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The Angiogenesis Inhibitor AGM-1470 Selectively Increases E-Selectin

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The levels of E-selectin mRNA and protein were analyzed in bovine capillary cells treated with or without the angiogenesis inhibitor AGM-1470 (also known as TNP-470). Cells treated with AGM-1470 had a two- to sevenfold (median fivefold) increase in E-selectin mRNA compared with little or no increase in P-selectin, PECAM-1 and VCAM-1 mRNA. E-selectin protein was also significantly increased after exposure to AGM-1470. In contrast, there was no detectable effect on PECAM-1 protein. The increase in E-selectin mRNA and protein was always greater with subconfluent growing cells than with confluent cells. This apparent resistance of confluent endothelial cells to AGM-1470 may be relevant to its specificity *in vivo.* The fact that the effect of AGM-1470 on E-selectin is relatively selective for subconfluent growing cells may provide a clue as to how AGM-1470 is able to both reversibly inhibit endothelial cell proliferation *in vitro* and inhibit tumor growth *in vivo* without apparent effects to quiescent endothelium.

Angiogenesis, the growth of new capillaries from pre-existing blood vessels, occurs during normal growth and development, and in adult life during wound healing and endometrial proliferation. Abnormal angiogenesis occurs in tumor growth, and in many other disease states including diabetic retinopathy, rheumatoid arthritis, and hemangiomas (1,2). Because cell adhesion molecules are known to mediate many cellular interactions in mammalian tissues (3), the role of E-selectin (also known as ELAM-1) in angiogenesis has been investigated.

E-selectin is expressed in dividing endothelial cells in many different angiogenic tissues *in vivo*, including hemangiomas, placenta, and neonatal foreskin (4). Monoclonal antibodies directed against E-selectin (but not P-selectin) inhibited capillary tube formation (5) in an *in vitro* model (6). Furthermore, when bovine capillary endothelial cells are induced to form tubes in this *in vitro* assay, E-selectin messenger RNA increases 7- to 10-fold compared with cells grown as monolayers (5). Recently, a soluble form of E-selectin has been found to induce neovascularization in the rat cornea (7).

AGM-1470 (also known as TNP-470) is a fungal-derived, non-toxic inhibitor of angiogenesis *in vivo* as well as a reversible inhibitor of endothelial cell proliferation *in vitro* (8,9). The mechanism of this inhibition is unknown. AGM-1470 had no observable effect on tube formation by the *in vitro* tube formation assay (10). The potential connection between E-selectin and the inhibitory effects of AGM-1470 has not been investigated.

In this study we demonstrate that AGM-1470 increases E-selectin messenger RNA and

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The abbreviations used are: BCE, bovine capillary endothelial; DMEM, Dulbecco's modified Eagle's medium; bFGF, basic fibroblast growth factor; TNF-α, tumor necrosis factor-α; SDS, sodium dodecyl sulfate; SSC, sodium citrate buffer, GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PBS, phosphate buffered saline; GPS, glutamine, penicillin, streptomycin; 10% CS, calf serum; cpm, counts per million; SDS-PAGE, SDS-polyacrylamide gel electrophoresis.

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protein in bovine capillary endothelial (BCE) cells, and has little or no effect on other cell adhesion molecules tested including P-selectin (also known as GMP-140), PECAM-1 (also known as CD31) and VCAM-1 (vascular cell adhesion molecule-1). Furthermore, we show that the effect of AGM-1470 on E-selectin is relatively confined to subconfluent, growing cells (versus confluent cells). To our knowledge, this increase of E-selectin is the first known effect of AGM-1470 on messenger RNA and protein levels. The fact that this effect is most pronounced in subconfluent, growing cells may help to explain the reversibility of AGM-1470 *in vitro* and its non-toxic effect *in vivo.*

