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In vitro inhibition of endothelial cell growth by the antiangiogenic drug AGM-1470 (TNP-470) and the anti-endoglin antibody TEC-11

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Angiogenesis plays a key role in tumor growth, progression and metastasis. The modulation of angiogenesis represents a potentially useful target for novel forms of anticancer therapy. Two such modulators are AGM-1470 (TNP-470, angioinhibin), which is a synthetic analog of the antibiotic fumagallin, and the monoclonal antibody TEC-11 to endoglin. We investigated the mechanisms of action of these modulators on human microvascular and macrovascular endothelial cells and on the transformed endothelial cell line ECV-304 *in vitro*. The administration of AGM-1470 or TEC-11 resulted in a significant inhibition of cell proliferation in all cell types used; this effect was reversible upon removal of these compounds from the culture medium. Furthermore, biochemical and morphological analyses showed that neither AGM-1470 or TEC-11 induce apoptosis. Both AGM-1470 and TEC-11 inhibited the production of urokinase-type plasminogen activator (u-PA), an enzyme involved in the early steps of neovascularization. Finally, the incubation of endothelial cells with both AGM-1470 and TEC-11 did not produce an additive effect on growth cell inhibition, apoptosis or u-PA production. Since both AGM 1470 and TEC-11 inhibit crucial events such as endothelial cell growth and protease production, our results provide a basis for their therapeutic use as angiostatic molecules in cancer.

Key words: Angiogenesis, angiogenesis inhibitors, human endothelial cells.

Introduction

There is considerable experimental evidence that angiogenesis plays a central role in tumor growth, progression and metastasis.[1–3] Recent clinicopathological studies have shown that the degree of angiogenic activity in primary solid human tumors, as determined by specific endothelial markers, immunocytochemistry and counting microvessel density, is associated with the biological aggressiveness of each individual tumor, and retains a significant and independent prognostic value.[4,5] Twenty five years ago, Folkman hypothesized that tumor angiogenesis may be a therapeutic target for novel forms of anticancer therapy.[6] At present, several specific angiogenesis inhibitors with antitumoral activity *in vivo* have been discovered and at least seven are under early clinical evaluation.[7–9] Thus, the therapeutic modulation of angiogenesis seems to be a realistic novel therapeutic approach to the treatment of malignant tumors.

Four main pharmacological strategies have been used to modulate angiogenesis. The first strategy is the development of antiangiogenic agents that inhibit endothelial cell proliferation and migration by a cytostatic mechanism.[8] One of the most promising agents is angioinhibin (*O*-[chloroacetyl-carbamoyl]-fumagillol, AGM-1470, TNP-470), a synthetic analog of fumagillin, which arrests endothelial growth *in vitro*[10] and induces regression of primary tumors and metastasis *in vivo*.[11–15] The second strategy is the direct vascular targeting using antibodies against tumor endothelial cells.[16] One example is TEC-11, an antibody to endoglin, a protein expressed preferentially on proliferating endothelial cells.[17] The third strategy is to block the action of angiogenic peptides by using neutralizing antibodies to basic fibroblast growth factor (bFGF)[18] or to vascular endothelial growth factor (VEGF).[19] The final strategy is to stimulate the synthesis of endogenous angiogenesis inhibitors such as thrombospondin-1 (TSP-1)[20] or angiostatin.[2]

The present experiments were performed to study the effect of AGM-1470 and TEC-11 on: (i) the

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growth of human normal microvascular and macrovascular endothelial cells as well as transformed human endothelial cells, (ii) apoptosis, (iii) the proteolytic activity of endothelial cells (e.g. production of the urokinase type plasminogen activator [u-PA]), and, finally, (iv) the activity of the concomitant exposure to TEC-11 and AGM-1470 on the aforementioned parameters in different types of endothelial cells.

Material and methods

Reagents

AGM-1470 (lot M 520-006) was kindly provided by Dr Terao (Takeda Chemical Industries, Osaka, Japan). The blocking IgG fraction against transforming growth factor (TGF)-β was purchased from R&D Systems (Minneapolis, MN).

Cell cultures

ECV-304 cells were kindly provided by Drs Mitsui and Imamura, and cloned to obtain a monoclonal population.[21] The cells are characterized by a cobblestone monolayer growth pattern, contact inhibition and high proliferative potential without any specific growth factor requirement. The cells have Weibel–Palade bodies and angiotensin-converting enzyme activity. ECV-304 cells were maintained by serial passage in medium M199 supplemented with 10% (v/v) fetal calf serum (FCS) and antibiotics.[22] Human umbilical vein endothelial cells (HUVEC) were derived from freshly delivered umbilical cords as described[23] and used up to the fourth passage. HUVEC were grown in M199 medium with 10% FCS, 10 U/ml heparin, 10 ng/ml recombinant acidic FGF and antibiotics in tissue culture dishes coated with purified fibronectin (5 μg/cm^2). Human dermal microvascular endothelial cells (HDMEC) were purchased from Invitrocyte (Seattle, WA) and cultured according to the manufacturers' instruction.

