Induction of Apoptosis in Proliferating Human Endothelial Cells by the Tumor-specific Antiangiogenesis Agent Combretastatin A-4

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Abstract

The antiangiogenic, tubulin-binding drug combretastatin A-4 exhibits a selective toxicity for proliferating endothelial cells in vitro and induces vascular shutdown in tumor models in vivo. The mechanism of combretastatin A-4 cytotoxicity has now been investigated with cultured proliferating human umbilical vein endothelial cells by examining various markers of apoptosis. Incubation of cells with 0.1 mM combretastatin A-4 induced the conversion (first detected after 6 h) of the CPP32 proenzyme to active caspase-3, a cysteine protease that plays an important role in apoptosis in many cell types; the drug also increased caspase-3 activity. Another early event observed was the binding of annexin V to 50% of the cells 8 h after drug treatment. Internucleosomal DNA fragmentation, another hallmark of apoptosis, was detected in cells incubated with 0.1 mM combretastatin A-4 for 24 h. Staining with Hoechst 33342 revealed that about 75% of cells exhibited a nuclear morphology characteristic of apoptosis after incubation with drug for 24 h. Incubation of cells for up to 8 h with combretastatin A-4 did not induce the release of lactate dehydrogenase or increase the uptake of propidium iodide, both indicators of membrane integrity. These results indicate that the selective cytotoxic effect of combretastatin A-4 is mediated by the induction of apoptosis rather than by necrosis and may provide an enhanced clinical strategy in cancer chemotherapy with this new agent.

Introduction

Angiogenesis is critical to both the survival and continued growth of solid tumors and therefore represents a prime target for new cancer treatments. We have shown recently that combretastatin A-4 exhibits selective toxicity toward endothelial cells of tumor vasculature (1). Our in vitro studies revealed a marked toxicity of this compound for proliferating endothelial cells but not for cells that were quiescent before and during drug exposure. A 93% reduction in functional vascular volume was also observed in an experimental cancer model in vivo 6 h after systemic drug administration. In our previous study, we assessed cell viability with the neutral red technique. This assay revealed a concentration-dependent toxic action of combretastatin A-4 on normal human proliferating endothelial cells in culture and, to a lesser extent, on cultured tumor cells (1). However, the neutral red assay cannot distinguish whether cell death occurs by necrosis or apoptosis. The morphological changes associated with apoptosis are highly similar among diverse cell types and include cytoplasmic vacuolization, cell shrinkage, plasma membrane blebbing, chromatin condensation and fragmentation, and the formation of membrane-bound apoptotic bodies. A family of cysteine proteases, known as caspases, is known to play an important role in apoptotic cell death (2). We previously contributed to the purification and characterization of one such human enzyme, now known as caspase-3, that catalyzes the cleavage of PARP (3) during apoptosis. PARP is a nuclear enzyme that is activated by DNA strand breaks and catalyzes the poly(ADP-ribose)ylation of various nuclear proteins. We have shown recently that a transient burst of poly(ADP-ribose)ylation of nuclear proteins early in apoptosis, which is followed by caspase-3-mediated cleavage and inactivation of PARP, is essential for subsequent progression of the death program in various cell types (4, 5). Given the potential clinical importance of combretastatin A-4 and related drugs, we have now investigated the mechanism by which this compound induces the death of proliferating endothelial cells. Specifically, we have examined various biochemical and morphological markers of apoptosis to determine whether the toxic effect of combretastatin A-4 results from the induction of programmed cell death.

Materials and Methods

Systemic Administration of Combretastatin and Measurement of Percentage of Vascular Shutdown. Six hours after i.p. administration of combretastatin A-4 (25 or 100 mg/kg), the mice were injected with Hoechst 33342 (10 mg/kg, i.v.) and, 1 min later, were euthanized. The tumors were excised and frozen at −20°C, sections were prepared and examined under UV illumination, and vascular volume was determined with a random point scoring system as described previously (1).

