VOL. 56 NO. 8, AUG. 2003 THE JOURNAL OF ANTIBIOTICS pp. 709 — 715

Anti-angiogenesis Effects of Borrelidin are Mediated through Distinct Pathways: Threonyl-tRNA Synthetase and Caspases are Independently Involved in Suppression of Proliferation and Induction of Apoptosis in Endothelial Cells

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(Received for publication April 4, 2003)

Borrelidin, an antibiotic with anti-angiogenic activity, not only suppresses new capillary tube formation, but also collapses formed capillary tubes in a rat aorta culture model. Since it selectively inhibits threonyl-tRNA synthetase, we examined the effect of threonine on its anti-angiogenic activity. We found that a high concentration of threonine (1 mM) attenuated the ability of borrelidin to inhibit both capillary tube formation in the rat aorta culture model and human umbilical vein endothelial cells (HUVEC) proliferation, yet did not affect the ability of borrelidin to collapse formed capillary tubes or to induce apoptosis in HUVEC. Borrelidin activated caspase-3 and -8, and inhibitors of both caspase-3 and -8 suppressed borrelidin-induced apoptosis in HUVEC. Taken together, these data suggest that the anti-angiogenic effects of borrelidin are mediated through at least two mechanisms, *i.e.* one threonine-dependent and the other threonine-independent, and borrelidin induces apoptosis in endothelial cells *via* the caspase-8/-3 pathway.

Angiogenesis is a complicated multistep process, which includes proliferation, adhesion, invasion, migration, and tube formation of endothelial cells[1]. One *in vitro* model that seems to accurately represent the biological phenomena is the rat thoracic aorta tube formation (RATF) model. In this model, a small piece of aorta is cultured in a matrix basement membrane. Using this model, screening of crude natural extracts and microbial metabolites for anti-angiogenic activity has led to the discovery of borrelidin in a culture broth of Actinomycetes[2]. Borrelidin showed anti-angiogenic and anti-metastatic effects in animal models[3]. The anti-angiogenic activity of borrelidin is unique in involving dual modes of action; inhibition of new capillary tube formation and collapse of formed capillary tubes *via* the cell death of capillary-forming endothelial cells[2].

Borrelidin was originally isolated as an antibiotic[4], and is produced by a variety of *Streptomyces*[5]. Its antibiotic effect is due to the specific inhibition of threonyl-tRNA synthetase; other aminoacyl-tRNA synthetases are not inactivated[5,6]. Borrelidin has been shown to inhibit the growth of mammalian cells as well as bacteria, and increased levels of threonyl-tRNA synthetase result in borrelidin resistance in Chinese hamster ovary (CHO) cells[7], suggesting that borrelidin inhibits cell proliferation in mammalian cells *via* the inhibition of threonyl-tRNA synthetase, as it does in bacteria. In contrast, the growth inhibition of CHO cells by borrelidin is antagonized by threonine in a dose-dependent manner[7]. These findings suggested that the involvement of threonyl-tRNA synthetase inhibition could be tested by using culture media containing a range of threonine concentrations.

In this paper, we present the results of our studies to examine the threonine dependence of borrelidin's anti-angiogenic effects, and to explore the mechanisms

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underlying borrelidin's anti-angiogenic activity.

Materials and Methods

Materials

HUVEC were obtained from Cascade Biologics, Inc. (Portland, OR, USA), and maintained in M-200 complete growth medium (Cascade Biologics, Inc.). The inhibitors of caspase, Z-VAD-FMK, Z-DEVD-FMK and Z-IETD-FMK, were purchased from Calbiochem (San Diego, CA, USA).

Rat Aorta Tube Formation Assay

Thoracic aorta were obtained from 8-week-old SD female rats sacrificed by exposure to CO_2 gas. The fibroadipose tissue around the aorta was carefully removed and one mm-long rings were cut and rinsed in cold PBS. Matrigel (BD Biosciences, Bedford, MA, USA) (0.5 ml) was added to each well of a 24-well plate, which was kept on ice. The aorta rings were embedded in Matrigel, followed by incubation at 37°C. After gel formation, 0.5 ml of M-200 complete medium with or without borrelidin was laid over the gel. The reaction medium was replaced with fresh medium every other day. Borrelidin was added on day 0 for the growth inhibition experiment, or on day 4 for the capillary tube collapse experiment. After incubation for the indicated time, aorta rings were stained with MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) and photographed. The number of capillary tubes was counted on a printout of the photograph.