Proliferation assay

To perform proliferation assays, ECV-304 were seeded at low density (5000 cells/cm^2) in M199 containing 10% FCS, and exposed to AGM-1470 and TEC-11 alone or in combination in 24-well plates. At the end of the experiment, cells were detached with trypsin and counted using a hemocytometer. HUVEC and HDMEC were seeded at low density (2000 and 3000 cells/cm^2, respectively) in the culture medium described above and treated with AGM-1470 or TEC-11. At the end of the experiments cells were trypsinized and counted using a hemocytometer.

Plasminogen activator assay

HUVEC and ECV cells were plated in 96-well microtiter plates. Subconfluent cultures were incubated in the presence of various agents for 16 h. After two washes with PBS, cells were lysed in 60 mM Tris–HCl, pH 8, containing 0.1% Triton-X100.[24] Then 10 μg of cell extract was used to measure urokinase activity using the Spectrozyme kit according to the manufacturer's instructions (American Diagnostica, Greenwich, CT). After incubation at 37°C, the plate was read at 405 nm with an automatic microplate reader.

Western blot

Cells were lysed in sample buffer containing β-mercaptoethanol. Proteins were separated by electrophoresis in an 8% polyacrylamide gel and transferred to nitrocellulose sheets. The blot was incubated with TEC-11 (2.5 μg/ml) and then with iodinated Protein A. After extensive washing, the blot was exposed to Kodak X-AR film.

SDS–PAGE zymography

For the determination of the molecular weight of the PA activity in endothelial cells cultured in the presence or in the absence of TEC-11 and AGM-1470, 100 μl of cell medium was run on SDS–8% PAGE under non-reducing conditions. Proteins were transferred to a nitrocellulose membrane at 300 mA for 3 h in 40 mM sodium phosphate buffer, pH 6.5. Zymography was carried out on a caseine–agarose gel as described.[25]

Results

AGM-1470 inhibits the proliferation of different types of human endothelial cells

As shown in Figure 1(A), the addition of AGM-1470 to both HUVEC and HDMEC resulted in an inhibition

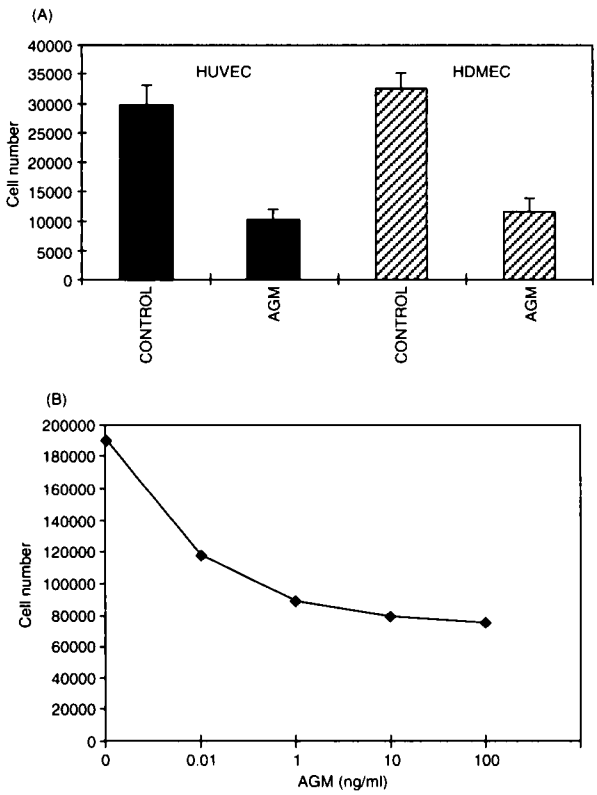

Figure 1. Effect of AGM 1470 on endothelial cell proliferation. (A) HUVEC and HDMEC were cultured as described and exposed to AGM-1470 (1 ng/ml). After 72 h, cells were trypsinized and counted with a hemocytometer as described. (B) ECV-304 cells were treated with different doses of AGM-1470 and counted after 72 h as described.

of cell proliferation with a maximal inhibition obtained with a drug concentration as low as 1 ng/ml. The addition of AGM-1470 6 or 12 h before the exposure to the growth factors did not exert any further inhibitory effect (not shown). Since two human transformed endothelial cell lines have been previously shown to be unaffected by AGM-1470, its effect on the growth of spontaneously transformed HUVEC (ECV)[22] was studied. ECV-304 cells were treated using different doses of AGM-1470. As shown in Figure 1(B), AGM-1470 potently inhibits the growth rate of these cells. Maximal inhibition of growth was obtained with ECV-304 cells incubated for 72 h with AGM-1470 at a concentration of 1 ng/ml.

