Original Article

# Effects of Macrolides on Angiogenesis Induced by Human Lung Cancer A549 Cells

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**Abstract**: **Objective** : We investigated the effects of two macrolides on the induction of angiogenesis by human bronchiolo-alveolar carcinoma A549 cells *in vitro*. **Methods** : We chose clarithromycin (CAM) and midecamycin (MDM) as 14—and 16—membered ring macrolide antibiotics, respectively. By using human umbilical vein endothelial cells (HUVEC), we performed a wounding cell migration assay, proliferation assay and tube formation assay with MATRIGEL® to investigate the effects of these macrolides on angiogenesis in both a direct manner, and an indirect manner mediated by A549 cells. We next measured the concentrations of angiogenic factors such as basic fibroblast growth factor (basic-FGF), vascular endothelial growth factor (VEGF) and interleukin-8 (IL-8) present in culture supernatant from macrolide-treated A549 cells by enzyme-linked immunosorbent assay (ELISA) and also measured the concentrations of urokinase plasminogen activator (u-PA) in culture supernatant from macrolide-treated HUVEC and macrolide-treated A549 cells by ELISA. **Results** : The culture supernatant from A549 cells promoted each step of HUVEC angiogenesis ; migration, proliferation, and tube formation. The culture supernatant from A549 cells treated with CAM and MDM at 0.1 and 1.0μg/ml which correspond to their concentrations in serum at clinical administration inhibited migration of HUVEC. However, the inhibitory effect of MDM was weak. The culture supernatant from A549 cells treated with CAM, but not with MDM, inhibited proliferation and tube formation of HUVEC. Both macrolides showed no direct effect on HUVEC angiogenesis. The concentrations of basic-FGF in culture supernatant from A549 cells were below the assay sensitivity limit. A549 cells produced interleukin-8 (IL-8) as well as VEGF, and both macrolides inhibited the production of these factors. The culture supernatant from MDM-treated A549 cells did not affect proliferation or tube formation of HUVEC. Both macrolides suppressed A549 cells from secreting u-PA, an endothelial cell migration factor, at 0.1 and 1.0μg/ml, but promoted them at 10μg/ml. This biphasic manner of the effect of macrolides on u-PA production was similar to that on HUVEC migration. **Conclusion** : The present results indicate that macrolides, especially a 14-membered ring macrolide, are a potential inhibitor of angiogenesis promoted by lung cancer cells. Their sites of action may be on cancer cells but not endothelial cells. However, the precise mechanism of the inhibitory effect of macrolides remains to be determined.

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**Key words** : Macrolide, Migration, Proliferation, Tube formation, A549, HUVEC

## Introduction

Fourteen-membered ring macrolide antibiotics have been shown to regulate cytokine production in various immunocompetent cells[1)2)], and are known to be highly effective in the treatment of chronic airway infections. Both erythromycin and roxithromycin inhibit biofilm synthesis in *Pseudomonas aeruginosa*, and prevent them from remaining in the lower respiratory tract[3)]. Macrolides are also known to be effective in the treatment of some types of bronchial asthma[4)5)]. Recently, macrolides have received attention as potential antitumor and antimetastatic agents. Hamada *et al*.[6)] reported that erythromycin prolonged survival time and depressed tumor growth in tumor-bearing mice, and that macrolide treatment enhanced the tumoricidal activity of both macrophages and natural killer cells by enhancing the production of interleukin-4 (IL-4). As the process of angiogenesis is required for tumor growth and metastasis, anti-angiogenesis therapy has received attention as a potential cancer therapy[7)]. Angiogenesis is a multistep process that involves

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breakdown of the extracellular matrix by endothelial cells, as well as their migration, proliferation and tube formation[8]. Recently, Yatsunami et al.[9] reported that clarithromycin (CAM) suppressed not only tumor-induced angiogenesis *in vivo*, but also tube formation of human umbilical vein endothelial cells (HUVEC) *in vitro*, suggesting a possible mechanism for the antitumor and antimetastatic effects of macrolides. In this study, we investigate the effects of macrolides on the proliferation of tumor cells, the production of angiogenic factors by tumor cells, and their effects on the three steps of angiogenesis ; migration, proliferation and tube formation of endothelial cells *in vitro*, to clarify the antitumor and antimetastatic effects of macrolides.

## Materials and Methods

### Cells

HUVEC were purchased from Clonetics (San Diego, California, USA) and cultured in Endothelial Cell Basal Medium 2 (EBM2) containing 2% fetal bovine serum (FBS), hydrocortisone, human basic fibroblast growth factor (FGF), vascular endothelial growth factor (VEGF), insulin-like growth factor-1, heparin, ascorbic acid, human epidermal growth factor, gentamicin and amphotericin-B. Human A549 bronchiolo-alveolar carcinoma cells were purchased from the American Type Culture Collection (Rockville, Maryland, USA) and cultured in Dulbecco's Modified Eagle Medium (D-MEM) containing 10% FBS. Both types of cultures were incubated at 37℃ in a humidified atmosphere of 5% carbon dioxide. In our preliminary experiments, we examined the angiogenic effects of various lung carcinoma cell lines such as A549 cells, human H69 small cell carcinoma cells, human PC-14 adenocarcinoma cells and human KB squamous cell carcinoma cells. The results showed that only culture supernatant from A549 cells significantly promoted tube-formation of HUVEC (unpublished observations). Therefore, we used A549 cells as a human carcinoma cell model in this study.