HUVEC Proliferation Assay

HUVEC (5×10^3/well) were seeded in 96-well plates with M-200 complete medium and cultured for 3 days. Cells were then serum-depleted by replacing the medium with M-200+0.5% FBS followed by overnight incubation. bFGF (10 ng/ml) and borrelidin were incubated with cells for 22 hours followed by the addition of 1 μCi of ^{3}H-thymidine to each well. After 2 hours, the cells were harvested using a 96-well cell harvester, and thymidine incorporation was measured with a scintillation counter.

HUVEC Apoptosis Assay

HUVEC (2×10^4/well) were seeded in a 96-well plate and cultured for 3 days. The medium was replaced with medium containing borrelidin, followed by incubation for the indicated time. For the caspase inhibitor experiment, each inhibitor was added 30 minutes before addition of borrelidin. Apoptosis was measured using the TiterTACS™ apoptosis detection kit (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions. This kit is designed to detect DNA fragments stained *in situ* by using TUNEL. Apoptosis index was defined as the relative absorbance value to a control sample after TUNEL staining. The apoptosis index value for DNase-treated HUVEC as a positive control was 7.09.

Caspase Activity Assay

The activities of caspases were measured using ApoAlert® caspase colorimetric assay kits (Clontech, Palo Alto, CA, USA) according to the manufacturer's instructions. Briefly, HUVEC were treated with 100 nM borrelidin for 24 hours and harvested. Cells (2×10^6) were resuspended in cell lysis buffer and incubated on ice for 10 minutes. The cell lysates were centrifuged and the supernatants collected. Following the addition of reaction buffer and substrate (DEVD-pNA for caspase-3 or IETD-pNA for caspase-8), the absorbance at 405 or 504 nm was measured in a microplate reader.

Statistical Analysis

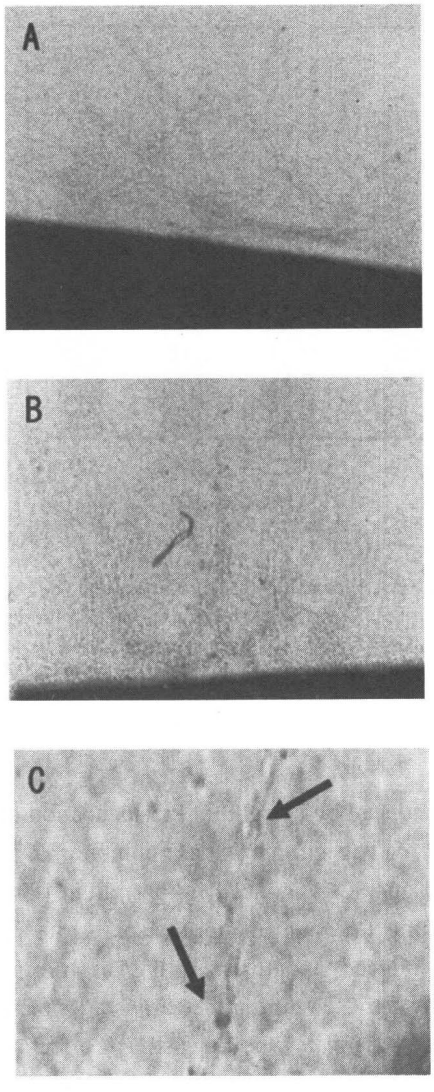
All values are expressed as mean±S.E.M. ANOVA with the Tukey test was used to identify significant differences in multiple comparisons, and paired data were analyzed for significant difference by paired Student's *t* test. A level of $P<0.05$ was considered statistically significant.

