect of ATRA on TGF-β_1 expression in MPM cells, and found an important role of NF-κB in this process (Fig. 3). These results suggest a possible mechanism whereby ATRA could reduce TGF-β_1 expression through an NF-κB-dependent pathway. Furthermore, it has been recently reported that the mechanism of asbestos-induced oncogenesis was associated with the activation of NF-κB (24), so the inhibitory effect of ATRA on NF-κB activation itself may be beneficial for the prevention of tumor growth in early MPM.

Next, we demonstrated in an in vivo study that intraperitoneal administration of ATRA three times per week throughout the course greatly inhibited MPM tumor growth 28 days after inoculation. The administration of ATRA inhibited TGF-β_1 mRNA expression in grown MPM tumors in SCID mice. A recent study reported that TGF-β_1 was significantly associated with the growth of MPM cells in a murine MPM tumor model through TGF-β_1/ TGF-β_1 receptor systems and TGF-β_1 signaling (25), and our study appears to support this.

It is well known that cell migration plays a pivotal role in the disease progression of cancer. PDGF acts as two types of peptide, A (16kDa) and B (14kDa) chains, with about 60% sequence identity, disulfide linked into three diametric molecules, PDGF-AA,-AB, and-BB (26). A and B chains bind to two cell-surface receptors which α receptor can bind all three dimmers (PDGF-AA, PDGF-AB, and PDGF-BB) with high affinity and β receptor can only recognize PDGF-BB with high affinity and PDGF-AB with lower affinity (27). It has been previously reported that MPM cells express PDGF- α and PDGF-β mRNA, whereas no PDGF-β and a low level of PDGF- α mRNA expression are detected in normal mesothelial cells. Moreover, PDGFR-β mRNA expression is detected in MPM cells, whereas only PDGFR- α mRNA expression is observed in mesothelial cells (28). In this study, we demonstrated that PDGFR-β mRNA expression in MPM cells was inhibited by ATRA (Fig. 6A), while ATRA had no effect on PDGF-β mRNA expression (data not shown). Moreover, MPM cells migrated by PDGF-BB, which was suppressed by ATRA, as shown in Fig. 6B.

PDGF-BB is also synthesized and released by several cells associated with MPM cells growth such as fibroblasts, vascular smooth muscle cells, and vascular endothelial cells (29). The administration of ATRA had no effect on PDGF-β mRNA expression in grown MPM tumor cells on SCID mice in the present study. However, the reduction of PDGFR-β mRNA expression on MPM cells by ATRA may be estimated to inhibit the PDGF-BB/PDGFR-β paracrine loop of MPM cells and PDGF-BB -producing surrounding cells, which might increase MPM cell migration and tumor invasion in cases of human MPM. As for cell migration, it has been reported that retinoic acid inhibited fibronectin and laminin synthesis and cell migration of human pleural mesothelioma in vitro (30).

de Cupis A, et al demonstrated that fenretinide (4HPR), a synthetic derivative of retinoic acid, induced apoptosis of malignant mesotheliama (MPM) cell line, ZL34 (31). However, in their report, 4HPR induced apoptosis not only tumor cells but Met5A, SV40-transformed normal mesothelial cells. Here we demonstrate the selective inhibitory effect of ATRA on growth of MPM cells, but not of Met5A. By Tunel assay, the increase of apoptosis was not observed in ATRA-treated MPM tumor cells in mice compared to untreated mice (data not shown). Next we examined the mRNA expression of CYP26A1 (32), a retinoic acid regulated gene, by real time RT-PCR, and found that mRNA levels of CYP26A1 were decreased in ATRA-treated MPM-tumor cells in mice compared to untreated mice, which suggested the increased amount of active retinoic acid, namely, autoregulation feedback loop

(data not shown). However, it is not clear whether the observed effect is specific to retinoic acid signaling. Although the precise cellular mechanism has not been fully investigated; we propose the possibility that TGF-β_1 and PDGF receptors play an important role in this mouse model and ATRA prevents MPM cell growth through the inhibition of these cytokine/cytokine receptor systems. Namely, we propose the dual inhibitory effect of ATRA on TGF-β_1-dependent proliferation and PDGF-BB-dependent migration of MPM cells, which may be the mechanism underlying the preventive and therapeutic effect of ATRA on MPM.