### Macrolide antibiotics

CAM was chosen as a 14-membered ring macrolide antibiotic, and Midecamycin (MDM) was chosen as a 16-membered ring macrolide in this study. Both were dissolved in methanol at a concentration of 2000μg/ml and then diluted to concentrations of 100,10 and 1.0μg/ml with sterile distilled water. The maximum serum drug concentrations in subjects who received CAM at a dose of 400 mg/day and MDM at 600 mg/day were reportedly 1.1 mg/ml and 2.3 mg/ml, respectively. Therefore, concentrations of both CAM (0.1～10 mg/ml) and MDM (0.1～10 mg/ml) used in this study correspond to the maximum concentration in serum.

### Preparation of macrolide-treated A549 cell culture supernatant

A total of $1\times10^6$ A549 cells were cultured in tissue culture flasks (area＝25cm$^2$). After 24 hours, the media was removed and the flasks were rinsed with phosphate buffered saline (PBS). For the tube formation assay, 9 ml of FBS-free D-MEM plus 1 ml of each macrolide dilution were added to the flasks. For the proliferation and cell migration assays, 4.5 ml of D-MEM plus 10% FBS and 0.5 ml of each macrolide dilution were added to the flasks. The concentrations of macrolides in each flask were adjusted to be 10, 1.0, 0.1, and 0μg/ml as a control. After 24 hours, cell culture supernatant was gathered, centrifuged and used in assays.

### HUVEC migration assay

A wounding migration assay was performed[10]. A total of $1x10^5$ HUVEC were cultured in EBM2 containing 2% FBS plus growth factors in 24-well culture plates. After 24 hours, the confluent endothelial monolayers were wounded across the diameter of the well with a 2 mm-wide cell scraper. The media and detached cells were aspirated and the wells rinsed with Hepes buffer. Then 500μl of EBM2 containing 2% FBS and growth factors and 500μl of the macrolide-treated A549 cell supernatant as macrolide-treated group or 500μl of A549 cell supernatant as a control were added to the wells together. We made another group in which 500μl of 10% FBS D-MEM and 500μl of EBM2 containing 2% FBS and growth factors were added to each well. To investigate the direct effect of macrolides on HUVEC migration, 900μl of EBM2 containing 2% FBS plus growth factors, and 100μl of diluted macrolide were added to the wells after wounding. Twelve hours after wounding, cells were stained with Diff-Quick (International Reagents Corp. Kobe, Japan) and photographed at ×40 magnification. Micrographs were scanned using a film scanner, analyzed using an image analyzer (NIH image), and the total numbers of migrated cells were counted. Each value represents the mean of quadruplicate experiments.

### Proliferation assay

3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrasodium

bromide（MTT）was purchased from Chemicon International Inc.(Temecula, CA, USA)[11]. The effect of macrolides on the proliferation of both HUVEC and A549 cells, and those of the macrolide-treated A549 cell supernatant on HUVEC proliferation, were investigated by MTT assay[11]. A total of $3\times10^4$ A549 cells were cultured in 96-well culture plates. After a 24 hour incubation, the media were removed and 90μl of D-MEM plus 10% FBS and 10μl of various concentrations of macrolides were added to each well. MTT assay was performed after 48 hours incubation. To examine HUVEC proliferation, two MTT assays were performed. A total of $1\times10^3$ HUVEC were cultured in 96-well culture plates. The media were removed after 24 hours and each well rinsed with Hepes buffer. In the first assay, either 50μl of the macrolide-treated A549 cell supernatant, or 50μl of 10% FBS D-MEM, were added to each well. In addition, 50μl of EBM2 plus 2% FBS containing growth factors were added to each well. In the second assay, 50μl of EBM2 plus 2% FBS containing growth factors, 40μl of normal A549 cell supernatant and 10μl of diluted macrolide were added to each well. After 48 hours incubation, MTT assays were performed. Each value represents the mean of quadruplicate experiments.

