COMP-Ang1 Potentiates the Antitumor Activity of 5-Fluorouracil by Improving Tissue Perfusion in Murine Lewis Lung Carcinoma

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Abstract
Chemotherapy is often hindered by abnormal tumor vasculature, which causes impaired delivery of drugs into the tumor. Angiopoietin-1 has potent roles in angiogenesis and vessel maturation. We report here that an angiopoietin-1 variant, COMP-Ang1, promotes vascular remodeling and pericyte coverage and thereby promotes the efficient delivery of a chemotherapeutic drug into tumors of murine Lewis lung carcinoma. The combination of COMP-Ang1 with the cytotoxic drug 5-fluorouracil potentiated the effect of 5-fluorouracil on tumor growth without increasing animal toxicity. Moreover, COMP-Ang1 increased perfusion into the tumor. Although COMP-Ang1 increased the functional vasculature in the tumor, COMP-Ang1 alone did not promote tumor growth, possibly due to its promotion of increased pericyte coverage. This study suggests that COMP-Ang1 may improve the microcirculation within a tumor by increasing functional vasculature and tissue perfusion and that the combination of chemotherapy together with COMP-Ang1 might be an advantageous therapeutic approach. (Mol Cancer Res 2009;7(12):OF1–8)

Introduction
Current treatments for solid tumors are often compromised by the impaired delivery of the therapeutic agents and the low drug sensitivity of the tumor cells. Elevated interstitial fluid pressure may be one of the causes of the poor availability of circulating therapeutic agents (1). In addition, the vasculature within tumors is often structurally and functionally abnormal, resulting in leakiness and heterogeneity in tumor blood perfusion (2-4). Because the abnormally high leakiness of the tumor vasculature may lead to an uneven distribution of delivered drugs and oxygen, the efficacy of the delivered drugs is also limited. A higher dose of the chemotherapeutic drug may increase its concentration in the tumor but may also produce unexpected cytotoxicity to normal tissues. To circumvent these problems, many approaches have been tried to correct the delivery system within tumors (5). Of note, remodeling of the tumor vasculature by anti–vascular endothelial growth factor treatment improves the delivery of drugs into tumors (6, 7). This treatment also produces increased coverage of pericytes and normal thickness of the basement membrane around the tumor vessels (6, 7). However, these changes to the vasculature brought about by anti–vascular endothelial growth factor treatment are transient (7). Thus, approaches to improve and prolong the remodeling of tumor vasculature may provide a useful strategy to improve the delivery of chemotherapeutics.

Angiopoietin-1 (Ang1) is a ligand of the Tie2 tyrosine kinase receptor (8). Ang1 signaling via the Tie2 receptor expressed on endothelial cells is involved in blood vessel formation and maturation (9, 10). By increasing the interaction between endothelial cells and pericytes, Ang1 is known to stabilize blood vessels (11, 12). In addition, overexpression of Ang1 produces enlarged and leakage-resistant vessels in adult mice (12). Given the potency of Ang1 in stabilization and leakage-resistant remodeling in blood vessels, Ang1 could improve tumor circulation by promoting vascular remodeling in tumors.

Conflicting results about the role of Ang1 in tumors have been reported. Some researchers have reported that overexpression of Ang1 improved tumor perfusion and growth (13-15), whereas others observed that Ang1 induced impaired angiogenesis and subsequent inhibition of tumor growth (16, 17). The diverse effects of Ang1 on tumor vasculature may be due to basic differences among different types of tumors, the extent of Tie2 expression in the tumors, or the availability and potency of exogenous Ang1. In addition, recent evidence showed that Tie2-expressing monocytes in tumors are involved in tumor growth (18, 19), implying that angiopoietin may play a role in tumor angiogenesis both through the Tie2 receptor on endothelial cells and through Tie2-expressing monocytes. Thus, the role of Ang1 in tumors is not well understood.

Recently, we developed a potent Ang1 variant, COMP-Ang1 (20). By replacing the NH2 terminus of Ang1 with the coiled-coil domain of cartilage oligomeric matrix protein (COMP), COMP-Ang1 is more soluble and more potent in Tie2.
phosphorylation and in its downstream Akt phosphorylation (20, 21). Adenoviral treatment of COMP-Ang1 enhanced blood flow by relatively lasting vascular remodeling in mouse tissue (22). By using the potency and the property of long-term vascular remodeling, we hypothesized that COMP-Ang1 may have a therapeutic benefit by promoting tumor perfusion and the delivery of chemotherapeutics. In the present study, we investigated the combined effects of COMP-Ang1 and 5-fluorouracil (5-FU)–based chemotherapy on the growth of implanted Lewis lung carcinoma (LLC).