Results

We have shown that borrelidin caused selective death of endothelial cells composing the capillary tubes when it was added to a rat aorta ring culture in which the aorta had formed new capillary tubes[2]. We first examined whether this cell death involved apoptosis. A culture with new capillary tubes was treated with borrelidin, followed by staining using the TUNEL method[2]. Apoptosis of some endothelial cells was induced by borrelidin (Fig. 1), suggesting that endothelial cell death *via* apoptosis leads to the collapse of newly formed capillary tubes. Based on this result, we examined the ability of borrelidin to induce apoptosis in cultured HUVEC. To consider the quiescent state of endothelial cells at pre-formed capillary tubes, HUVEC were confluently seeded for apoptosis assay. Borrelidin induced apoptosis in HUVEC, starting within 3 hours and progressing for up to 24 hours (Fig. 2).

We then investigated whether threonine affects the anti-angiogenic effect of borrelidin. Borrelidin at 3~30 nM was added to the rat aorta culture on day 0, followed by incubation for 4 days. Borrelidin inhibited sprouting of new

Fig. 1. Apoptosis of endothelial cells composing the capillary tubes induced by borrelidin.

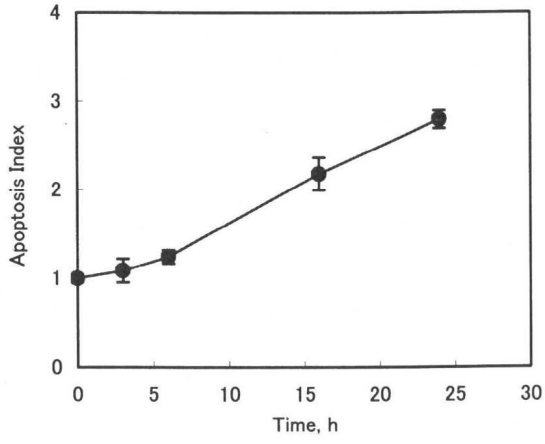


Aortic culture was treated with borrelidin (6 nM) after capillary tubes had formed: not treated (A) or treated for 4 hours (B) or 6 hours (C).

Apoptotic cells were stained by using the TUNEL method. Arrows indicates apoptotic cells.

Magnification: (A, B); ×100, (C); ×200.

Fig. 2. Time course of apoptosis induced by 100 nM borrelidin in HUVEC.



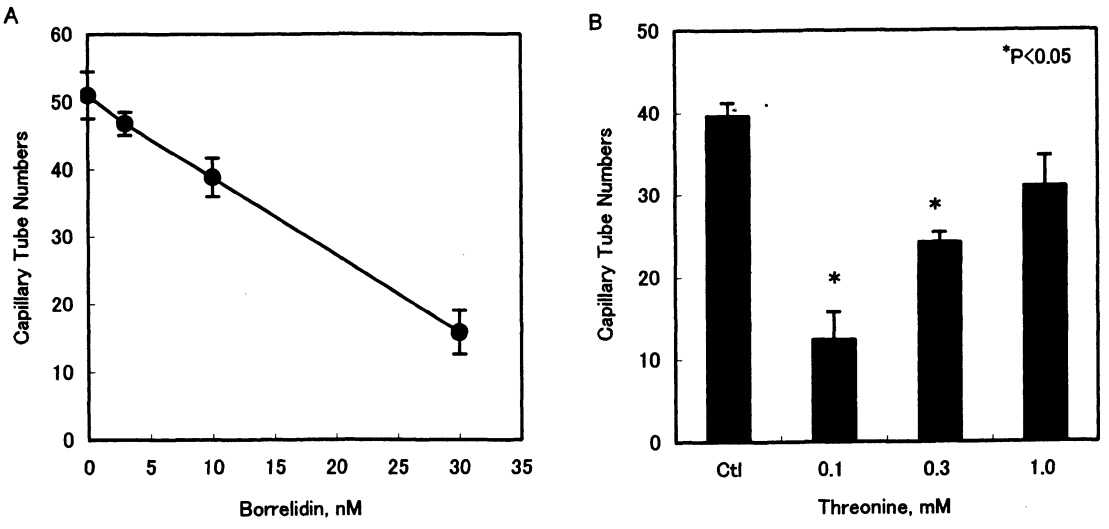
The results are shown as means±S.E.M. (n=5).