### Tube formation assay

Growth factor-reduced MATRIGEL®-coated 24-well plates（Becton Dickinson Labware, Bedford, MA, USA）were used in this assay[12)13)]. A total of 900μl of either macrolide-treated A549 cell supernatant, or FBS-free D-MEM, were added to each well, along with 100μl of HUVEC（$3\times10^5$/ml suspended with Hepes buffer）. To investigate the direct effect of macrolides on HUVEC tube formation, 800μl of FBS-free and growth factor-free EBM 2,100μl of diluted macrolide, and 100μl of HUVEC were added to each well. After 3 hours incubation, the formation of tube-like structures by HUVEC was examined microscopically and photographed. These micrographs were scanned using a film scanner and analyzed using an image analyzer（NIH image）. The total length of tube formation was measured as an index of this assay. Each value represents the mean of triplicate experiments. Interleukin-8（IL-8）is known as a potent angiogenic factor in vivo[14)15)], and to examine its effects on HUVEC tube formation, 100 μl of various concentrations of human recombinant IL-8（Dainippon Pharmaceutical Co. Ltd. ,Osaka, Japan）, 800μl of FBS-free and growth factor-free EBM2 and 100μl of HUVEC were added to the wells. The concentrations of IL-8 in each well were adjusted to be 100, 10, 1.0, 0.1, 0.01 or 0 ng/ml. After 3 hours incubation, the formation of tube-like structures by HUVEC was examined as described above.

### Quantitative analysis of the angiogenic factors（basic FGF, VEGF and IL-8）in macrolide-treated A549 cell supernatant by ELISA

The levels of basic FGF and IL-8 in the supernatant from A549 cells prepared for the tube formation assay were determined by enzyme-linked immunosorbent assay（ELISA）. ELISA of VEGF requires FBS at a concentration of at least 1% in the supernatant to stabilize VEGF. Therefore, the FBS-free cell culture supernatant used in the tube formation assay could not be used in the VEGF ELISA. Therefore supernatant from macrolide-treated A 549 cells made from D-MEM plus 10% FBS was prepared for VEGF ELISA. ELISA kits were obtained from R&D Systems（Minneapolis, MN, USA）.

### Quantitative analysis of urokinase plasminogen activator in macrolide-treated A549 and HUVEC cell supernatant by ELISA

A total of $1\times10^6$ A549 cells were cultured in tissue culture flasks（area＝25 $cm^2$）. After 48 hours when the cells had almost reached confluence, the flasks were rinsed with PBS, and 9 ml of FBS-free D-MEM and 1 ml of diluted macrolide were added. After 48 hours incubation, the supernatants were collected, centrifuged, and used in this assay.

A total of $5\times10^5$ HUVEC were cultured in tissue culture flasks. After 48 hours when HUVEC had almost reached confluence, the flasks were rinsed with Hepes buffer, and 9 ml of EBM2 without FBS or supplemental growth factors and 1 ml of macrolide dilution were added. After 24 hours incubation, the supernatants were collected, centrifuged and used in this assay. Quantitative analysis of urokinase plasminogen activator（u-PA）was performed by ELISA.

### Statistical analysis.

Statistical significance was established by Student's t-test or the Welch test when dispersion between groups was not equal in F-test. The data were considered statistically significant if the p value was less than 0.05.

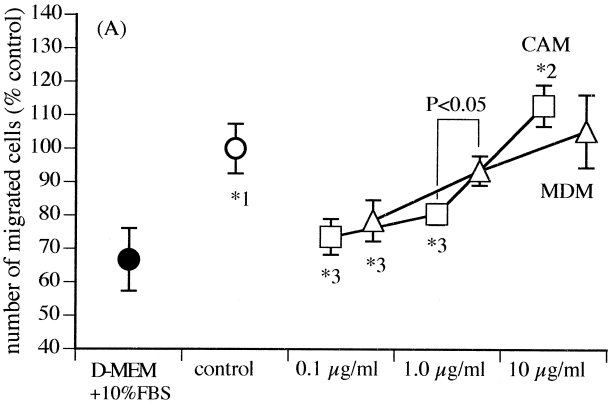
## Results

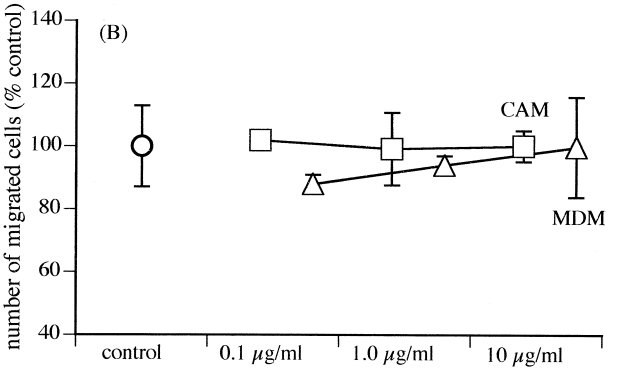
### HUVEC migration assay

A wounding migration assay was performed with the

culture supernatants from macrolide-untreated A549 cells as a control (Fig. 1A). The culture supernatants from macrolide-untreated A549 cells promoted the migration of HUVEC compared with 10% FBS D-MEM. Culture supernatant from A549 cells treated with each macrolide at a concentration of 0.1μg/ml inhibited the migration caused by the A549 cells, whereas prior treatment with CAM at 10μg/ml promoted migration. Another assay was performed to investigate the direct effect of macrolides on migration (Fig. 1-B). Neither macrolide affected migration of HUVEC when added directly to culture, suggesting that macrolides may only affect the migration of HUVEC indirectly via the effect on A549 cells.