Cells. HUVECs were obtained from Clonetics and maintained in EGM-2 BulletKit medium in a 5% CO2 incubator. Cells were grown in tissue culture dishes, and assays were performed when they reached a density of 1.3 × 10⁶ cells/cm².

Drug Treatment. HUVECs were incubated with 0.1 mM combretastatin A-4 (Oxigene, Inc.) for various times and then assayed for various markers of apoptosis or necrosis. Jurkat cells were exposed to antibiotics to Fas (50 nm/g/ml; Kamiya Biologicals) as a control for cells undergoing apoptosis.

Analysis of DNA Fragmentation. Total DNA was isolated from cells as described previously (4) and analyzed by electrophoresis in 1.5% agarose gels and ethidium bromide staining.

PARP-Cleavage Assay. Caspase-3 activity in cytosolic extracts of cells was measured by assaying the cleavage of ³²°S)methionine-labeled recombinant PARP as described previously (4). Briefly, 5 μg of cytosolic protein were incubated for 1 h at 37°C in a reaction mixture (25 μl) containing ³²°S)PARP (~5 × 10⁶ cpm), 50 mM PIPES-KOH (pH 6.5), 2 mM EDTA, 0.1% 3-(3-cholamidopropyl)dimethylammonio)-1-propanesulfonate detergent, and 5 mM DTT. The reaction was terminated by the addition of an equal volume of 6X SDS sample buffer [0.25M Tris-Cl (pH 6.8), 2% SDS, 8% BME, Bromophenol blue 0.02%], and PARP-cleavage products were detected by SDS-PAGE on 10% gel followed by fluorography.

Hoechst or Propidium Iodide Staining. HUVECs were isolated by exposure to trypsin followed by resuspension in serum-containing media. The cells were centrifuged (800 × g for 5 min), and fixed in 4% formalin for 10 min. After washing twice with PBS, the cells were stained either with Hoechst 33258 (24 μg/ml) in PBS containing 80% (v/v) glycerol or with propidium iodide.
iodine (according to the manufacturer's specifications) in PBS, mounted on a slide, and observed with a fluorescence microscope.

**Annexin-V and Propidium Iodide Staining.** HUVECs were isolated by exposure to trypsin and centrifugation, washed with ice-cold PBS, and stained for 15 min in the dark at room temperature with propidium iodide and FITC-labeled annexin-V (both, according to the manufacturer's specifications; Trevigen) in a solution containing 10× binding buffer and water. The cells were then examined with a fluorescence microscope.

**Measurement of Levels of Toxicity.** Levels of cell toxicity were assessed by measuring the release of the cytosolic enzyme lactate dehydrogenase into the medium with the use of a CytoTox 96 kit (Promega).

**Immunoblot Analysis.** Cytosolic extracts prepared from HUVECs for the PARP-cleavage assay were also subjected to immunoblot analysis for measurement of CPP32 processing to caspase-3. Samples containing 30 µg of protein were fractionated by SDS-PAGE on 4–20% gradient gels, and the separated proteins were transferred to a nitrocellulose membranes. Membranes were stained with Ponceau S to confirm equal loading and transfer of proteins among lanes. After blocking of nonspecific sites using 5% nonfat milk in PBS + 0.05% Tween 20, the membrane was incubated for 1 h at room temperature with rabbit polyclonal antibodies that recognize both CPP32 and the p17 subunit of caspase-3 (1:10,000 dilution; kindly provided by Dr. D. Nicholson, Merck Labs, Pointe Claire-Dorval, Quebec, Canada). Immune complexes were then detected with horseradish peroxidase-conjugated goat antibodies to rabbit immunoglobulin G (1:2000 dilution; Amersham Life Sciences) and enhanced chemiluminescence (ECL; Pierce).