It is noteworthy that in this report we showed the “late”, namely “therapeutic”, effect of ATRA in MPM cell growth (Fig. 5A) in addition to the “throughout”, namely “preventive”, effect, because in clinical use, the “therapeutic” effect is often more important when clinicians find that MPM is already progressive in their patients.

ATRA is known to affect cell differentiation, proliferation and development. Clinically, ATRA has been widely used in differentiating therapy for acute promyelocytic leukemia (APL) (33). Furthermore, oral administration of the drug results in good compliance. Our data may lead to the development of novel strategies incorporating ATRA for the prevention and treatment of MPM.

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FIGURE LEGENDS

Fig. 1. Inhibitory effect of ATRA on proliferation of MPM cells

H28 (A), H2052 (B), H2452 (C), MSTO-211H (D) MPM cells and human mesothelial cell line MeT-5A (E) were cultured in 96-well flat-bottomed culture plates for 48 hours in serum-free medium with or without (DMSO alone) various concentrations (10^{-7}, 10^{-6}, 10^{-5} M) of ATRA for 48 hours, and cell proliferation was assayed as described in Materials and Methods. The results are indicated as the mean ± SD of three separate experiments in triplicate.

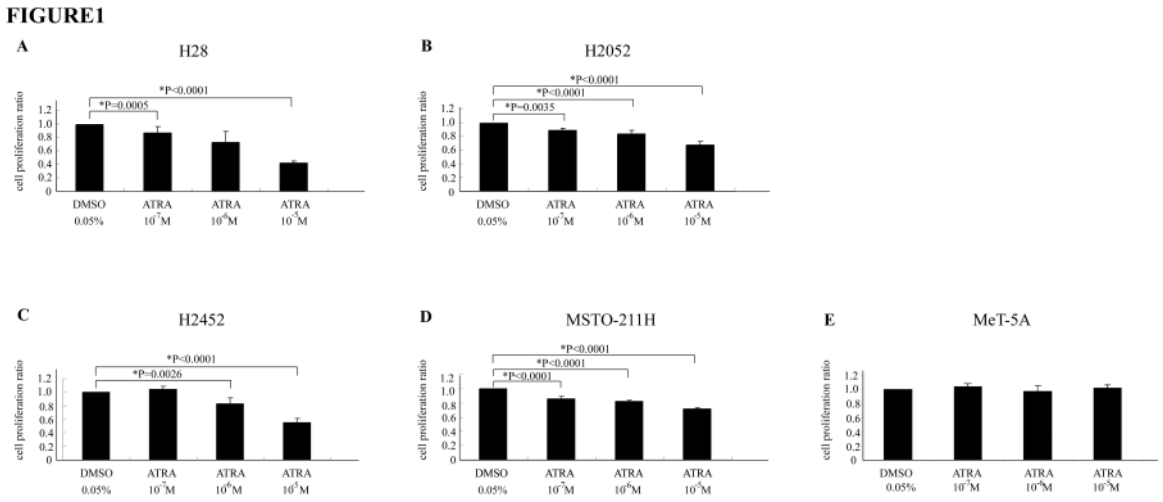


Fig. 2. Effect of ATRA on TGF-β_1/ TGF-β_1 receptor mRNA expression of MPM cells

(A-C) Real-time RT-PCR was performed to determine the changes in mRNA levels for TGF-β_1 / TGF-β_1 receptors as described in Materials and Methods. H28, H2052, H2452, MSTO-211H MPM cells and human mesothelial cell line MeT-5A were cultured in the presence or absence (DMSO alone) of 10^{-5} M of ATRA for 7 hours. The levels of mRNA for TGF-β_1 (A), TGF-β_1 type 1 receptor (B), and TGF-$\beta_1$type 2 receptor (C) are represented as the

ratio to 18SrRNA, an endogenous control. The results are indicated as the mean ± SD of three separate experiments in triplicate.