capillary tubes in a dose-dependent manner in the presence of 0.1 mM threonine (Fig. 3A). Further, higher concentrations of threonine concentration-dependently attenuated the inhibitory effect of borrelidin on sprouting of new capillary tubes (Fig. 3B). Increase in the threonine concentration in the medium (0.1 to 1 mM) also attenuated the inhibitory effect of borrelidin on thymidine incorporation in cultured HUVEC stimulated with bFGF (Fig. 4). This attenuation was specific for threonine: other amino acids, such as serine, glutamic acid, valine, isoleucine and glycine, did not influence the inhibitory effect of borrelidin (data not shown).

We next investigated whether the higher threonine concentrations antagonize the ability of borrelidin to promote collapse of newly formed capillary tubes. Borrelidin was added to rat aorta culture on day 4, when new capillary tubes were formed. After 24 hours, the newly formed capillary tubes in the borrelidin-treated samples were found to have collapsed (Fig. 5A), and the collapse was observed at concentrations similar to those that caused inhibition of new capillary tube formation in Fig. 3A. In another experiment, borrelidin 30 nM was added together with threonine at various concentrations on day 4. Interestingly, higher concentrations of threonine had no effect on the collapse of new capillaries by borrelidin (Fig. 5B). As borrelidin induced apoptosis in HUVEC, we next investigated the effect of threonine concentration on the induction of apoptosis by borrelidin in HUVEC. We found that borrelidin treatment for 15 hours induced apoptosis in a dose-dependent manner, but in contrast to the earlier result on thymidine incorporation (Fig. 4), induction of apoptosis was independent of threonine concentration (Fig. 6).

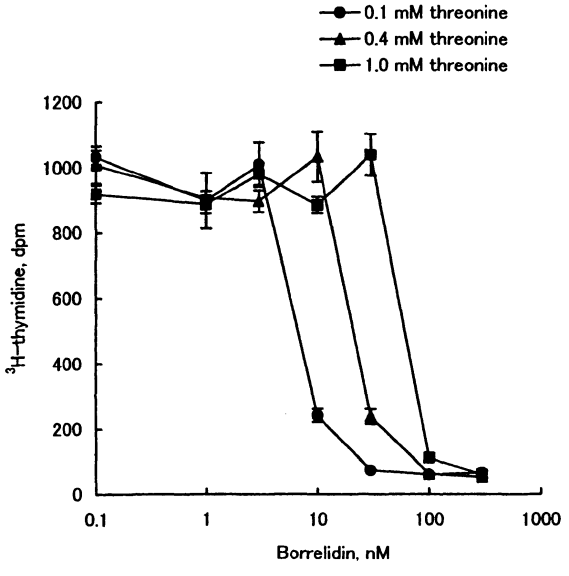
To study further the mechanism of apoptosis induced by borrelidin, we investigated whether caspases are involved in

Fig. 3. Inhibitory effect of borrelidin on capillary tube formation and effect of threonine concentration on the inhibitory activity of borrelidin.



(A) Inhibitory effect of borrelidin on capillary tube formation in the presence of 0.1 mM threonine, and (B) effect of threonine concentration on the inhibitory effect of 30 nM borrelidin in RATF assay. Borrelidin was added on day 0 and the number of formed capillary tubes was counted on day 4. The results are shown as means±S.E.M. (n=5). $P<0.05$ *versus* Ctl: borrelidin-untreated samples in the presence of 0.1 mM threonine.

Fig. 4. Effect of threonine concentration on the activity of borrelidin to inhibit proliferation of HUVEC.