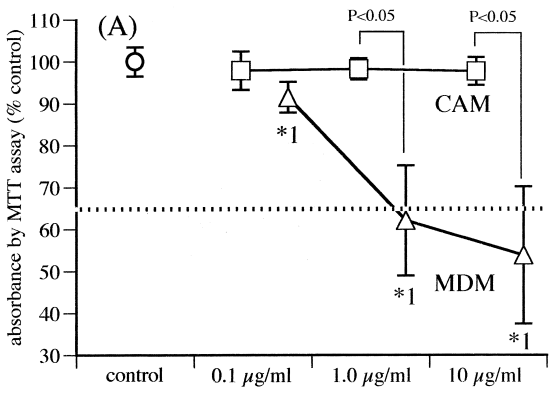
**Fig. 1.** Wounding cell migration assay of HUVEC.
(A) The effects of culture supernatant from macrolide-treated A549 cells on HUVEC migration. Cells were cultured with D-MEM supplemented with 10% FBS (D-MEM+10% FBS) (●), culture supernatant from macrolide-untreated A549 cells (○), culture supernatant from CAM-treated A549 cells (0.1, 1.0, 10 mg/ml) (□) or culture supernatant from MDM-treated A549 cells (0.1, 1.0, 10 mg/ml) (△). Results are expressed as the mean±SD of 4 different experiments. *1 ; $p<0.01$ compared with number of migrated cells cultured with D-MEM+10% FBS. *2 ; $p<0.05$ compared with number of migrated cells cultured with macrolide-untreated A549 culture supernatant. *3 ; $p<0.01$ compared with number of migrated cells cultured with macrolide-untreated A549 culture supernatant. (B) The direct effects of macrolides on HUVEC migration. Cells were cultured with D-MEM supplemented with 10% FBS (○), various concentrations of CAM (□) or various concentration of MDM (△).

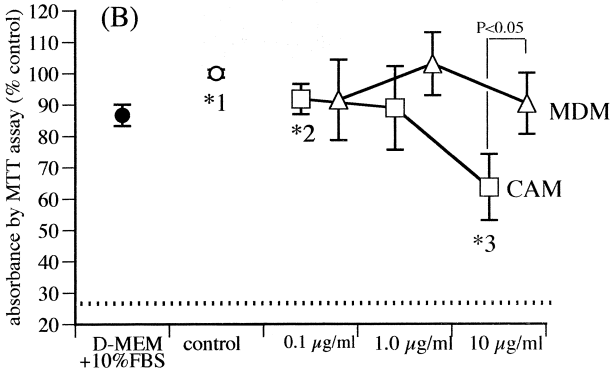


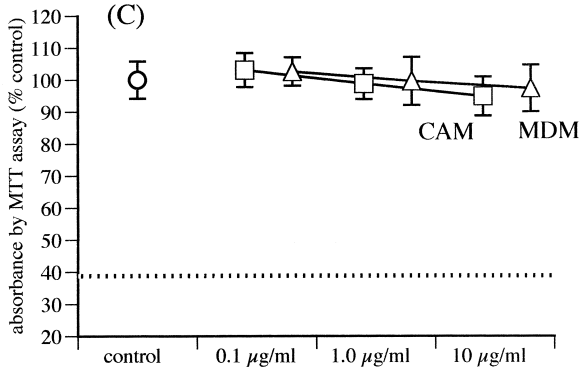




## Proliferation assay

The effect of macrolides on proliferation of A549 cells was investigated by MTT assay (Fig. 2A). CAM did not affect the proliferation of A549 cells at concentrations of 0.1-10μg/ml. However, compared with controls, MDM did significantly inhibit proliferation of A549 cells at the same concentrations, in a dose-dependent manner. At both 1.0 and 10μg/ml concentrations, a statistically significant difference in proliferation between CAM and MDM treated