Results
Systemic COMP-Ang1 Treatment Increases Functional Vascular Density and Pericyte Coverage in LLC Tumors

We selected the LLC cell line for studying the role of COMP-Ang1 in tumor angiogenesis because LLC cells only weakly express Ang1 (23) and are a well-characterized murine tumor model. To systemically overexpress COMP-Ang1 protein, we used adenoviral vectors encoding COMP-Ang1 for COMP-Ang1 treatment and LacZ for control treatment, unless otherwise noted. On day 5 after tumor implantation, COMP-Ang1 was i.v. administered by tail vein injection. On day 21, we examined the vascular remodeling in response to the treatment by measuring the vascular area and the coverage of pericytes (Fig. 1). Confocal microscopic images by anti-CD31 antibody staining showed that blood vessels in the tumor displayed a more organized structure, with many branches and a broader lumen in COMP-Ang1–treated tumors than in control-treated tumors (Fig. 1A and B). Quantitative analysis of vascular area showed that COMP-Ang1 treatment significantly increased average vessel diameter from 4.8 ± 0.1 μm to 5.9 ± 0.2 μm per image (P < 0.01; Fig. 1E). In addition, the number of blood vessel segments in each section was slightly lower in COMP-Ang1–treated tumors compared with control-treated tumors, but this difference was not statistically significant (Fig. 1E). This result indicates that COMP-Ang1 induces structural changes in tumor vessels, resulting in broader lumens and increased connections among the blood vessel segments in each section.

Previous studies suggest that pericytes are involved in vessel maturation (24-26) and that Ang1 may have a role in vessel maturation by promoting the interaction between endothelial cells and supporting pericytes (9, 10). To assess the status of tumor vessel maturation, we investigated the pericycle coverage of tumor vessels. The pericycle coverage of tumor vessels was evaluated by staining pericytes with an antibody to α-smooth muscle actin (27) and endothelial cells with anti-CD31 antibody. Confocal microscopic images showed that COMP-Ang1 treatment resulted in increased colocalization of pericytes and endothelial cells, whereas several pericytes were detached or loosely attached from the endothelial cells in the control-treated tumors (Fig. 1A and B). Quantitative analysis of pericycle coverage was done by a distance-based colocalization method, which is part of JACoP plug-in in ImageJ software. This method shows the percentage at which the intensity centers or centroids of pericytes colocalize with blood vessels. COMP-Ang1 treatment significantly increased the percentage of pericytes overlaying blood vessels from 57.2 ± 5.4% to 86.0 ± 4.1% (P < 0.01; Fig. 1F).

Although the increases in the blood vessel areas and pericycle coverage imply that there was a normalization of tumor vasculature, these results do not show whether these architectural changes were also accompanied by a functional change in tumor blood perfusion. To assess blood perfusion, tumor-bearing mice were i.v. injected with rhodamine-labeled lectin to label functional blood vessels (Fig. 1C and D). As expected, only 56.7 ± 7.3% of blood vessels were perfused in control tumors. In contrast, COMP-Ang1 significantly increased tumor perfusion to 73.7 ± 2.5% of blood vessels (P < 0.05; Fig. 1G).

Taken together, these results suggest that COMP-Ang1 increases functional microvessel density and increases the interaction between pericytes and endothelial cells in tumor vessels, and these effects improve perfusion in the tumor.

Combined Treatment of COMP-Ang1 and 5-FU Suppresses Tumor Growth In vivo

To investigate the efficacy of tumor suppression and animal toxicity of 5-FU, mice bearing an LLC tumor were i.p. treated daily with 1, 5, or 20 mg/kg of 5-FU or with saline for the control mice. Drug treatment started on day 5 when tumor volumes had reached 21 ± 11 mm3 and ended on day 21. No apparent suppression of tumor growth or toxicity, as defined by weight loss, was observed at doses of 1 or 5 mg/kg of 5-FU (Fig. 2A and B). In comparison, tumor growth was significantly inhibited from day 9 at 20 mg/kg of 5-FU (P < 0.05; Fig. 2A). Moreover, significant weight loss was also observed in the 20 mg/kg 5-FU–treated group, an indication of animal toxicity (P < 0.05; Fig. 2B). This result suggests that suppression of tumor growth could be achieved by a higher dose of 5-FU, but increasing the drug dosage may be harmful to normal tissues. Thus, we selected 1 to 5 mg/kg of 5-FU for further studies.