A decrease in cell number may be due to a failure of the cells to enter the cell cycle or to the induction of apoptosis, or both. We therefore evaluated whether AGM-1470 induced apoptosis in endothelial cells. Morphologic analysis of the nuclei stained with DAPI as well as the biochemical analysis on agarose gel revealed no significant induction of apoptosis in all the endothelial cell types studied (not shown).

AGM-1470 inhibitory effect is reversible upon removal of the drug from the culture medium

HUVEC were incubated in the presence of AGM-1470 for 48 h and then counted. At this time point, some samples were extensively washed and incubated in normal culture medium, whereas other samples were kept in the presence of the drug. After 3 days, cells were trypsinized and counted. Upon removal of AGM-1470 from the culture medium, cells quickly began to regrow, whereas growth in the sister culture kept in the presence of the drug remained inhibited (Figure 2). Similar results were obtained in HDMEC and ECV cells (not shown).

TEC-11, a monoclonal antibody against endoglin, inhibits the proliferation of human endothelial cells

Endoglin is an essential component of the TGF-β receptor system of the endothelial cells and is a proliferation-linked endothelial cell marker, being preferentially expressed on dividing endothelial cells *in vitro* and on vascular endothelial cells of solid tumors *in vivo*.[17] ECV-304, HUVEC and HDMEC express endoglin to a similar level, as detected by Western blot analysis (Figure 3A). Moreover, the

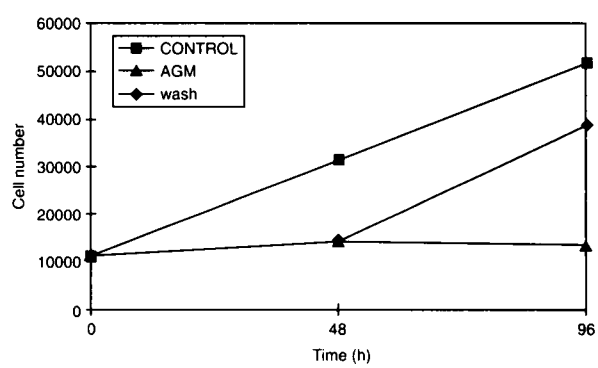

Figure 2. Reversibility of AGM 1470 effect. HUVEC cells were left either untreated or treated with AGM-1470 (1 ng/ml). Cells were counted after 48 h. At this time point some samples were extensively washed to remove AGM-1470 and exposed to normal growth medium. Cells were counted 48 h later. The results are the mean of three experiments performed in triplicate.

Figure 3. Expression of endoglin by endothelial cells. (A) Cells were lysed in sample buffer and separated on a 8% SDS–PAGE. Western blot analysis was performed as described in the text using the anti-endoglin monoclonal antibody TEC-11. Lane 1, HUVEC; lane 2, HDMEC; lane 3, ECV-304. (B) Quiescent and proliferating HUVEC were lysed and separated on SDS–PAGE. Lane 1, proliferating cells; lane 2, quiescent cells.

levels of endoglin were higher in proliferating HUVEC cells versus those quiescent (Figure 3B). We then evaluated the modulation of endothelial cell growth by TEC-11. As shown in Figure 4(A and B), both HUVEC and ECV proliferation was dramatically inhibited by the addition of TEC-11 (10 μg/ml) to the culture medium. This concentration of TEC-11 was cytotoxic on HDMEC within 24 h. When exposed to 2 μg/ml of TEC-11, a marked inhibition of HDMEC proliferation rate was observed in the absence of cytotoxicity (Figure 4A). The peculiar sensitivity of HDMEC to TEC-11 remains obscure. Interestingly, the inhibitory effect of TEC-11 was reversible within 72 h (data not shown). An IgM antibody of irrelevant specificity used as control did not exert any effect.

Since TEC-11 mimics the AGM-1470 effect on endothelial cells, we evaluated whether AGM-1470 modulated endoglin. Western analysis showed no alteration of endoglin levels after incubation with AGM-1470 (not shown).