FIGURE2

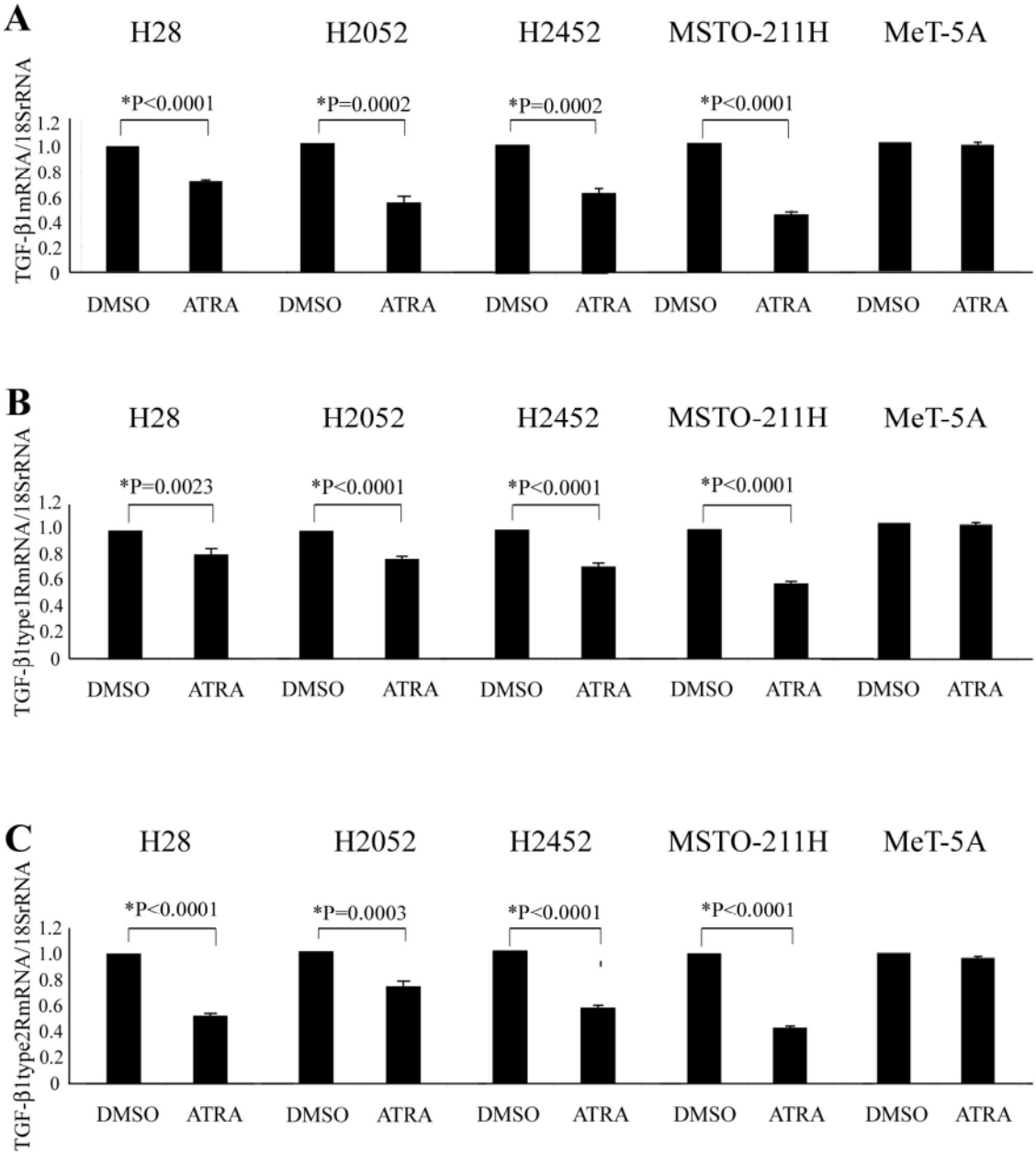


Fig. 3. Involvement of NF-κB in the suppressive effect of ATRA on TGF-β_1 mRNA expression

(A) H28, H2052, H2452, MSTO-211H MPM cells were cultured in the presence or absence of 5 μM MG-132 for 7 hours. Real-time RT-PCR was performed to determine changes in TGF-β_1 mRNA levels. (B) The activities of NF-κB were analyzed as described in Materials and Methods. H28, H2052, H2452, MSTO-211H cells were treated with or without (DMSO only) ATRA (10^{-5} M) for 1 hour and NF-κB p65 amounts in nuclear protein extracts were analyzed. The results are indicated as the mean ± SD of three separate experiments in triplicate.