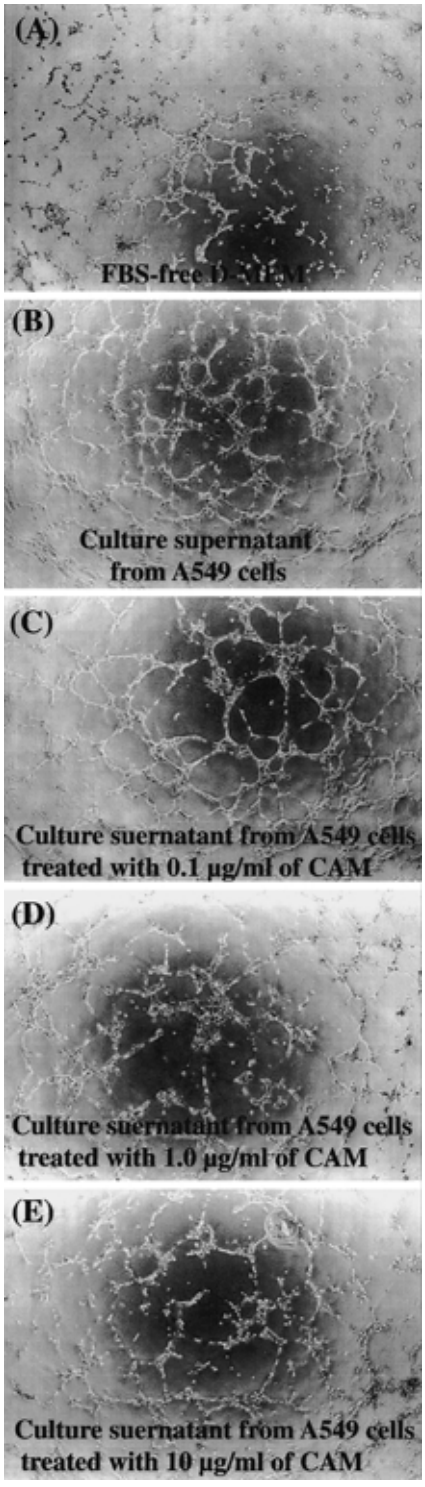



cells was observed. We also examined the effects of macrolide-treated A549 culture supernatant on the proliferation of HUVEC（Fig. 2B）. The culture supernatant from macrolide-untreated A549 cells promoted proliferation of HUVEC, compared with 10% FBS D-MEM. The proliferation of HUVEC cultured with A549 culture supernatant that had been treated with 10μg/ml CAM was lower than that cultured with 10% FBS D-MEM, indicating that the culture supernatant from the CAM-treated A549 cells inhibits the proliferation of HUVEC. In contrast, the culture supernatant from MDM-treated A549 cells showed the same promoting effect on HUVEC proliferation, as macrolide-untreated A549 cell supernatant. At a concentration of 10μg/ml, there was a significant difference in the proliferation of HUVEC between CAM and MDM treatments. As the culture supernatant from macrolide-treated A549 cells may have contained macrolide antibiotics, the possibility that CAM present in the supernatant directly inhibited HUVEC proliferation could not be ruled out. Therefore, a second MTT assay was performed（Fig. 2C）, to test the direct effects of macrolides on HUVEC proliferation. As the macrolides were added separately in a final step, A549 cells were not macrolide-treated. Neither macrolide showed any direct effect on the proliferation of HUVEC.

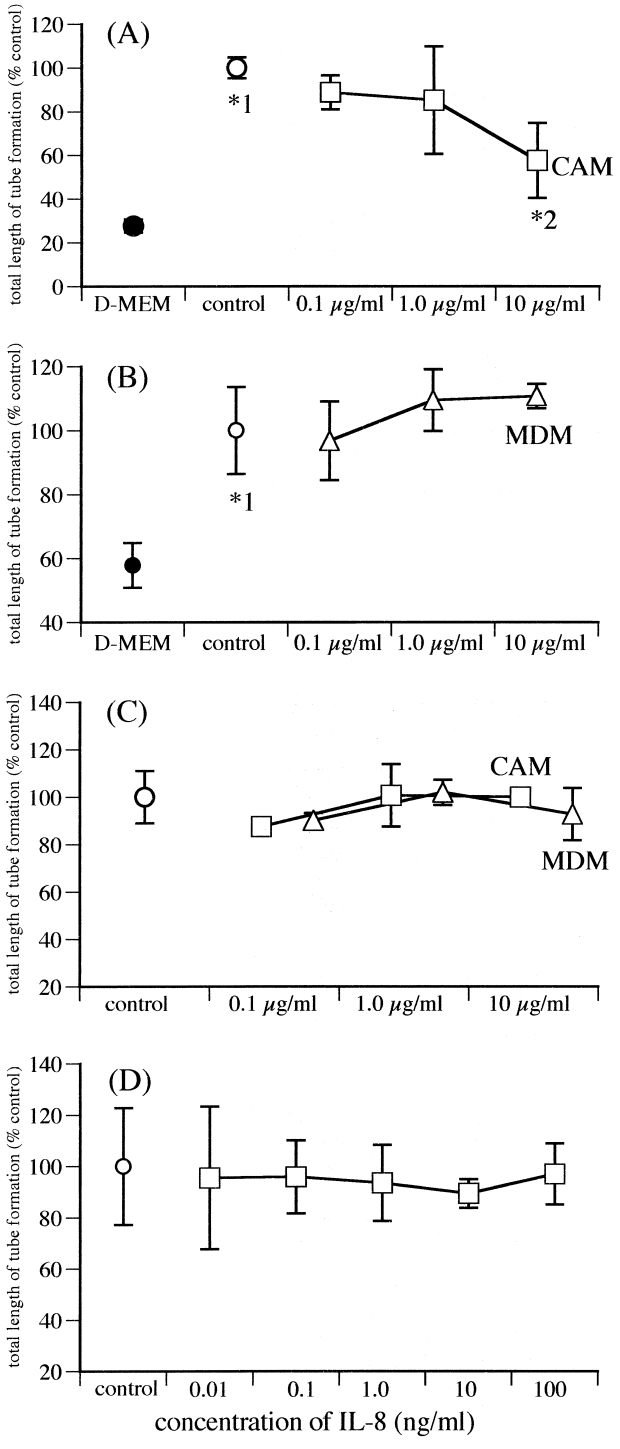
**Fig. 2.** Proliferation of A549 and HUVEC.
（A）The effects of macrolides on proliferation of A549 cells. Cells were cultured with D-MEM supplemented with 10% FBS（○）, various concentration of CAM（□）or various concentration of MDM（△）. Results are expressed as the mean±SD of 4 different experiments. *1 ; $p<0.01$ compared with proliferation of cells cultured with D-MEM＋10% FBS.（B）The effects of culture supernatant from macrolide-treated A549 cells on HUVEC proliferation. Cells were cultured with D-MEM supplemented with 10% FBS（D-MEM＋10% FBS）（●）, culture supernatant from macrolide-untreated A549 cells（○）, culture supernatant from CAM-treated A549 cells（0.1,1.0,10 mg/ml）（□）or culture supernatant from MDM-treated A549 cells（0.1, 1.0, 10mg/ml）（△）. Results are expressed as the mean±SD of 4 different experiments. *1 ; $p<0.01$ compared with cell proliferation cultured with D-MEM＋10% FBS. *2 ; $p<0.05$ compared with cell proliferation cultured with macrolide-untreated A549 cell culture supernatant *3 ; $p<0.01$ compared with cell proliferation cultured with macrolide-untreated A549 cell culture supernatant.（C）The direct effect of macrolides on HUVEC proliferation. Cells were cultured without any macrolide（○）, with various concentration of CAM（□）or various concentrations of MDM（△）. Results are expressed as the mean ±SD of 4 different experiments.