EXPERIMENTAL PROCEDURES

Materials. Random-primed DNA labeling kit and recombinant TNF-α (tumor necrosis factor-α), Boehringer Mannheim; Klenow fragment of DNA polymerase I, Pharmacia LKB Biotechnology Inc.; oligo(dT)-cellulose type 3, Collaborative Research; L-[^{35}S]cysteine (>800 Ci/mM), and 5′α-[^{32}P]dCTP (>1000 Ci/mM), NEN; Nytran Plus, Schleicher & Schuell; formamide, Fluka Chemika; Ecolume, ICN Biomedicals; Dulbecco's modified Eagle's medium (DMEM) and Protein-A-Sepharose Cl-4B, Sigma; calf serum, Hazelton; fetal calf serum, Hyclone; gelatin, Difco. Recombinant basic fibroblast growth factor (bFGF) and AGM-1470 were kindly provided by Takeda Chemical Industries, Osaka, Japan. Restriction enzymes and DNA modifying enzymes were obtained from reputable suppliers. All other chemicals were of reagent grade.

Cell culture. Capillary endothelial cells from bovine adrenal cortex (BCE cells) at passage 9-13 were plated in 60-mm and 150-mm dishes previously coated with 1.5% gelatin. Cultures were grown at 37°C in 10% CO_2 and fed with DMEM and 10% calf serum (10% CS) supplemented with L-glutamine (2 mM), penicillin (100 UI/ml), streptomycin (100 μg/ml) (GPS), and 3 ng/ml basic fibroblast growth factor (growth medium). Cells were trypsinized to obtain single cells and then plated at an average density of 1.25×10^4 cells per cm^2, and exposed to AGM-1470 (100 ng/ml from a 3 mg/ml aqueous stock of AGM-1470/β-cyclodextrin) or control for 24 hours at either day 2 or 7 after splitting. (Preliminary experiments were carried out using AGM-1470 alone in an ethanol stock versus ethanol as control; the results obtained were identical to those of AGM-1470/β-cyclodextrin solution). Cells were treated with TNF-α (200 units/ml) for 4 hours.

Isolation of RNA and Northern blot analysis. Poly-A selected RNA of BCE cells was isolated using a proteinase K/SDS lysis method (11) and fractionated by formaldehyde-1% agarose gel electrophoresis. RNA was transferred to Nytran Plus by capillary action in 20× SSC (sodium citrate buffer, 3M NaCl, 0.3M Na citrate, pH 7.0) and baked in an oven for 30 minutes at 80°C (12). A 1.7 kb *KpnI* fragment of the bovine E-selectin cDNA (5), a 2.0 kb fragment (*BamH1*/*HindIII*) of the bovine P-selectin cDNA (13), a 1.8 kb *EcoRI* fragment of the human PECAM-1 cDNA (kindly provided by Peter Newman, The Blood Center of Southeastern Wisconsin, Milwaukee, WI) and a 2.0 kb *HindIII* fragment of the human VCAM-1 cDNA (kindly provided by Tucker Collins, Dept. of Pathology, Brigham and Women's Hospital, Boston, MA) were isolated and radiolabeled with 5′α-[^{32}P]dCTP to a specific activity of 1-3×10^9 cpm/μg of DNA. Northern blots were incubated with the [^{32}P]-labeled probe (1×10^6 cpm/ml) in 50% formamide, 50 mM $NaPO_4$ pH 7.4, 750 mM NaCl, 1 mM EDTA, 5× Denhardt's (12), 0.1% SDS, 100 μg/ml poly(A), and 100 μg/ml heat-denatured salmon sperm DNA at 42°C for 16 hours. The blots were then washed in 0.1× SSC, 0.1% SDS, 4 × 15 minutes at 50°C. The blots were probed in a similar fashion with a 1.3 kb fragment of radiolabeled rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA to serve as an internal standard (14). The signals were detected and quantitated by Phosphor Imager analysis.