### Results

**Effect of Combretastatin A-4 on Perfused Tumor Vascular Volume in Mice.** We determined previously the time course for the effect of systemic administration of combretastatin A-4 at a dose of 100 mg/kg on the perfused vascular volume of the murine carcinoma NT growing s.c. in CBA/Gy fTO mice (1). To confirm this action for the present study, we assessed the effects of two doses of the drug (25 and 100 mg/kg) 6 h after i.p. administration (Fig. 1). At the lower dose, combretastatin A-4 reduced functional vascular volume in the tumor mass by ~30%. At the higher dose, the drug induced essentially complete vascular shutdown, consistent with the results of our previous study (1).

**Effects of Combretastatin A-4 on CPP32 Processing and Caspase-3 Activity in HUVECs.** Similar to other members of the caspase family, caspase-3 is synthesized in cells as an inactive Mr 32,000 proenzyme (CPP32). Early during apoptosis, CPP32 is cleaved at specific aspartate residues to yield the active enzyme (caspase-3), which is a heterodimer of a Mr 17,000 subunit (p17) and a Mr 12,000 subunit (p12). To determine whether CPP32 is proteolytically processed to p17 during exposure of HUVECs to combretastatin A-4, we incubated the cells with the drug for various times and then subjected cytosolic extracts to immunoblot analysis with antibodies to CPP32.

![Figure 1](image-url) **Fig. 1.** Effect of combretastatin A-4 on the perfused vascular volume of the murine carcinoma NT growing s.c. in CBA/Gy fTO mice. Six h after i.p. administration of combretastatin A-4 (25 or 100 mg/kg), the mice were injected with Hoechst 33342 (10 mg/kg; i.v.) and, 1 min later, killed. The tumors were excised and frozen at −20°C; sections were prepared and examined under UV illumination, and vascular volume was determined with a random point scoring system as described previously (1). Data are expressed as percentage vascular shutdown relative to the mean vascular volume of untreated tumors and are means for 5 to 13 tumors; bars, SE.

![Figure 2](image-url) **Fig. 2.** Effects of combretastatin A-4 on the proteolytic processing of CPP32 (A), on caspase-3 activity (B), and on internucleosomal DNA fragmentation (C) in HUVECs. A. HUVECs (1 × 10⁶) were incubated in the presence of 0.1 mM combretastatin A-4 for 6 or 24 h or in the absence of drug for 24 h, as indicated. The cells were then washed with serum-free medium and homogenized in 1 ml of a solution containing 10 mM HEPES-KOH (pH 7.4), 2 mM EDTA, 0.1% 3-[[(3-cholamidopropyl)dimethylamino]-1-propanesulfonic acid, 5 mM DTT, 1 mM phenylmethylsulfonyl fluoride, 10 µg/ml pepstatin A, 20 µg/ml leupeptin, and 10 µg/ml aprotinin. The homogenate was centrifuged at 100,000 × g, and the resulting supernatant was subjected to immunoblot analysis with antibodies to CPP32. The positions of CPP32 (Mr 32,000) and p17 (Mr 17,000) are indicated. B. HUVECs (1 × 10⁶) were incubated with 0.1 mM combretastatin A-4 for the indicated times or in its absence for 24 h. Cytosolic extracts were prepared as described in A and assayed for caspase-3 activity as described in "Materials and Methods." The positions of the full-length PARP substrate (Mr 116,000) and its Mr 89,000 and Mr 24,000 cleavage products are indicated. C. HUVECs (1 × 10⁶) were incubated with 0.1 mM combretastatin A-4 for the indicated times or in its absence for 48 h. As a positive control, Jurkat cells were treated with antibodies to Fas for 6 h. DNA was extracted from the cells, subjected to electrophoresis on a 1.5% agarose gel, and stained with ethidium bromide.
Fig. 3. Effects of combretastatin A-4 on staining of HUVECs by Hoechst 33258 (A) or propidium iodide (B). A. cells were incubated in the absence of drug for 24 h or in its presence (0.1 mM) for 6, 16, or 24 h, as indicated. They were then isolated by exposure to trypsin and centrifugation. The cells were fixed with formalin, washed with PBS, and stained with Hoechst 33258. Arrows, cells exhibiting chromatin condensation. B. cells were incubated with 0.1 mM combretastatin A-4 for 24 h (upper panels) or 48 h (lower panels), after which they were exposed to trypsin, collected by centrifugation, fixed with formalin, washed with ice-cold PBS, and incubated with propidium iodide for 10 min in the dark. Left panels, propidium iodide staining; right panels, phase-contrast micrographs. ×40.
Fig. 4. Effect of combretastatin A-4 on staining of HUVECs by annexin-V and propidium iodide. Cells were incubated with 0.1 mM combretastatin A-4 for 0, 8, or 24 h as indicated, after which they were exposed to trypsin, collected by centrifugation, washed with ice-cold PBS, and incubated with annexin-V and propidium iodide for 15 min in the dark. Left panels, phase-contrast micrographs; right panels, annexin-V (green) and propidium iodide (red) staining. Filled arrows, late apoptosis; unfilled arrows, early apoptosis. X40.