We next investigated the association of antitumor activity between COMP-Ang1, 5-FU, and a combination of the two agents. COMP-Ang1 treatment alone did not induce a significant change in tumor growth (Fig. 2C) or animal weight compared with control treatment (data not shown). Treatment with 5-FU (5 mg/kg) alone had no effect on tumor growth, as observed in the previous experiment (Fig. 2C). In contrast, the combination of COMP-Ang1 and 5-FU (5 mg/kg) showed significant suppression of tumor growth compared with control (P < 0.05; Fig. 2C). Although 5 mg/kg 5-FU with COMP-Ang1 was effective in suppressing tumor growth, we observed no effect from the combination of 1 mg/kg 5-FU and COMP-Ang1 on tumor growth (data not shown). These results suggest that COMP-Ang1 may somehow increase 5-FU delivery into the tumor or increase the cytotoxicity of 5-FU on tumor cells.

COMP-Ang1 Potentiates the Effect of 5-FU on Tumor Apoptosis In vivo

Tumor suppression by the COMP-Ang1 and 5-FU treatment may result from either increased apoptosis or decreased proliferation of the tumor. To investigate the mechanisms of tumor suppression, we examined tumor apoptosis and proliferation by immunostaining of active caspase-3 and phospho-histone H3, respectively. Confocal microscopic images showed that 5-FU (5 mg/kg) alone did not induce any change in apoptosis or...
proliferation of tumor cells (Fig. 3A). However, when 5-FU (5 mg/kg) was cotreated with COMP-Ang1, apoptotic cells in the tumor increased significantly from 158.3 ± 8.5 cells per mm² area to 627.1 ± 137.8 cells per mm² area ($P < 0.05$; Fig. 3B). Whereas proliferating cells also increased slightly in tumors treated with 5-FU (5 mg/kg) and COMP-Ang1, the ratios of apoptotic cells to proliferating cells in the 5-FU/COMP-Ang1–treated tumors was remarkably higher than those in control-treated tumors (Fig. 3B). These results indicate that the effect of combined treatment of 5-FU and COMP-Ang1 on tumor suppression was mediated by enhanced apoptosis in the tumor, not by decreased proliferation of tumor cells.

The enhancement of the antitumor effect of 5-FU brought about by cotreatment with COMP-Ang1 might be due to improved 5-FU delivery by COMP-Ang1–induced vascular remodeling in the tumor. To verify the enhanced delivery of the drug, we tried to directly measure the level of 5-FU in tumors by high-performance liquid chromatography. However, only

FIGURE 1. Effect of systemic COMP-Ang1 treatment on tumor vasculature. A and B. Representative confocal microscopic images showing that COMP-Ang1 treatment increased vascular density (CD31, red) and pericyte coverage ($\alpha$-smooth muscle actin ($\alpha$-SMA), green) on day 21 after tumor implantation. Note that detached or loosely attached pericytes were remarkably decreased in COMP-Ang1–treated tumor compared with control tumor (arrows). C and D. Tissue perfusion measured by rhodamine-labeled lectin injection. Note that nonperfused blood vessels (CD31, green) were observed in control tumor (C), whereas COMP-Ang1 treatment increased lectin perfusion (lectin, red) in tumor vessels (D). Scale bars, 100 μm. E. Quantitative analysis of microvessel density (gray) and average vessel diameter (black) per section. F. Quantitative analysis of pericyte coverage made by measuring the percentage of pericytes (green) overlapping blood vessels (red). G. Quantitative analysis of lectin perfusion. Columns, mean of each value ($n = 15$); bars, SE. *, $P < 0.05$; **, $P < 0.01$, Student’s t test.
the high-dose treatment of 5-FU (50 mg/kg) could be detected in tumor tissue (data not shown). We were unable to measure lower concentrations of 5-FU (5 mg/kg) in tumors by this method. Considering that the endothelial toxicity of 5-FU (28-30) may limit its concentration within tumors, we could not extrapolate the increased 5-FU delivery by COMP-Ang1 from the analysis. Instead, we evaluated drug delivery indirectly by measuring the tissue accumulation of Evans blue dye. On day 21 after tumor implantation, Evans blue dye in the tumor was evaluated after circulation for 1 hour. Quantitative analysis showed that COMP-Ang1 treatment significantly increased Evans blue contents from 2.58 ± 1.01 μg/g of tumor weight to 10.59 ± 3.24 μg/g of tumor weight (P < 0.05; Fig. 3C).