Endoglin seems to modulate the response of endothelial cells to TGF-β. Therefore, we evaluated whether the suppressive effect of TEC-11 on endothelial cell growth requires the presence of TGF-β. As shown in Figure 4(A and B), incubation in the presence of neutralizing antibodies against TGF-β (50 μg/ml) did not alter endothelial cell response to TEC-11. This suggests that the inhibitory effect of

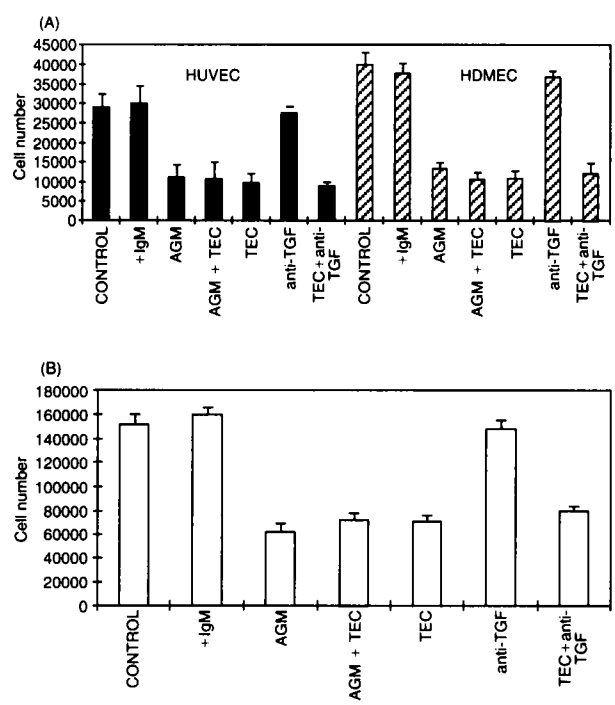

Figure 4. Effect of TEC-11 on endothelial cell growth. (A) HUVEC and HDMEC were treated with AGM-1470 (1 ng/ml) and TEC-11 (10 and 2 μg/ml, respectively) alone or in combination for 72 h. Cells were counted using a hemocytometer. In some experiments cells were cultured in the presence of a neutralizing antibody against TGF (50 μg/ml) and treated with TEC-11 at the aforementioned concentrations. (B) ECV-304 were treated and counted as above.

TEC-11 is not mediated through an activation in TGF-β signaling in such an experimental model. Figure 4(A and B) also shows that the simultaneous addition of TEC-11 and AGM-1470 did not give any additional inhibitory effect in the cells studied.

AGM-1470 and TEC-11 inhibit urokinase production by endothelial cells

Neovascularization requires both proliferation and proteolytic activity of endothelial cells.[24] We therefore evaluated whether AGM-1470 and TEC-11 modulate u-PA activity in cultures of endothelial cells. Figure 5(A and B) shows that 16 h treatment with AGM-1470 or TEC-11 significantly reduced u-PA activity. This reduced activity was confirmed in HUVEC by a zymographic assay (Figure 5C). No additive effect was observed when cells were concurrently incubated in the presence of the two agents.

Discussion

AGM-1470, which has been shown to be less toxic than its parent compound fumagallin,[10,14] is an angiogenesis inhibitor capable of suppressing the growth of a wide variety of experimental primary tumors and metastasis *in vivo*.[11,12,14,15,26] Although AGM-1470 is in phase I/II clinical trials, its mechanisms of action have not been completely clarified. Some studies indicate that AGM-1470 arrests endothelial cell proliferation in G_1 phase.[27,28] Abe *et al.*[29] extended these studies, showing that AGM-1470 inhibits the action of *cdc-2* and the cyclin-dependent kinase (*cdk*)-*2* as well as the phosphorylation of Rb, thus indicating that the drug acts mainly in the late G_1 phase of the cell cycle.

In agreement with previous observations, we found that AGM-1470 inhibits the growth of human microvascular and macrovascular endothelial cells as well as of the transformed endothelial cell line ECV. Interestingly, the cytostatic effect of AGM-1470 was reversible upon removal of the drug from the culture medium. Recent evidence shows that antagonists of integrin $\alpha_v\beta_3$ inhibit angiogenesis by selectively promoting apoptosis of vascular cells.[30] Therefore, a tightly controlled balance between endothelial proliferation and cell death seems to be a crucial event in controlling angiogenesis.[31] Our data indicate that AGM-1470 does not cause apoptosis in endothelial cells. Since angiogenesis requires not only proliferation but also induction of proteolytic activity in endothelial cells, the observation that AGM-1470 reduces the u-PA activity is of relevance.