As a positive control, we induced apoptosis in human Jurkat T cells by incubation with antibodies to Fas; such treatment resulted in the generation of p17 from CPP32. Whereas incubation of HUVECs in the absence of drug for 24 h did not result in the conversion of CPP32 to p17, incubation of the cells with 0.1 mM combretastatin A-4 induced CPP32 processing in a time-dependent manner (Fig. 2A). Processing of CPP32 was substantial after incubation of HUVECs with the drug for 6 h and appeared maximal at 24 h.

We next examined the effect of combretastatin A-4 on caspase-3 activity (Fig. 2B). HUVECs were incubated with 0.1 mM drug for various times, after which cytosolic extracts were prepared and assayed for their ability to catalyze cleavage of the caspase-3 substrate [35S]PARP into M, 89,000 and M, 24,000 products. Extracts prepared from cells incubated in the absence of drug for 24 h or those derived from cells incubated in the presence of drug for 1 h contained negligible caspase-3 activity. However, cells treated with combretastatin A-4 for 4 h showed a marked increase in caspase-3 activity, which increased further in cells exposed to the drug for 6 or 24 h. These data are consistent with the time course of CPP32 proteolysis in combretastatin A-4-treated HUVECs (Fig. 2A).

Effects of Combretastatin A-4 on Internucleosomal DNA Fragmentation and Nuclear Morphology in HUVECs. The effect of combretastatin A-4 on internucleosomal DNA fragmentation, a biochemical hallmark of apoptosis, in HUVECs was examined by agarose gel electrophoresis of extracted DNA (Fig. 2C). As a positive control, Jurkat cells treated with antibodies to Fas for 6 h exhibited marked internucleosomal DNA degradation, as revealed by the characteristic ladder pattern of DNA fragments on electrophoresis. HUVECs incubated in the absence of drug for 48 h or those exposed to combretastatin A-4 for 6 h showed no evidence of nuclear DNA fragmentation. In contrast, HUVECs treated with drug for 24 or 48 h exhibited substantial DNA fragmentation. The time course of DNA fragmentation in drug-treated cells is consistent with previous data suggesting that the processing of CPP32 and cleavage of PARP by caspase-3 are required before substantial DNA fragmentation can occur (4).
Combretastatin A-4 also induced changes in nuclear morphology characteristic of apoptosis. Hoechst 33258 staining and fluorescence microscopy revealed that 75% of HUVECs treated with combretastatin A-4 for 24 h exhibited chromatin condensation (Fig. 3A), compared with only ~1% of cells incubated in the absence of drug. Nuclear fragmentation and chromatin condensation were also detected by propidium iodide staining of cells treated with combretastatin A-4 for 24 or 48 h (Fig. 3B); no such changes were observed in cells incubated without drug.