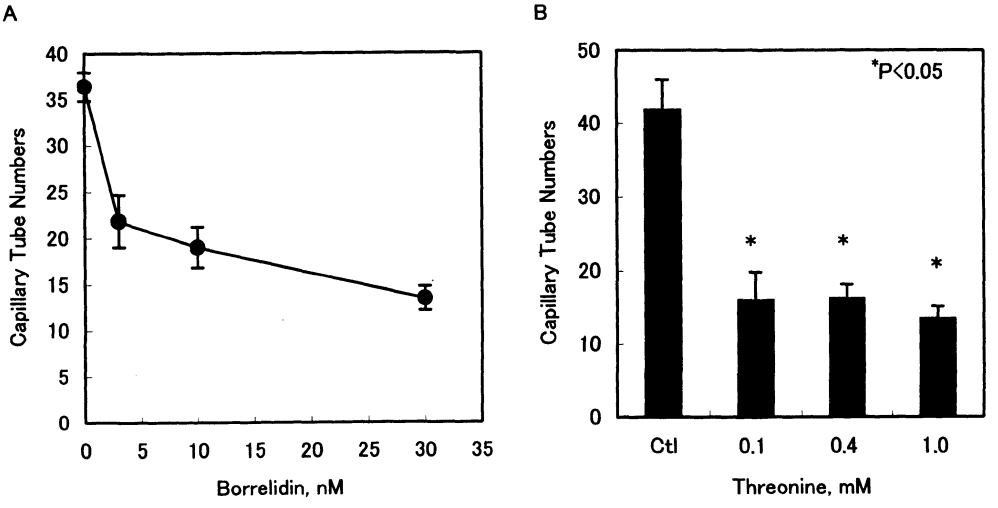
The proliferation of HUVEC was stimulated by the addition of bFGF. The cells were incubated with borrelidin for 24 hours in the presence of various concentrations of threonine (0.1~1 mM) and thymidine incorporation activity was measured. Results are shown as means±S.E.M. (n=4).

apoptosis induced by borrelidin. Pre-incubation of HUVEC with the broad-spectrum caspase inhibitor Z-VAD-FMK suppressed apoptosis induced by borrelidin ($P<0.05$, Fig. 7), suggesting that one or more caspases are involved in borrelidin-induced apoptosis. It has been reported that caspase-3 is activated during the early stages of apoptosis[8], and caspase-8 activates the downstream effector caspases, including caspase-3[9,10]. We therefore assessed the effect of the specific caspase-3 inhibitor Z-DEVD-FMK and the specific caspase-8 inhibitor Z-IETD-FMK. As shown in Fig. 7, both Z-DEVD-FMK and Z-IETD-FMK suppressed borrelidin-induced apoptosis in HUVEC ($P<0.05$). The involvement of caspase-3 and -8 was confirmed in caspase-3 and -8 enzyme assays using HUVEC. Borrelidin (100 nM) induced a 3.1- ($P<0.05$) and 1.8-fold ($P<0.01$) increase in the activities of caspase-3 and -8, respectively, on 24 hours treatment (Fig. 8).

Discussion

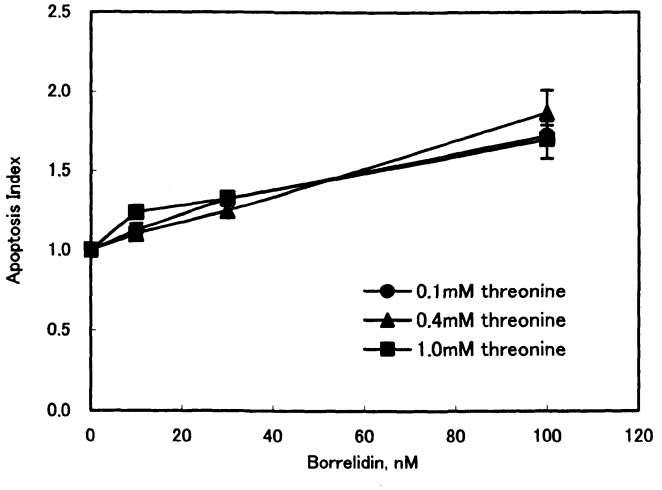
In this work, we have investigated the mechanisms through which borrelidin exerts its anti-angiogenic effects. The antibiotic action of borrelidin is mediated by inhibition

Fig. 5. Collapsing effect of borrelidin on formed capillary tubes and effect of threonine concentration on the collapsing activity of borrelidin.