**COMP-Ang1 Does Not Change the Sensitivity of LLC Cells in Response to 5-FU In vitro**

Although COMP-Ang1 enhances the availability of 5-FU and subsequent apoptosis in the tumor by improving tissue perfusion, we could not exclude the possibility that COMP-Ang1 itself increased the sensitivity of tumor cells to the cytotoxic 5-FU treatment. To examine the sensitivity of LLC cells in response to 5-FU and COMP-Ang1, we measured active caspase-3 expression in LLC cells in vitro following treatment with 5-FU and COMP-Ang1 protein (Fig. 4A). The induction of active caspase-3 in response to the indicated amount of 5-FU was not significantly different from that induced by COMP-Ang1 protein treatment (Fig. 4A and B). A similar result was observed by fluorescence-activated cell sorting analysis of LLC cells stained with Annexin V (data not shown). These results suggest that COMP-Ang1 does not alter the sensitivity of LLC cells in response to 5-FU, and the *in vivo* effect of COMP-Ang1 on 5-FU–induced cytotoxicity is probably due to the improved delivery of 5-FU into the tumor through the effects of COMP-Ang1 on tissue perfusion.

**Discussion**

Solid tumors require angiogenesis for their growth. This is the reason why antiangiogenic drugs, which prevent nutritive flow into the tumor, have received a lot of attention in cancer therapy (31). However, some antiangiogenic drugs cause unexpected effects on vascular remodeling to generate mature and stabilized vessels in tumors (6, 7), and these effects may decrease the resistance of tumors to the antiangiogenic therapy. The “normalization” of tumor vessels, accompanied by pericyte recruitment, improves drug delivery into tumors (6, 7). This rationale provides a basis for the combined use of antiangiogenic drugs with radiation therapy or chemotherapy (7, 32). In this study, we added COMP-Ang1, a more potent Ang1 variant...
(20, 22), to chemotherapy with 5-FU in LLC tumors. Because Ang1 has been implicated to play a role in angiogenesis and vascular maturation through pericyte recruitment (9-12), we hypothesized that COMP-Ang1 could enhance drug delivery into tumors by inducing a sustained vascular remodeling and ameliorating abnormal tumor vasculatures. COMP-Ang1 treatment produced more organized blood vessels with more branching and increased pericyte recruitment (Fig. 1). Moreover, tumor perfusion measured by fluorescence-labeled lectin was also increased by the COMP-Ang1 treatment (Fig. 1). In agreement with our previous report (22), circulating COMP-Ang1 protein increased to 5.7 ± 0.8 μg/mL at 3 days after adenoviral treatment and declined by half but was still significantly greater at 3 weeks after the treatment (Supplementary Fig. S1). Because the level of endogenous Ang1 in tumors was undetectable or negligibly detectable by Western blot analysis (data not shown), the observed changes in tumor vasculatures were mediated by the COMP-Ang1 treatment, not by endogenous Ang1. These results corroborate the hypothesis that COMP-Ang1 produces vascular remodeling to improve microcirculation within the tumor.

A question may arise here as to how COMP-Ang1 increases tumor perfusion and drug delivery. In tumors, the abnormal leakiness of blood vessels prevents normal blood

![Image of Figure 3](https://mcr.aacrjournals.org/)

**FIGURE 3.** Effect of 5-FU and COMP-Ang1 on proliferation and apoptosis. **A,** Immunohistochemical staining of blood vessels (CD31, red), apoptotic cells (caspase-3, green in top images), and proliferating cells (phospho-histone H3, green in bottom images) on day 21 after tumor implantation. Scale bars, 100 μm. **B,** Quantitative analysis of apoptotic cells (gray) and proliferating cells (black). Columns, mean of apoptotic (gray) and proliferating (black) cells (n = 15); bars, SE. *, P < 0.05, one-way ANOVA on ranks followed by SNK test. **C,** Mice were injected with Evans blue solution. After 1 h of circulation, Evans blue was extracted from tumor tissue and quantitatively measured by spectrophotometry. Columns, mean ratio of Evans blue content (μg) to tumor volume (g; n = 5); bars, SE. *, P < 0.05, Student’s t test.
the tumor. One possible explanation for the increased Evans blue dye accumulation by COMP-Ang1 treatment is that COMP-Ang1 might inadequately restore the vascular permeability in the tumor. If COMP-Ang1 partially reduces the vascular leakage of highly permeable vessels in the tumor margin, it may increase the number of perfused blood vessels, which are less permeable to large molecules than tumor vessels, but still much higher than normal blood vessels. In this case, the tumor would have more vessel area with intermediate permeability, and the accumulation of drugs in a limited area within tumor would be relieved by the reduction of local hyperpermeability. Indeed, normalized vessels in a tumor are less leaky than the other tumor vessels, but they seem to be still more permeable than the vessels of normal tissues (3, 36).