Metabolic labeling of BCE cells. BCE cells were plated in 60-mm dishes with 4 ml DMEM 10%CS GPS with 3 ng/ml bFGF and treated with AGM-1470 for 24 hours or TNF-α for 4 hours at 37°C in 10% CO_2. Next the cells were rinsed twice with phosphate buffered saline (PBS) and then preincubated in 4 ml of DMEM devoid of cysteine but supplemented with 10% dialyzed fetal calf serum and GPS (Cys-free DMEM) for 30 minutes at 37°C, 10% CO_2. After the preincubation, the cells were labeled with 200 μCi of [^{35}S]cysteine in 1 ml of Cys-free DMEM and 3 ng/ml bFGF at 37°C in 10% CO_2 for 1 hour before complete growth media was added back for 30 min.

Immunoadsorption of E-selectin. All steps were carried out on ice or at 4°C. Total cell lysates were prepared by rinsing cell monolayers twice with 5 ml of ice-cold PBS, and then solubilizing the monolayer with 0.5 ml of PBS, 1% Triton X-100, 0.5% deoxycholate, 0.005% SDS, 1 mM EDTA, 0.2 TIU/ml aprotinin, and 0.1 mM leupeptin (cell lysis buffer). Cell extracts were vortexed, incubated on ice for 15 minutes, centrifuged at $16,000 \times g$ for 10 minutes, and precleared with 100 μl of a 1:1 suspension of Sepharose Cl-4B for 30 minutes on a rotating platform. Beads were removed by centrifugation at $16,000 \times g$ for 10 minutes. Protein assays and tricarboxycylic acid precipitations (to determine radioactivity incorporated into protein, i.e. specific activity) were performed at this point. (It was previously determined that cell number was directly proportional to total protein.) The amounts of control, AGM-1470 and TNF-α treated cell extracts used were adjusted to obtain equal levels of radioactivity (cpm) in equal volumes prior to immunoadsorption. Cell extracts in cell lysis buffer, adjusted to 2.5 mg/ml bovine serum albumin, were incubated on ice for 16 hours with 1 μl of either rabbit polyclonal anti-E-selectin antibody or pre-immune serum

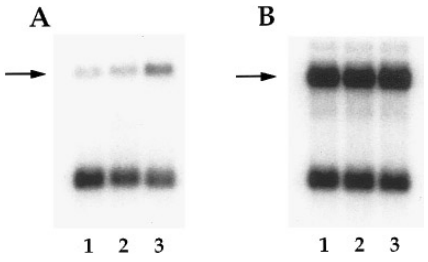

FIG. 1. Effect of AGM-1470 on E- and P-selectin mRNA. Poly-A mRNA was isolated from BCE cells treated without AGM-1470 (lane 1), with AGM-1470 for 5 hours (lane 2), and with AGM-1470 for 24 hours (lane 3). Panel A was probed with E-selectin and GAPDH cDNAs; panel B was probed with P-selectin and GAPDH cDNAs.

from the same rabbit. Immune complexes were adsorbed with 20 μl of Protein A-Sepharose for 1 hour at 4°C. The adsorbed complexes were sedimented and washed sequentially with 1 ml of the following solutions: twice with 20 mM Tris, pH 8, 1 M NaCl, 1% deoxycholate, 1% Nonidet P-40, 1 mM EDTA; twice with 20 mM Tris, pH 8, 1 M KCL, 0.1% Triton X-100; once with 10 mM Tris, pH 8, 0.2% Nonidet P-40; once with 20 mM Tris, pH 8, 0.1 M NaCl, 1% Triton X-100, 0.5% deoxycholate, 0.5% SDS; and then once with 10 mM Tris, pH 8, 0.2% Nonidet P-40. The antigen-antibody complexes were eluted from the Protein A-Sepharose and denatured by boiling for 5 minutes in 60 μl of 125 mM Tris, pH 6.8, 2% SDS, 20% glycerol, 2% 2-mercaptoethanol, 20 mM dithiothreitol, and 0.002% bromophenol blue and then subjected to 7.5% SDS-PAGE (15). The signals were detected and quantitated by Phosphor Imager analysis. PECAM-1 was immunoadsorbed from the cell lysate supernatants after removal of E-selectin/antibody complexes with Protein A-Sepharose using an anti-bovine PECAM-1 polyclonal antibody (kindly provided by Dr. Steven M. Albelda, University of Pennsylvania).