**Fig. 3.** Photomicrographs of the tube formation assay of HUVEC cultured with FBS-free D-MEM（A）, culture supernatant from A549 cells（B）, culture supernatant from A549 cells treated with 0.1μg/ml（C）, 1.0μg/ml（D）and 10μg/ml（E）of CAM. Original magnification : ×40. Results are representative of three experiments.



### Tube formation assay

The effects of culture supernatant from macrolide-treated A549 cells on the tube formation of HUVEC were investigated. The culture supernatant from macrolide-untreated A549 cells promoted tube formation compared with FBS-free D-MEM (Fig. 3A & B, 4A & B). However, CAM-treated A549 culture supernatant inhibited tube formation in a dose-dependent manner (Fig. 3B～E, 4A), whereas culture supernatant from MDM-treated A549 cells had no effect (Fig. 4B). In contrast, neither macrolide directly suppressed tube formation (Fig. 4C). Since IL-8 is known to be a potent angiogenic factor[14)15)], we investigated the effect of IL-8 on HUVEC tube formation. The results showed that IL-8 did not affect tube formation at any concentration (Fig. 4D).




### Quantitative analysis of basic FGF, VEGF and IL-8 as angiogenic factors in the culture supernatant from macrolide-treated A549 cells

Macrolide-untreated A549 culture supernatant promoted tube formation in HUVEC (Fig. 3 and Fig. 4), but the culture supernatant from A549 cells treated with CAM, but not MDM, inhibited tube formation (Fig. 3 and Fig. 4). Since basic FGF, VEGF and IL-8 are known to promote angiogenesis[14)～17)], the concentration of these factors, as well as the effect of macrolides on their production, in A549 culture supernatant were determined. The concentration of basic FGF in A549 cell supernatant was below the assay sensitivity limit of the ELISA kit used (5 pg/ml). The concentration of VEGF in macrolide-untreated A549 cell supernatant was 1.3 ng/ml, and IL-8 was present at 2.6 ng/ml. The production of VEGF was inhibited by both macrolides, but to a lesser degree by CAM (Fig. 5A). The production of IL-8 was inhibited by CAM

**Fig. 4.** Tube formation of HUVEC.
(A) The effects of culture supernatant from CAM-treated A549 cells on HUVEC tube formation. Cells were cultured with D-MEM (●), culture supernatant from A549 cells (○) or culture supernatant from CAM-treated A549 cells (0.1, 1.0, 10 mg/ml) (□). Results are expressed as the mean±SD of three different experiments. *1 ; $p<0.01$ compared with tube formation in cells cultured with D-MEM. *2 ; $p<0.01$ compared with tube formation in cells cultured with the A549 culture supernatant. (B) The effects of culture supernatant from MDM-treated A549 cells on HUVEC tube formation. Cells were cultured with D-MEM (●), culture supernatant from A549 cells (○) or culture supernatant from MDM-treated A549 cells (0.1, 1.0, 10 mg/ml) (□). Results are expressed as the mean±SD of three different experiments. *1 ; $p<0.01$ compared with tube formation in cells cultured with D-MEM. (C) The direct effects of macrolides on tube formation of HUVEC. Cells were cultured without any macrolide (○), with various concentrations of CAM (□) or various concentrations of MDM (△). Results are expressed as the mean±SD of three different experiments. (D) The effects of IL-8 on tube formation of HUVEC. The cells were cultured with EBM2 (○) or various concentrations of IL-8 (□). Results are expressed as the mean±SD of three different experiments.