Controversies about the role of Ang1 in tumor angiogenesis and growth have been reported. Overexpression of Ang1 results in promotion of tumor angiogenesis and growth in human cervical cancer xenografts and a glioma model (14, 15). However, in many other tumor models, Ang1 transfection of tumor cells leads to decreased tumor growth by stabilization of vessels with increased pericyte recruitment (13, 17, 37, 38). One of the reasons for these discrepancies is the complex microenvironment of tumors, which includes a diverse collection of growth factors and cytokines. Presumably, the other reason could be the extent of ectopic expression of Ang1 in tumor cells. Whatever the nature of the role of Ang1 in tumors, vascular density seems to be importantly related to tumor growth. Our results show that COMP-Ang1 increased the functional network of tumor vessels, as evidenced by increased functional vasculatures and pericyte coverage. However, COMP-Ang1 alone did not produce any significant changes in tumor growth. This might be related to the ability of Ang1 to inhibit vascular permeability. Because pericyte recruitment decreases vascular permeability and results in the decline of plasma extravasation, COMP-Ang1–induced pericyte coverage may partly inhibit tumor growth. Thus, we speculate that COMP-Ang1 may induce tumor growth by increasing functional blood vessels but at the same time counteract this effect through increased pericyte recruitment.

The use of an angiogenic factor during chemotherapy causes a concern that increased vascular density may provide a route for tumor metastasis. Although blood vessels as well as lymphatic vessels are common routes for tumor metastasis, we can exclude the possibility of any contribution of lymphatic vessels to metastatic activity because lymphatic vessels of rapidly growing solid tumor such as LLC are only located at the tumor margin and rarely penetrated into the tumor (39). Consistently, we also did not find any changes in the lymphatic structure of tumors treated with COMP-Ang1.
not shown). However, the correlation between microvascular density in the tumor and metastatic activity is less clear. Given that the loss of pericytes increases the possibility of tumor cell dissemination (40), pericyte coverage may be better correlated with tumor metastasis than microvascular density is. Indeed, we did not observe a greater propensity for metastatic nodule formation in lung tissue of mice receiving COMP-Ang1 treatment, although 3 weeks of tumor implantation was not long enough to form metastatic nodules. Thus, we cannot fully exclude the possibility of changes in tumor metastasis caused by COMP-Ang1 treatment after a long period of tumor cell implantation.

In summary, we have shown that COMP-Ang1 increases the functional vasculatures in tumor and pericyte coverage of tumor vessels. Furthermore, combined treatment by COMP-Ang1 and the chemotherapeutic drug 5-FU enhances the tumor toxicity of 5-FU without increasing animal toxicity by improving tissue perfusion into tumors. Our results suggest that COMP-Ang1 might improve the delivery of chemotherapeutic drugs into tumors, and this combination strategy might be useful for potentiating conventional chemotherapy.

Materials and Methods

Cell Lines
The LLC cell line was purchased from the American Type Culture Collection. The cells were cultured in DMEM with 10% fetal bovine serum, 100 μg/mL streptomycin sulfate, and 100 U/mL penicillin sulfate (Sigma-Aldrich).

Animals
C57BL/6 mice (male, ∼25 g, 7-8 wk old) were purchased from SamTako, Inc. Animal care and experimental procedures were done under approval from the Animal Care Committees of Chungnam National University (CNU-COM 2007-011).

Tumor Model and Treatment
Mice were randomly divided into groups, each consisting of five mice. Approximately 1 × 10⁶ LLC cells were s.c. implanted in the flank of all mice. For adenoviral treatment, 1 × 10⁹ plaque-forming units of Ad-COMP-Ang1 (COMP-Ang1 groups) or Ad-LacZ (control groups) were injected i.v. through the tail vein, as previously described (22). For 5-FU treatment, mice were injected daily i.p. with the indicated amount of 5-FU (Sigma-Aldrich). The tumor-bearing mice received adenosine starting on day 3 and 5-FU starting on day 5 after tumor implantation.