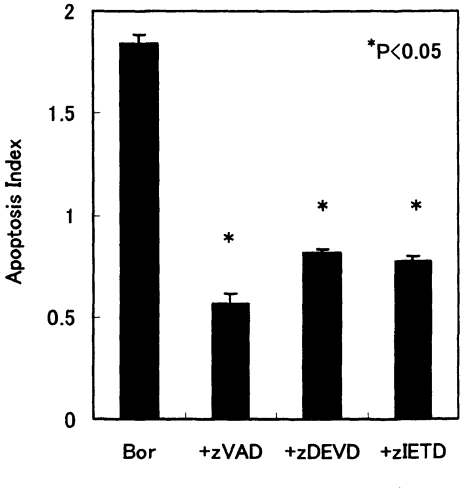
(A) Collapsing effect of borrelidin on formed capillary tubes in the presence of 0.1 mM threonine, and (B) effect of threonine concentration on the ability of 30 nM borrelidin to collapse formed capillary tubes in RATF assay. Borrelidin was added on day 4 and the number of formed capillary tubes was counted after 24 hours. The results are shown as means±S.E.M. (n=5). $P<0.05$ *versus* Ctl: borrelidin-untreated samples in the presence of 0.1 mM threonine.

Fig. 6. Effect of threonine concentration on borrelidin-induced apoptosis of HUVEC.



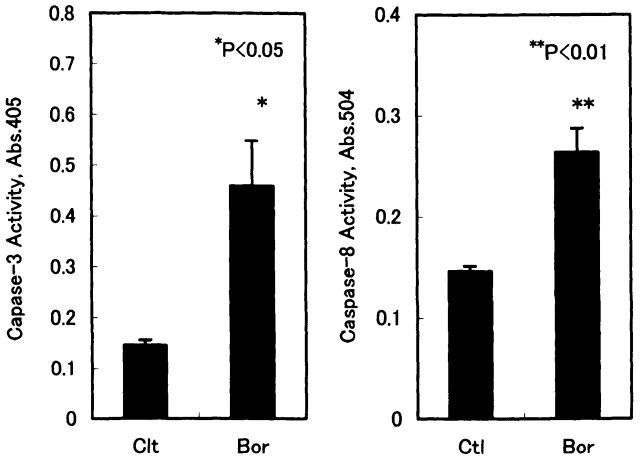
HUVEC were treated with borrelidin for 15 hours in the presence of various concentrations of threonine (0.1~1 mM) and apoptosis was measured. The results are shown as means±S.E.M. (n=5).

Fig. 7. Effect of caspase inhibitors on borrelidin-induced apoptosis in HUVEC.



Each inhibitor (100 μM) was added 30 minutes before the addition of borrelidin (100 nM), and then incubatation with borrelidin was continued for 15 hours. The results are shown as means±S.E.M. (n=5). $P<0.05$ *versus* Bor: borrelidin-treated samples without an inhibitor.

Fig. 8. Effect of borrelidin on caspase-3 (left) or caspase-8 (right) activity.



The results are shown as means±S.E.M. (n=5). The statistical significance of differences between untreated (Ctl) and borrelidin-treated samples (Bor) was analyzed.