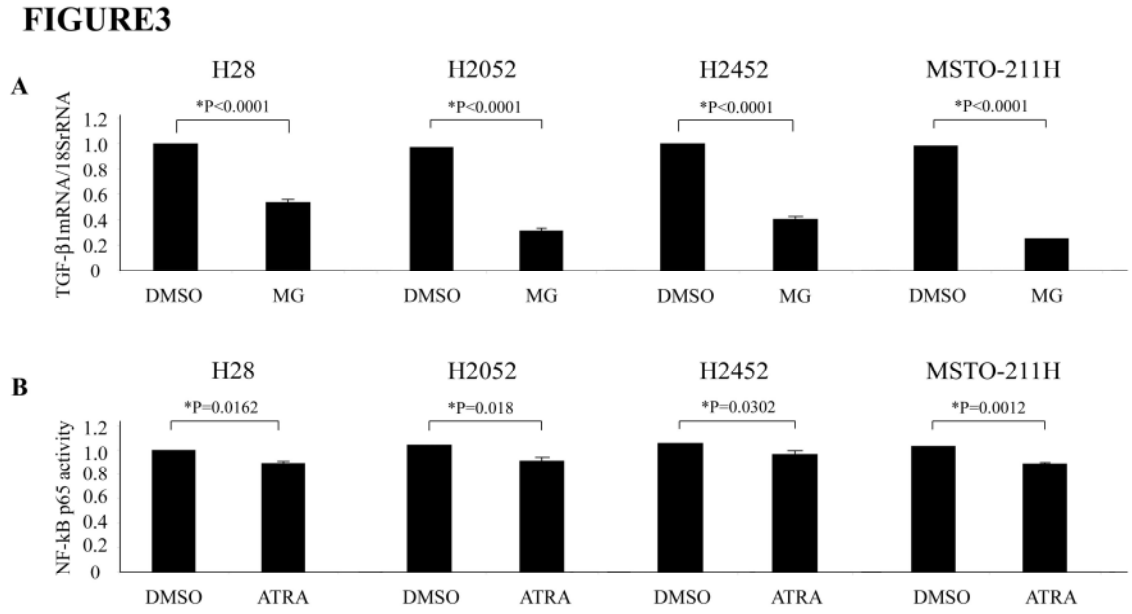


Fig. 4. Effect of ATRA on TGF-β_1 mediated proliferation of MPM cells

(A) H28, H2052, H2452, MSTO-211H MPM cells and human mesothelial cell line MeT-5A were cultured in 96-well flat-bottomed culture plates for 48 hours in serum-free medium

with indicated (0 to 1000 pg/ mL) concentrations of TGF-β_1 and cell proliferation was assayed as described in Materials and Methods. (B) H28, H2052, H2452, MSTO-211H MPM cells were cultured in the presence of 1000 pg/ mL TGF-$\beta_1$with or without (DMSO only) ATRA (10^{-5} M), and cell proliferation was assayed. The results are indicated as the mean ± SD of three separate experiments in triplicate.

FIGURE 4

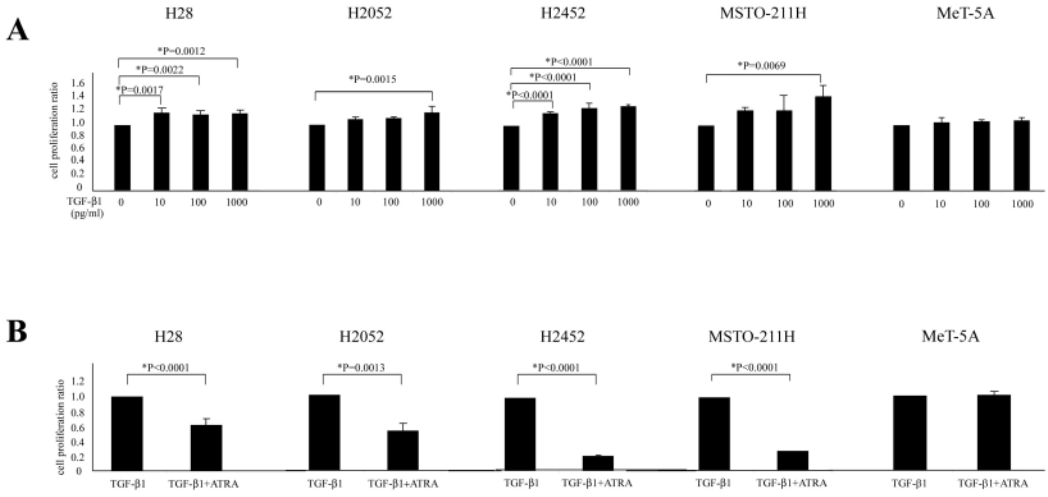


Fig. 5. Antitumor efficacy with ATRA in subcutaneous xenograft model

(A) MSTO-211H cells with a viability of > 95% were implanted subcutaneously into the back of SCID mice. The tumors were measured and calculated as described in Materials and Methods. (B, C) Real-time RT-PCR was performed to determine changes in mRNA levels of tumors for TGF-β_1 (B) and PDGFR-β (C) as described in Materials and Methods. The levels of mRNA are the ratio to 18SrRNA. The results are indicated as the mean ± SD of three separate experiments in triplicate.