**Fig. 5.** Quantitative analysis of VEGF and IL-8 in macrolide-treated A549 cell supernatant. (A) Quantitative analysis of VEGF. A549 cells were cultured with D-MEM supplemented with 10% FBS (○), various concentrations of CAM (□) or various concentrations of MDM (△). (B) Quantitative analysis of IL-8. A549 cells were cultured with D-MEM (○), various concentrations of CAM (□) or various concentrations of MDM (△).

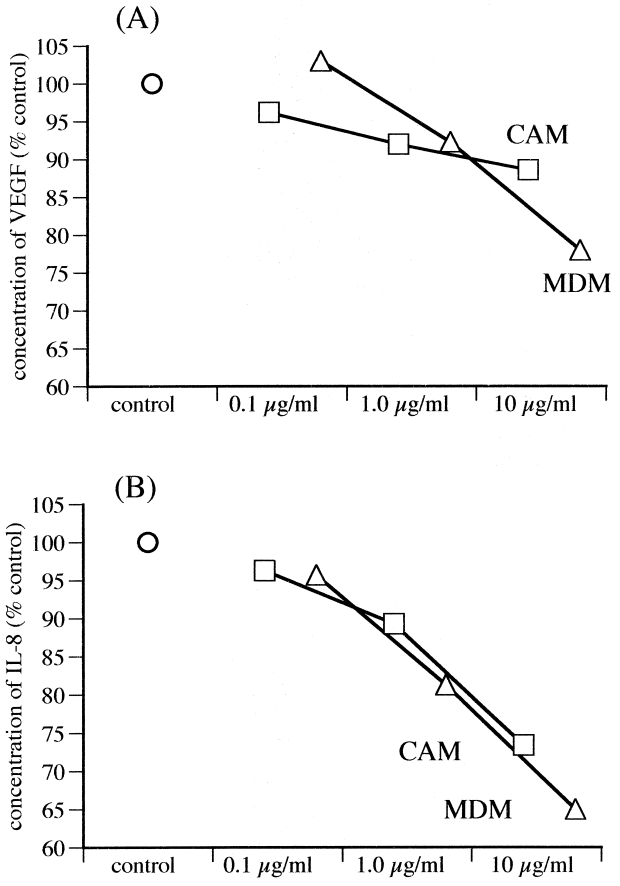
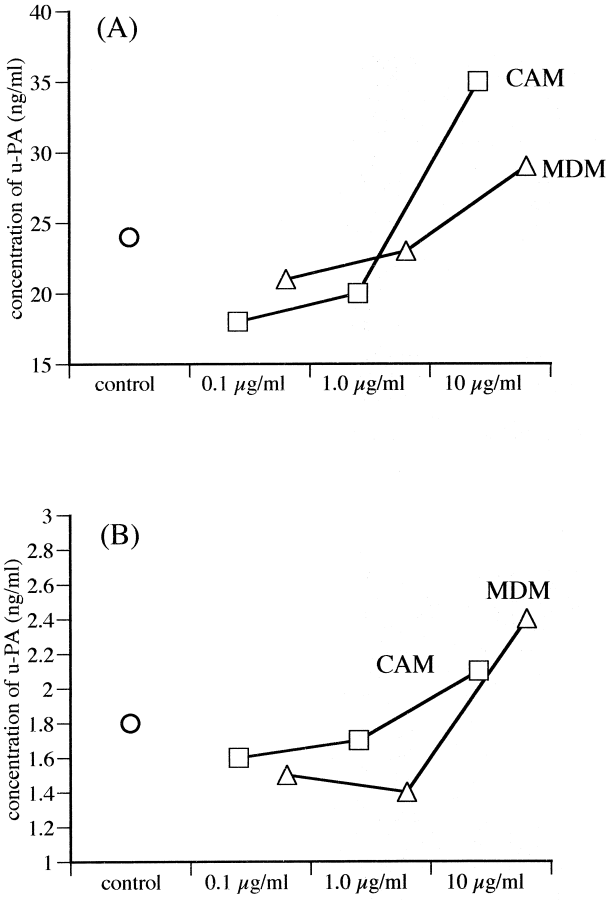



**Fig. 6.** Quantitative analysis of u-PA in macrolide-treated A549 and HUVEC supernatant. (A) A549 cells were cultured with D-MEM (○), various concentrations of CAM (□) or various concentrations of MDM (△). (B) HUVEC were cultured with EBM2 (○), various concentrations of CAM (□) or various concentrations of MDM (△).




and MDM in a dose dependent manner (Fig. 5B).

## Quantitative analysis of u-PA in culture supernatant from macrolide-treated A549 cells and HUVEC

At concentrations of 0.1-1.0μg/ml, the macrolides inhibited the u-PA secretion by both A549 cells and HUVEC, but at 10μg/ml, they acted to promote u-PA secretion in both cell types (Fig. 6A, 6B). The concentration of u-PA in A549 control was 24 ng/ml and that in the HUVEC control was 1.8 ng/ml.