of threonyl-tRNA synthetase[5], an enzyme that belongs to the group of aminoacyl-tRNA synthetases (EC 6.1.1.) catalyzing the attachment of threonine to tRNA to synthesize threonyl-tRNA. The biosynthesis of threonyl-tRNA synthetase is regulated by feedback mechanisms. For example, growth of *E. coli* under conditions of threonine deficiency leads to depression of the transcription of the threonyl-tRNA synthetase gene[11]. On the other hand, the product of threonyl-tRNA synthetase, namely threonyl-tRNA can act as a derepressor and activate the biosynthesis of threonyl-tRNA synthetase. The synthesis of threonyl-tRNA proceeds in a two-step reaction through formation of a tertiary complex intermediate of L-threonine, AMP and threonyl-tRNA synthetase. L-Threonine is a substrate in the first reaction[12]. Higher concentrations of L-threonine kinetically push the reaction forward to the synthesis of threonyl-tRNA and produce larger amounts of threonyl-tRNA as a final product. This threonyl-tRNA activates the biosynthesis of threonyl-tRNA synthetase *via* the feedback mechanism. Thus, higher concentrations of threonine result in higher levels of threonyl-tRNA synthetase. Such higher levels of threonyl-tRNA synthetase might antagonize the threonyl-tRNA synthetase-inhibitory effect of borrelidin. For instance, CHO cells overexpressing threonyl-tRNA synthetase are resistant to growth inhibition by borrelidin[13], and CHO cells grown in medium containing high threonine concentrations are also resistant to the growth-inhibitory effect of borrelidin[7]. We therefore considered that high threonine concentrations may abrogate the inhibitory effects of borrelidin on angiogenesis. Interestingly, although the inhibitory effect of borrelidin on new capillary tube formation was suppressed by higher threonine concentrations, the ability of borrelidin to collapse newly formed capillary tubes was not affected. Since we observed apoptosis in endothelial cells at collapsed capillary tubes, it appears that apoptosis of endothelial cells induced by borrelidin is not affected by high threonine concentrations. This notion prompted us to examine the situation in HUVEC, and a similar pattern of threonine dependency was observed. That is, the inhibitory effect of borrelidin on proliferation of HUVEC was suppressed by higher threonine concentrations, but induction of apoptosis by borrelidin was not affected. Thus, these results suggested that the anti-angiogenic effect of borrelidin is the result of at least two distinct modes of action, one threonine-dependent and one threonine-independent. The threonine-dependent mechanism is involved in the growth inhibition of endothelial cells, while the threonine-independent mechanism is involved in apoptosis induction. The apoptosis by borrelidin may depend on the cell cycle with endothelial cells, since we have observed that borrelidin induced apoptosis in endothelial cells at pre-formed capillary tubes or confluent cultures of HUVEC. Aminoacyl-tRNAs are the products of the first committed step in protein biosynthesis, and inhibition of aminoacyl-tRNA synthesis leads to inhibition of protein synthesis. We have observed that protein synthesis is inhibited by borrelidin in HUVEC[2]. Thus, it is likely that the inhibition of threonyl-tRNA synthetase is responsible for the growth inhibition of endothelial cells.

To understand the threonine-independent induction of apoptosis of HUVEC by borrelidin, we investigated possible pathways that might mediate borrelidin-induced apoptosis. Our data showed that a caspase-3 or -8 inhibitor blocked borrelidin-induced apoptosis, and that borrelidin activated caspase-3 and -8, suggesting that activation of caspase-3 by caspase-8 is responsible, at least in part, for borrelidin-induced apoptosis. It is noteworthy that thrombospondin-1 (TSP-1), a natural inhibitor of angiogenesis, induces apoptosis *via* caspase-3 and -8 in endothelial cells[14,15]. Caspase-8 is a key initiator of the apoptotic cascade from Fas[9], and TSP-1 upregulates Fas/Fas ligand on endothelial cells[15]. On the other hand, we have observed that borrelidin enhances phosphorylation levels of p38 MAPK (mitogen-activated protein kinase) in HUVEC (data not shown), and Fas has been reported to

activate p38 MAPK[16]. Therefore, borrelidin may stimulate Fas/Fas ligand, thereby leading to apoptosis of endothelial cells.

The dual anti-angiogenic activities of borrelidin appear to have potential for use as a combination therapy with conventional anti-cancer drugs. The *in vivo* anti-angiogenic activity of borrelidin has been confirmed in a quantitative mouse dorsal air sac model, and its inhibitory effect on spontaneous lung metastasis of B16-BL6 melanoma has also been proved[3]. Our experiments suggest that threonyl-tRNA synthetase inhibition is responsible for the growth-inhibitory effects of borrelidin, but the molecular target(s) involved in the induction of apoptosis by borrelidin has yet to be elucidated, although the caspase-8/-3 cascade appears to be involved. Further studies will be needed to investigate the molecular target(s) of borrelidin, other than threonyl-tRNA synthetase, that mediate the anti-angiogenic activity of borrelidin.

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