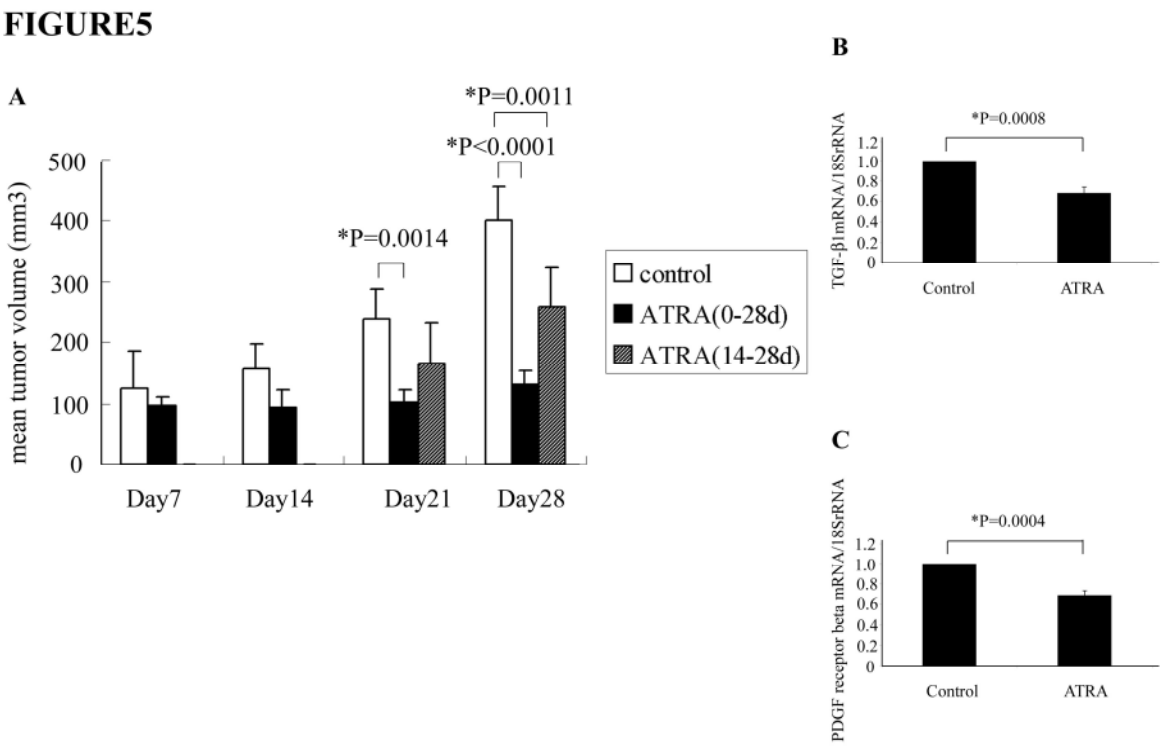


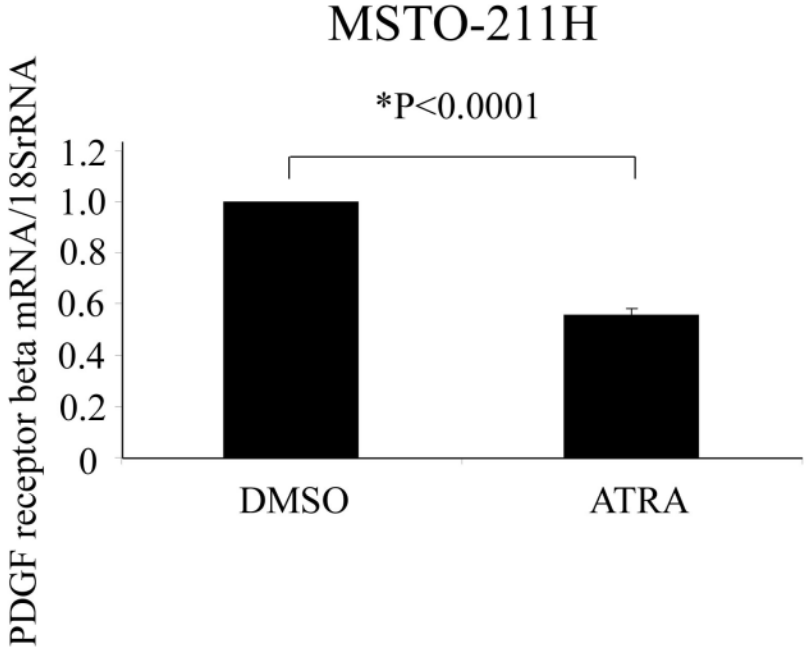
Fig. 6. Inhibitory effect of ATRA on PDGFR-β expression and PDGF-BB induced migration of MPM cells

