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Copper Stimulates Proliferation of Human Endothelial Cells Under Culture

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Abstract Copper ions stimulate proliferation of human umbilical artery and vein endothelial cells but not human dermal fibroblasts or arterial smooth muscle cells. Incubation of human umbilical vein endothelial cells for 48 h with 500 μM $CuSO_4$ in a serum-free medium in the absence of exogenous growth factors results in a twofold increase in cell number, similar to the cell number increase induced by 20 ng/ml of basic fibroblast growth factor under the same conditions. Copper-induced proliferation of endothelial cells is not inhibited by 10% fetal bovine serum or by the presence of antibodies against a variety of angiogenic, growth, and chemotactic factors including angiogenin, fibroblast growth factors, epidermal growth factor, platelet-derived growth factor, tumor necrosis factor-α, transforming growth factor-β, macrophage/monocyte chemotactic and activating factor, and macrophage inflammatory protein-1α. Moreover, despite the previous observations that copper increased total specific binding of ^{125}I-angiogenin to endothelial cells, binding to the 170 kDa receptor is not changed; hence, the mitogenic activity of angiogenin is not altered by copper. Copper-induced proliferation, along with early reports that copper induces migration of endothelial cells, may suggest a possible mechanism for the involvement of copper in the process of angiogenesis. J. Cell. Biochem. 69:326–335, 1998.

Key words: copper; human endothelial cells; angiogenesis; growth factors; cell proliferation

Copper is known to modulate angiogenesis, the process by which new blood vessels are formed [Gullino, 1992]. This is the only tissue ion that undergoes a consistent increase in the cornea of rabbit eyes implanted with an angiogenic stimulus [Gullino et al., 1990; Raju et al., 1982; Ziche et al., 1982]. Moreover, rabbits fed a copper-deficient diet that reduces serum copper to about half of its normal level are unable to mount an angiogenic response regardless of the nature of the angiogenic stimulus applied [Raju et al., 1982; Ziche et al., 1982]. Thus, copper may in fact be essential for the angiogenic process. Several lines of evidence suggest that copper is also involved in tumor growth. The concentration of ceruloplasmin, a plasma protein that contains seven copper atoms and accounts for about 95% of circulating copper [Mukhopadhyay et al., 1996], increases in human patients with osteosarcoma or primary brain tumors [Shifrine and Fisher, 1976] and in rabbits bearing the VX-2 carcinoma [Ungar-Waron et al., 1978]. Upon removal of the tumor, the serum levels of ceruloplasmin and copper decrease rapidly [Shifrine and Fisher, 1976; Ungar-Waron et al., 1978]. Ceruloplasmin mRNA, whose expression is controlled by copper levels, is increased threefold in human cancer cells and neoplastic tissues [Kunapuli et al., 1987].

Not only is copper an essential participant in angiogenesis, but it has also been recognized to be angiogenic itself [McAuslan and Gole, 1980; McAuslan and Reilly, 1980; McAuslan et al., 1983]. Copper ions, at 10^{-4} to 10^{-6} M, induce

Abbreviations: aFGF, acidic fibroblast growth factor; bFGF, basic fibroblast growth factor; EGF, epidermal growth factor; FBS, fetal bovine serum; HE-SFM, human endothelial–serum-free medium; HUAE, human umbilical artery endothelial cells; HUVE, human umbilical vein endothelial cells; MCAF, macrophage/monocyte chemotactic and activating factor; MIP-1α, macrophage inflammatory protein-1α; mAb, monoclonal antibody; pAb, polyclonal antibody; PBS, phosphate-buffered saline; PDGF, platelet-derived growth factor; TGF-β, transforming growth factor-β; TNF-α; tumor necrosis factor-α.

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neovascularization in the rabbit cornea pocket assay [McAuslan et al., 1983]. Pure copper salt was also reported to induce angiogenesis in mouse kidney and in the anterior chamber of the rat eye [McAuslan and Reilly, 1980]. Cu(II) has been demonstrated to be the active principle for the angiogenic and endothelium-stimulating activities in preparations from bovine parotid gland and liver [Hoffman et al., 1976]. Moreover, Cu(II) supplementation enables molecules that are originally nonangiogenic to become angiogenic. For example, heparin and the tripeptide glycyl-L-histidyl-L-lysine became angiogenic when bound to Cu(II) [Raju et al., 1982]. Copper-bound ceruloplasmin and a peptide fragment of the protein are able to induce angiogenesis [Gullino, 1992; Raju et al., 1982], while removal of copper from these molecules nullified their angiogenic capacity. These data convincingly demonstrate that copper is involved in the angiogenic process. However, the mechanism by which it participates has remained obscure.

Cu(II) has been reported to mediate the interaction between endothelial cells and the potent angiogenic agent angiogenin, a protein originally isolated from the conditioned medium of tumor cells [Fett et al., 1985]. Addition of 100 μM Cu(II) increases the specific binding of ^{125}I-labeled human angiogenin to calf pulmonary artery endothelial cells fourfold [Badet et al., 1989] and of ^{125}I- labeled bovine angiogenin to bovine brain capillary endothelial cells by 25% [Chamoux et al., 1991]. Cu(II) also seems to abolish the effect of placental ribonuclease inhibitor, a tight-binding inhibitor for both the ribonucleolytic and angiogenic activities of angiogenin [Shapiro and Vallee, 1987], on the binding of ^{125}I-angiogenin to endothelial cells [Badet et al., 1990]. How Cu(II) enhances binding of angiogenin to endothelial cells is also unclear.