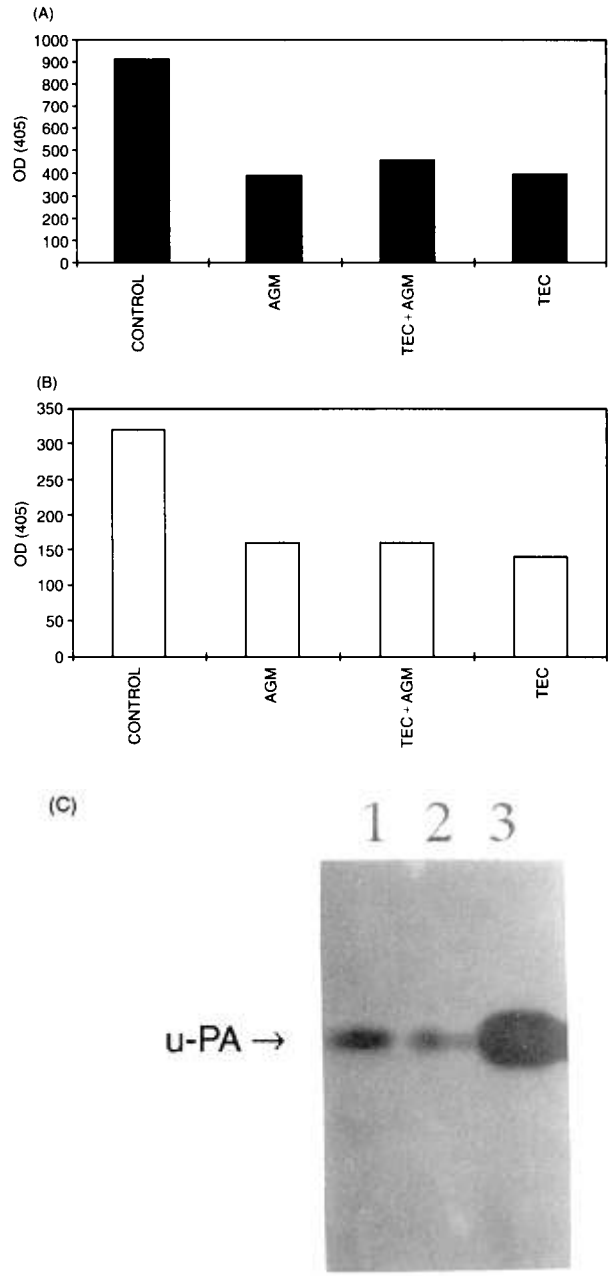

Figure 5. Modulation of u-PA activity. (A) HUVEC and (B) ECV-304 were treated with AGM-1470 and TEC-11 alone or in combination for 16 h. u-PA activity was evaluated as described. (C) Zymography on conditioned media collected from untreated HUVEC (lane 3), HUVEC exposed to AGM-1470 (lane 2) or TEC-11 (lane 1).

Endoglin is a dimeric membrane glycoprotein (M_r 180 000)[32] which binds TGF-β1 and 3, and modu-

lates the response of endothelial cells to TGFs.[33] This is relevant to endothelial pathophysiology because TGF-β1 and 3 inhibit endothelial growth, whereas TGF-β2 does not.[34–36] Because endoglin is overexpressed in tumor vasculature and in dividing as opposed to non-proliferating endothelial cells, it has been described as an endothelial cell proliferation-associated marker.[17] We found that the monoclonal antibody TEC-11 against endoglin is able to reversibly inhibit the growth and u-PA production of HUVEC and ECV-304 cells. Moreover, experiments performed in the presence of neutralizing antibodies against TGF-β indicate that the suppressive effect of TEC-11 on endothelial growth does not interfere with TGF-β signaling. Worthy of note is that TEC-11 is rapidly internalized and recycled to the cell surface by growing endothelial cells (Maier *et al.*, unpublished results). The different route of TEC-11 within the cell in proliferating versus quiescent endothelial cells may give insights into the mechanism of action of TEC-11.

Conclusion

We conclude that AGM-1470 and TEC-11 exert similar effects on endothelial cells. We can rule out the possibility that the action of AGM-1470 is somehow related to the levels of endoglin since AGM-1470 does not modulate the expression of this glycoprotein. We speculate that these two compounds may act through a common intracellular pathway or may target common events, as suggested by the evidence that the simultaneous addition of AGM-1470 and TEC-11 did not produce an additive effect on cell growth or u-PA production *in vitro*.

Our results provide evidence that TEC-11 and AGM-1470 may represent a powerful approach for treatment of neoplasia or other diseases characterized by abnormal angiogenesis. Suitable *in vivo* models[5,13] are needed to study the potential antitumor activity of these agents or of their combination with conventional anticancer drugs.

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