(A) Real-time RT-PCR was performed to determine changes in mRNA levels for PDGFR-β as described in Materials and Methods. MSTO-211H MPM cells were cultured in the presence or absence (DMSO only) of 10^{-5} M of ATRA for 7 hours. The levels of mRNA for PDGFR-β are the ratio to 18SrRNA, an endogenous control. The results are indicated as the mean ± SD of three separate experiments in triplicate. (B) Cell migration assay was performed as described in Materials and Methods. MSTO-211H cells were pre-cultured overnight with or without (DMSO only) ATRA (10^{-5} M), and further cultured in the presence or absence of PDGF-BB (10

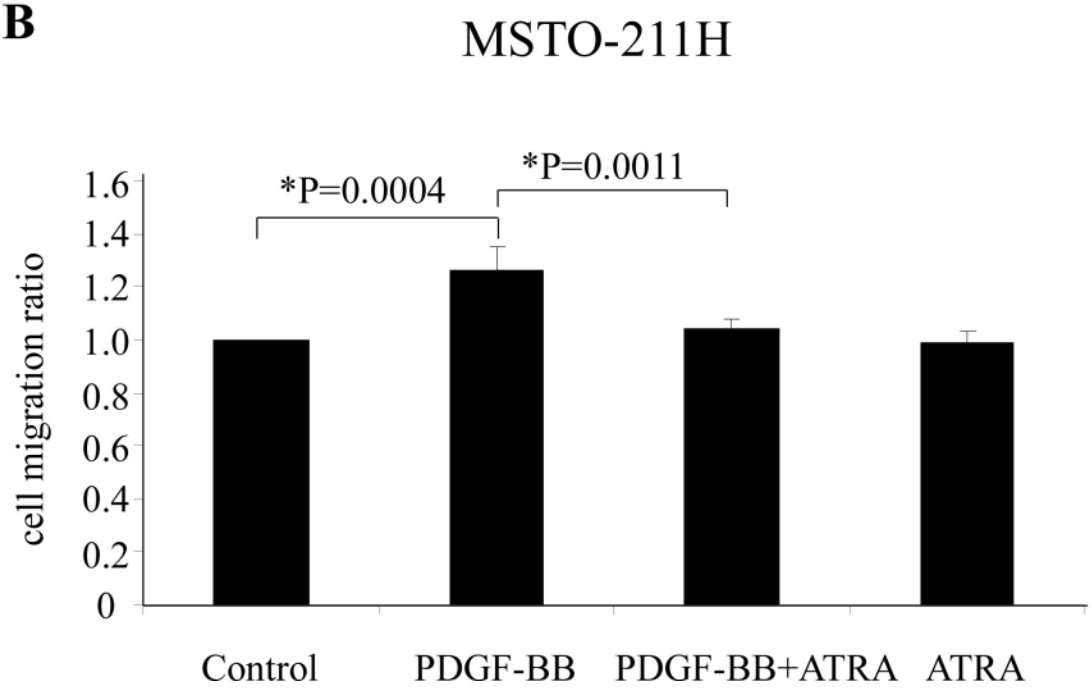
ng/mL) with or without ATRA. The results are indicated as the mean ± SD of three separate experiments in triplicate.

FIGURE6

A


MSTO-211H
*P<0.0001
PDGF receptor beta mRNA/18SrRNA
1.2
1.0
0.8
0.6
0.4
0.2
0
DMSO
ATRA

B


MSTO-211H
*P=0.0004
*P=0.0011
cell migration ratio
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
Control
PDGF-BB
PDGF-BB+ATRA
ATRA