McAuslan et al. [1983] showed that both Cu(II) and Cu(I) at 10^{-6} M induce migration of bovine aorta endothelial cells. Other transition metal ions and Zn(II), at concentrations ranging from 10^{-7} to 10^{-4} M, had no effect on cell motility [McAuslan and Reilly, 1980]. This is interesting since the primary responses to angiogenic inducers appear to be chemotactic attraction and migration and invasion of endothelial cells into basement membrane and extracellular matrix, each of which is a prerequisite for angiogenesis. It has therefore been postulated that the angiogenic activity of copper is due to its ability to induce endothelial cell migration [McAuslan and Reilly, 1980]. Indeed, it is known that the initial phase of angiogenesis (i.e., vascular sprouting and elongation) can be achieved merely by migration and redistribution of existing endothelial cells and does not require cell proliferation [Sholley et al., 1984]. This would be consistent with the findings that 1) Cu(II) concentrations that stimulate migration do not induce ^{3}H-thymidine incorporation into endothelial cells [McAuslan and Reilly, 1980], and 2) the complex of heparin and Cu(II), while angiogenic in the rabbit cornea, does not induce the growth of bovine adrenal endothelial cells in culture [Gullino et al., 1990]. However, in order to establish an extensive vascular network, proliferation of endothelial cells is essential since otherwise subsequent vascular ingrowth will not proceed.

We have investigated the effect of copper on endothelial cell growth and report that copper stimulates proliferation of human endothelial cells in culture, suggesting a positive mechanism by which Cu(II) induces or mediates an angiogenic response.

MATERIALS AND METHODS

Materials

Human angiogenin, a recombinant product isolated from an *Escherichia coli* expression system, was provided by R. Shapiro. The antihuman angiogenin monoclonal antibody (mAb) was provided by K. A. Olson. Human recombinant basic fibroblast growth factor (bFGF), human fibronectin, antihuman acidic fibroblast growth factor (aFGF) polyclonal antibody (pAb), antihuman epidermal growth factor (EGF) mAb, antihuman macrophage inflammatory protein-1α (MIP-1α) mAb, antihuman macrophage/monocyte chemotactic and activating factor (MCAF) pAb, antihuman platelet-derived growth factor (PDGF) pAb, antihuman transforming growth factor-β (TGF-β) pAb, and antihuman tumor necrosis factor-α (TNF-α) pAb were from Promega (Madison, WI); anti-bFGF mAb was from Sigma (St. Louis, MO); carrier-free Na^{125}I (17.4 Ci/mg) and methyl-^{3}H-thymidine (6.7 Ci/mmol) were from DuPont/New England Nuclear (Boston, MA); excellulose GF-5 desalting columns and Iodo-beads iodination reagent were from Pierce (Rockford, IL); (disulfosuccinimidyl) suberate was from CalBiochem (San Diego, CA); cupric sulfate ($CuSO_4$), zinc sulfate ($ZnSO_4$), and

cupric bromide ($CuBr_2$) were from Fisher (Pittsburgh, PA); cuprous bromide (CuBr) was from MCB (Norwood, Ohio); ferrous ammonium sulfate ($Fe(NH_4)_2(SO_4)_2$) and ferric sulfate ($Fe_2(SO_4)_3.6H_2O$) were from Merck (Darmstadt, Germany).

Cell Cultures

Human umbilical vein endothelial (HUVE) and umbilical artery endothelial (HUAE) cells, and human dermal fibroblasts and arterial smooth muscle cells were purchased from Cell Systems Corp. (Kirkland, WA) as primary cultures. HUAE cells were cultured on attachment factor (Cell Systems)–coated flasks in endothelial cell growth medium CS 3.0 (Cell Systems) containing 10% fetal bovine serum (FBS). HUVE cells were cultured on fibronectin-coated dishes in human endothelial–serum-free medium (HE-SFM) (Gibco/BRL-Life Technology, Gaithersburg, MD) containing 20 ng/ml bFGF or on uncoated dishes but in HE-SFM + 10% FBS + 20 ng/ml bFGF. Human arterial smooth muscle cells were cultured on uncoated dishes in CSC medium (Cell Systems) supplemented with 10% FBS and 20 ng/ml bFGF. Human dermal fibroblasts were cultured in uncoated dishes in HE-SFM + 10% FBS + 20 ng/ml bFGF. Cells were cultured at 37°C under humidified air containing 5% CO_2 and subcultured at a 1:3 split ratio. HUVE cells between passages 3 and 15, HUAE cells between passages 3 and 8 inclusive, human arterial smooth muscle cells at passage 8, and dermal fibroblasts at passage 6 were used in these experiments. Cell numbers were determined with a Coulter counter, and cell viability was measured by the trypan blue exclusion assay.