Tumor growth was monitored every day by measuring the length (a) and width (b) of the tumor with a Vernier caliper. Tumor volume was calculated as (a × b²)/2. The mice were sacrificed on day 21 and perfused with 1% paraformaldehyde fixative. Tumors were harvested for immunohistochemistry.

Immunohistochemistry
Tumors were sectioned at 30-μm thickness by using a cryostat (Leica). For staining of blood vessels, the sections were incubated with anti-CD31 antibody (Millipore) followed by secondary antibody, cy3-conjugated anti-hamster IgG antibody (Jackson ImmunoResearch). For staining of pericytes, FITC-conjugated anti-α-smooth muscle actin antibody (Sigma-Aldrich) was used. Signals were visualized and obtained using a confocal microscope (Zeiss LSM 510, Carl Zeiss). For staining of apoptotic and proliferating cells, anti–caspase-3 (Cell Signaling) and anti–phospho-histone H3 (Millipore) antibodies were used, respectively, and FITC-conjugated anti-rabbit IgG antibody (Jackson ImmunoResearch) was used as secondary antibody.

Quantification of immunostained images was done by using ImageJ software. For quantification of blood vessel diameters in tumors, we measured total blood vessel area (in square micrometers, Av) and total vessel length (in micrometers, Lv) from the binary and skeletonized images by using an ImageJ plug-in program. Average vessel diameter (in micrometers, Dv) was then calculated as Dv = Av/Lv.

A distance-based colocalization from the JACoP plug-in in ImageJ was used for the analysis of pericyte coverage (41). Briefly, the method compares the position of the centroid or intensity center of one fluorescent object with another fluorescent structure. Two objects are considered to be colocalized if the centroid of an object falls into the area of the other object. Results are expressed as percentage of pericytes colocalizing with blood vessels.

Measurement of Tissue Perfusion
To measure tissue perfusion, rhodamine-labeled lectin (Griffonia simplicifolia lectin I, Vector Laboratories, Inc.) was i.v. injected (8 mg/kg). One hour after lectin injection, mice were perfused with saline and subsequently fixatives. Tumors were then harvested and cryosectioned at 30-μm thickness. Sections were immunostained with anti-CD31 antibody as mentioned above. To quantitate lectin perfusion, the total blood vessel area labeled by CD31 immunostaining was defined as a region of interest with ImageJ software. After superimposing the region of interest on the lectin-stained image of same section, the area and intensity of the rhodamine fluorescence were measured.

To measure Evans blue dye accumulation, Evans blue dye (2% saline, 3 mL/kg; Sigma-Aldrich) was administered i.v. 1 h before perfusion with saline. Tumors were harvested and weighed after drying. The tumors were homogenized in formamide solution and incubated for 18 h at 60°C. After centrifugation at 10,000 rpm for 20 min, the supernatants were collected and quantified using a spectrophotometer at wavelengths of 620 nm for Evans blue and 740 nm for reference. The values were compared with those of standards. Results were expressed as micrograms of Evans blue per gram of tumor tissue.

Western Blot Analysis
To measure the sensitivity of LLC cells to 5-FU treatment, LLC cells were treated with the indicated concentration of 5-FU for 24 h. For COMP-Ang1 groups, 300 ng/mL of COMP-Ang1 protein were cotreated with 5-FU. Cells were then lysed in buffer containing 20 mmol/L Tris (pH 7.5), 150 mmol/L NaCl, 1% Triton X-100, and protease inhibitor cocktail. Fifty-microgram amounts of cell lysate were separated...
by 12% SDS-PAGE and electrobotted to nitrocellulose membrane. Blots were incubated with anti-active caspase-3 antibody (1:1,000) and anti–β-actin antibody (1:3000). Horseradish peroxidase–conjugated anti-rabbit antibody was used as secondary antibody. The blots were then developed and visualized by using the Enhanced Chemiluminescence Detection kit (Pierce).

**Statistical Analysis**

Data are presented as mean ± SE. The data were processed using SigmaStat software. Student’s t test was applied for comparison of two means and one-way ANOVA on ranks followed by the Student-Newman-Keuls (SNK) test for pairwise multiple comparisons. Differences were considered significant when P < 0.05.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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