Protein determination. The Pierce Bicinchoninic acid protein assay reagent was used to determine protein concentrations as described by the manufacturer. Bovine serum albumin was used as a standard.

RESULTS AND DISCUSSION

The expression of E- and P-selectin mRNAs was examined in subconfluent BCE cells treated either with or without 100 ng/ml of AGM-1470 (Fig. 1). E-selectin mRNA was elevated 4.2 fold in cultures exposed to AGM-1470 for 24 hours compared to control (Panel A, compare lanes 1 and 3). The degree of up-regulation varied from 1.9 to 7.0 in a series of 6 experiments with slightly varied conditions. Five hours of exposure to AGM-1470 produced only a modest 1.6 fold increase of E-selectin mRNA (Panel A, lane 2). In contrast, there was little or no change in the level of P-selectin mRNA exposed to AGM-1470 in the same experiment (Panel B). The degree of up-regulation of P-selectin mRNA varied from none detectable to 1.4 fold in 4 experiments. Similar experiments found little or no change in the levels of PECAM-1 and VCAM-1 mRNA when exposed to AGM-1470 (data not shown).

The increase of E-selectin mRNA by a 24 hour exposure to AGM-1470 was next examined in subconfluent (2 day) versus confluent (7 day) cultures (Fig. 2). E-selectin mRNA was increased 3.0 fold in the 2 day cultures (compare lanes 1 and 2) versus 1.8 fold in the 7 day culture (compare lanes 4 and 5). As expected, TNF-α increased E-selectin mRNA in both subconfluent (lane 3) and confluent (lane 6) BCE cells. In a series of 4 additional experiments, the increase of E-selectin mRNA in subconfluent cultures varied from 1.9 to 7.0 fold. In contrast, the increase in response to AGM-1470 treatment varied from 0 to 1.8 fold in confluent cultures.

The level of E-selectin and PECAM-1 protein was analyzed in BCE cells treated with or without 100 ng/ml of AGM-1470 in both subconfluent and confluent cultures (Fig. 3). Treatment with AGM-1470 increased E-selectin protein 1.7 fold in 2 day subconfluent cultures (Panel A, compare lanes 2 and 4), but there was essentially no change of E-selectin protein in 7 day confluent cultures (0.8 fold, compare lanes 6 and 8). TNF-α increased E-selectin protein as expected, 2.0 and 9.4 fold for subconfluent and confluent cultures respectively.

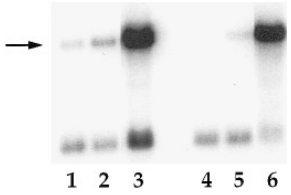

FIG. 2. Effect of AGM-1470 on E-selectin mRNA in subconfluent and confluent cultures. Poly-A mRNA was isolated from BCE cells which were either subconfluent (2 days after trypsin passage, lanes 1–3) cultures or confluent (7 days after trypsin passage, lanes 4–6). Cultures were treated without (lanes 1 and 4) or with (lanes 2 and 5) AGM-1470 for 24 hours. For comparison, BCE cells were also treated with TNF-α for 4 hours (lanes 3 and 6).

While one additional experiment yielded similar results, another experiment revealed a slightly more robust response to AGM-1470, with increases of E-selectin protein in subconfluent and confluent cultures of 4.1 and 2.0 fold, respectively. Again the increase in E-selectin protein in subconfluent cultures of 4.1 and 2.0 fold, respectively. Again the increase in E-selectin protein in subconfluent cultures was usually at least twice that of confluent cultures, with no significant difference seen between subconfluent and confluent cells in the level of TNF-α inducible E-selectin. There was no significant change in PECAM-1 protein with exposure to either AGM-1470 (1.2 and 0.8 fold) or TNF-α (1.0 and 1.2 fold) in both subconfluent and confluent cells (Fig. 3, Panel B). Some degradation of the 130 Kd PECAM-1 occurred in these samples.