## Discussion

Macrolides have been shown to inhibit tumor growth in animal models. Hamada et al. reported that erythromycin activated natural killer cells in tumor-bearing mice, but not in normal mice[6]. In the present study, we not only show that culture supernatant from A549 cells promotes the three major steps in angiogenesis, but also that culture supernatant from macrolide-treated A549 cells, especially those that were CAM-treated, inhibited these steps. The inhibitory effect of macrolides on angiogenesis was indirect, since the macrolide-treated A549 culture supernatant, but not macrolides by themselves, inhibited HUVEC angiogenesis. These results indicate that the antiangiogenic effect of macrolides is mediated via tumor cells.

Previous studies on the antiangiogenic or immunomodulatory effects of macrolides have only examined 14-membered ring macrolides[6]. Herein, we also examine the effect of a 16-membered ring macrolide, MDM, on angiogenesis. While we observed the indirect inhibition of mi-

gration, tube formation and proliferation of HUVEC by CAM, MDM only caused inhibition of HUVEC migration at a concentration of 0.1μg/ml. As angiogenesis is a multi-step process in vivo, inhibiting only one step can block it. It would therefore be interesting to examine the *in vivo* effects of MDM on angiogenesis.

Since basic FGF, VEGF and IL-8 are shown to promote angiogenesis[14)~17)], concentrations of these factors in A549 culture supernatant and the effect of macrolides on the production of these factors by A549 cells were examined. The concentration of basic FGF was found to be below the assay sensitivity limit of 5 pg/ml, and this is unlikely to significantly promote angiogenesis. However, A549 cells produce both VEGF and IL-8, which were found to be present in the ng/ml range. These results suggest that VEGF and IL-8 in the culture supernatant from A549 cells may have promoted the angiogenesis of HUVEC. However, this possibility could be ruled out based on the following evidence : 1) CAM and MDM both inhibited the production of VEGF and IL-8 by A549 cells, whereas MDM did not affect proliferation or tube formation of HUVEC, and 2)CAM and MDM both inhibited the production of IL-8 by A549 cells, whereas IL-8 did not promote tube formation of HUVEC. These results suggest that A549 cells produce other angiogenic factors, the production of which is inhibited by CAM treatment, but not by MDM-treatment. Furthermore, a number of factors that inhibit the proliferation of endothelial cells have been described, including IL-1[18)], platelet factor-4[19)], growth-related oncogene[20)], a 16-kDa prolactin fragment[21)], the tissue inhibitor of metalloproteinase-2[22)] and angiostatin[23)]. It would be of interest to determine whether macrolide-treated A549 cells produce any of these factors.

IL-8 is known as a strong angiogenic factor *in vivo*[14)15)]. Koch et al.[24)] reported that IL-8 promoted both migration and proliferation of HUVEC. Alternatively, Petzelbauer et al.[25)] found that HUVEC lacked the IL-8 receptor and did not respond to IL-8 *in vitro*. We also found that IL-8 was not associated with any of the steps involved in angiogenesis. The effects of this cytokine on angiogenesis may therefore be mediated via some unknown immune cell, rather than be exerted directly on endothelial cells.

It is also known that u-PA promotes migration of endothelial cells[26)]. Kobayashi et al.[27)] reported that an antibody against u-PA suppressed tumor cell invasion *in vitro* as well as the formation of lung metastasis following subcutaneous tumor cell inoculation, but not following intravenous inoculation. The culture supernatant from A549 cells treated with 0.1μg/ml of each macrolide inhibited migration of HUVEC, whereas A549 culture supernatant treated with 10μg/ml of CAM promoted this migration slightly. These effects are correlated with those of macrolides on u-PA production by A549 (Fig. 1A, 6A), suggesting that the effect of macrolides on HUVEC migration may be mediated via the effect of macrolides on the production of u-PA by A549 cells. However, further studies are necessary to clarify this point.

In conclusion, we have clearly shown that neither macrolide affect the three major steps of angiogenesis directly, that CAM, a 14-membered ring macrolide, may reduce proliferation and tube formation of HUVEC via an effect on carcinoma cells, and that 0.1μg/ml of CAM and MDM, may reduce HUVEC migration via their effect on A549 cells. To clarify the anti-angiogenic effects of macrolides, further studies of the effects of macrolides on the production of angiogenic and anti-angiogenic factors by A549 cells are needed.

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## Footnotes

The abbreviations used are : HUVEC, human umbilical vein endothelial cell ; CAM, clarithromycin ; MDM, midecamycin ; u-PA, urokinase plasminogen activator ; IL, interleukin ; VEGF, vascular endothelial growth factor ; FGF, fibroblast growth factor ; D-MEM, Dulbecco's modified Eagle medium ; PBS, phosphate buffered saline ; FBS, fetal bovine serum.