**Effects of Combretastatin A-4 on Cell Membrane Asymmetry, Integrity, and LDH Release in HUVECs.** The loss of plasma membrane asymmetry is an early event in apoptosis in many different cell types, resulting in the exposure of phosphatidyl serine on the outer surface of the membrane. Because annexin-V reacts with high affinity with phosphatidyl serine in the presence of calcium, it is a useful assay for apoptosis. We therefore stained unfixed HUVECs with annexin-V and propidium iodide. Untreated HUVECs did not stain with either annexin-V or propidium iodide (Fig. 4). However, after incubation with the drug for 8 h, 50% of the cell membranes were bound to annexin-V, while 99% of the cells excluded propidium iodide, supporting the idea that apoptosis is the primary mechanism of cell death in this system. Only after a 24-h exposure of cells to combretastatin A-4 do annexin-V-positive cells begin to take up propidium iodide, indicating that loss of membrane integrity occurs late in apoptosis, after caspase activation.

We also investigated the effect of combretastatin A-4 on the release of LDH from HUVECs into the culture medium. The medium of cells incubated in the absence or presence of combretastatin A-4 for up to 24 h consistently contained ~10% of total cellular LDH (data not shown), again indicating that the drug does not impair membrane integrity at these time points and that it induces cell death by apoptosis rather than by necrosis.

**Discussion.**

We have demonstrated previously that combretastatin A-4, a tubulin-binding drug, selectively reduces the viability of proliferating HUVECs in culture as well as induces vascular shutdown in tumor models in vivo (1). However, the neutral red assay used in these experiments to assess the viability of cultured cells could not determine whether the cytotoxic effect of the drug resulted in cell death by necrosis or by apoptosis. We have now shown that combretastatin A-4 induces the death of proliferating HUVECs, in large part if not completely, by apoptosis. An important difference between apoptosis and necrosis is that the contents of the dying cell are efficiently disposed of in the former and are released into the surrounding environment in the latter; necrosis, unlike apoptosis, is thus associated with a risk of inflammation and inappropriate exposure of self-antigens.

We have shown that combretastatin A-4 induced the proteolytic conversion of CPP32 into caspase-3 in proliferating HUVECs. Furthermore, with the use of an in vitro PARP-cleavage assay, we showed that combretastatin A-4 induced a marked increase in caspase-3-like activity after incubation with the cells for only 4 h. Various lines of evidence indicate that caspase-3 is both necessary and sufficient to trigger apoptosis: (a) disruption of the caspase-3 gene in knockout mice results in excessive accumulation of neuronal cells, due to lack of apoptosis in the brain (6); (b) various strategies for inducing apoptosis, including Fas activation, serum withdrawal, and exposure to ionizing radiation or pharmacological agents, also trigger activation of caspase-3 as a result of proteolytic cleavage of CPP32 (7-11); (c) a tetrapeptide aldehyde inhibitor of caspase-3 (Ac-DEVD-CHO) blocks initiation of the apoptotic program in response to various signals (3, 12); and (d) the addition of active caspase-3 to a cytosolic fraction derived from normal cells triggers biochemical events characteristic of apoptosis (13). In addition to PARP, substrates for the protease activity of caspase-3 include U1-70K, DNA-dependent protein kinase, the retinoblastoma protein (Rb), fodrin, actin, lamin, gelsolin, and an inhibitor of a caspase-activated DNAse, all of which play roles in DNA repair, mRNA splicing, regulation of the cell cycle, or the morphological changes or DNA fragmentation associated with apoptosis (3, 12-17).

In addition to triggering the cytosolic and membrane events associated with apoptosis, combretastatin A-4 induced internucleosomal DNA cleavage, chromatin condensation, and nuclear fragmentation in proliferating HUVECs, all of which are nuclear hallmarks of cells in the final stages of apoptosis. Although we have identified the mechanism of cell death induced by combretastatin A-4 in endothelial cells, it remains to be determined why proliferating cells, such as those contributing to tumor vascularization, are sensitive to the cytotoxic action of this drug, whereas quiescent cells, which constitute the majority of endothelial cells in the body, are not. The signaling pathway by which combretastatin A-4 triggers caspase-3 activation and the other apoptotic phenomena described here also remains to be identified. Nevertheless, our data should contribute to the development of combretastatin A-4 or related drugs as potential cancer chemotherapeutic agents.

**References.**

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