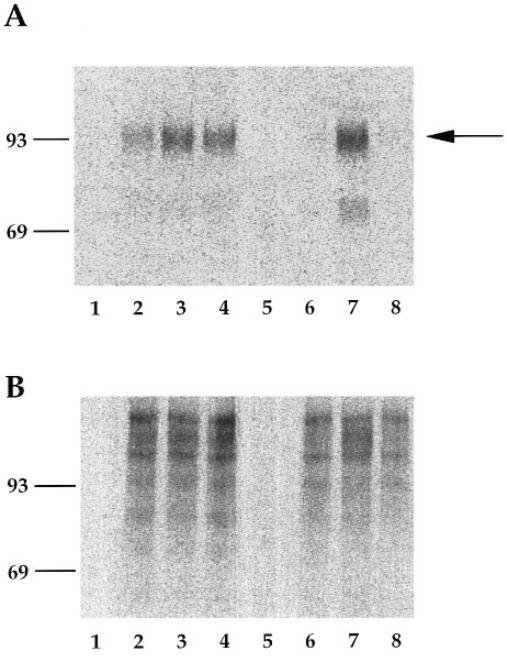

FIG. 3. Effect of AGM-1470 on E-selectin and PECAM-1 protein in subconfluent and confluent cultures. E-selectin and PECAM-1 were immunoadsorbed sequentially from BCE cells which were either subconfluent (2 days after trypsin passage, lanes 1–4) or confluent (7 days after trypsin passage, lanes 5–8). Cultures were treated without (lanes 1, 2, 5, and 6) or with (lanes 4 and 8) AGM-1470. BCE cells were also treated with TNF-α for 4 hours (lanes 3 and 7). Lanes 1 and 5 are control rabbit serum. Lanes 2–4 and 6–8 are polyclonal anti-E-selectin antibody (Panel A) or polyclonal anti-PECAM-1 antibody (Panel B).

Although previous studies have shown that AGM-1470 is a reversible inhibitor of endothelial cell proliferation *in vitro* (8,9), its mechanism of action remains unknown. In this study we demonstrate that while having no significant effect on P-selectin, PECAM-1 or VCAM-1, AGM-1470 increases E-selectin mRNA in BCE cells. Similarly, AGM-1470 increases E-selectin protein while having no significant effect on PECAM-1 protein. Although it is not clear whether this increase is the cause or the effect of its inhibition of endothelial cell proliferation, the selectivity of AGM-1470 for E-selectin suggests that its effect on E-selectin is relevant to its mechanism of action. It is noteworthy that the AGM-1470-induced increase in E-selectin is comparable to the induction observed with TNF-α in subconfluent BCE cells. The increase in E-selectin induced by AGM-1470, an *anti*-angiogenic drug, may seem counter-intuitive given the important role of E-selectin in tube formation (5) (a critical step in angiogenesis), the detection of E-selectin in the dividing endothelial cells of angiogenic tissues *in vivo* (4), and the ability of E-selectin to induce neovascularization in the rat cornea (7). However, little is known about the mechanism of action of AGM-1470 and its effects on gene expression in endothelial cells.

By demonstrating that the increase in E-selectin mRNA and protein is relatively confined to subconfluent cultures, we can hypothesize that the reason that the inhibition of AGM-1470 is reversible *in vitro* and non-toxic *in vivo* is because its effects are relatively confined to growing endothelial cells. This hypothesis is supported by the observation that AGM-1470 has no observable effect on the *in vitro* tube formation assay (10), another situation in which endothelial cells are in a quiescent state (6). By learning more about the mechanism of action of AGM-1470 we hope both to further the rational design of future angiogenic inhibitors, and to gain a greater understanding of the process of angiogenesis itself.

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