Cell Proliferation Assay

All cell types were seeded in attachment factor–coated 35 mm dishes in HE-SFM at 5×10^3 cells/cm^2. Test samples were added immediately after the cells were seeded. When combinations of samples were tested, they were premixed and always adjusted to a constant final volume with HE-SFM before being added to the cells. The cells were incubated at 37°C in humidified atmosphere of 5% CO_2 in air for 48 h. The medium was aspirated, and the cells were washed once with 1 ml phosphate-buffered saline (PBS) and detached with 0.25 ml of trypsin-versene (0.05%) solution. Cell numbers were determined with a Coulter counter.

^{3}H-Thymidine Incorporation Assay

All cells were seeded in 35 mm dishes at 5×10^3 cells/cm^2 and cultured in HE-SFM containing 10% FBS and 20 ng/ml bFGF at 37°C under humidified air containing 5% CO_2 for 24 h. The medium was removed, and the cells were washed three times with prewarmed HE-SFM and serum-starved in HE-SFM for 18 h. The culture medium was removed, and the cells were incubated in fresh HE-SFM with test samples in the presence of 1 µCi/ml ^{3}H-thymidine for 14 h. At the end of the incubation, the cells were washed three times with PBS, precipitated with 10% trichloroacetic acid at room temperature for 30 min, washed two times with ethanol, and solubilized with 0.2 M NaOH + 0.2% sodium dodecyl sulfate (SDS). After the solublizate was neutralized with 1/5 volume of 1 M HCl and stabilized with 0.1 M Tris-HCl, pH 7.5, radioactivity was determined by liquid scintillation counting.

Iodination of Angiogenin

^{125}I-angiogenin was prepared with the use of Iodo-beads. One Iodo-bead was added to 175 µl of 0.1 M phosphate buffer, pH 6.5, containing 0.5 mCi Na^{125}I and incubated at room temperature for 5 min. Angiogenin, 20 µg in 25 µl of 0.1 M phosphate buffer, pH 6.5. was added, and the mixture was incubated at room temperature for 15 min. The reaction was terminated by removing the Iodo-bead, and iodinated angiogenin was separated from free iodine by a GF-5 desalting column equilibrated in 0.1 M phosphate buffer, pH 6.5.

Chemical Cross-Linking

HUVE cells, seeded at 5×10^3 cells/cm^2, were cultured in HE-SFM + 10% FBS + 20 ng/ml bFGF at 37°C under 5% humidified CO_2 for 24 h and starved in HE-SFM for another 24 h. The cells were then cooled to 4°C, washed twice with PBS or PBS + 100 µM $CuSO_4$, and incubated with 50 ng/ml ^{125}I-angiogenin in PBS or PBS + 100 µM $CuSO_4$ at 4°C for 30 min. At the end of the incubation, unbound ^{125}I-angiogenin was removed by washing three times with PBS or PBS + 100 µM $CuSO_4$. Bound ^{125}I-angiogenin was cross-linked to the cell surface by treatment with 0.1 mM Sulfo-DSS in PBS or PBS + 100 µM $CuSO_4$ at 4°C for 10 min. Unreacted Sulfo-DSS was quenched by 5 mM Tris-HCl, pH 7.5. The cells were then washed twice

with PBS and solubilized in 100 µl of 1× SDS-PAGE sample buffer. The entire sample was subjected to SDS-PAGE and autoradiography.

RESULTS

Copper Stimulates ^{3}H-Thymidine Incorporation and Cell Proliferation of Human Endothelial Cells

The growth-stimulating activity of copper toward HUVE cells was determined both by ^{3}H-thymidine incorporation and by cell proliferation assays. Addition of $CuSO_4$ to the culture medium in the absence of serum and growth factors induced cell proliferation as well as ^{3}H-thymidine incorporation into the acid-insoluble fractions of the cells. Copper-induced cell growth is concentration dependent, as shown in Figure 1. HUVE cells cultured in HE-SFM do not proliferate in the absence of growth factors. However, a 48 h incubation with increasing concentration of $CuSO_4$ up to 500 µM results in a progressive increase of cell numbers which reaches 216% of the control. ^{3}H-thymidine uptake by the cells increased in a similar pattern in response to $CuSO_4$ and reached a maximum of 204% of the control. Under the same conditions, 20 ng/ml bFGF and 10 ng/ml EGF increased cell numbers to 248 and 160% of the control and ^{3}H-thymidine incorporation to 900 and 150% of the control, respectively. The cell number increase induced by bFGF, one of the best endothelial cell growth factors, at its optimal concentration was in the same range as that induced by 500 µM $CuSO_4$.

Serum Does Not Inhibit Copper-Induced Cell Proliferation

Copper binding proteins, such as ceruloplasmin, transferrin, and albumin, exist in serum at high concentration [Halliwell and Gutteridge, 1988]. To investigate the effect of these proteins on the proliferative activity of copper, HUVE cells were cultured in serum-supplemented medium and stimulated with $CuSO_4$. Table I showed that copper-induced proliferation of

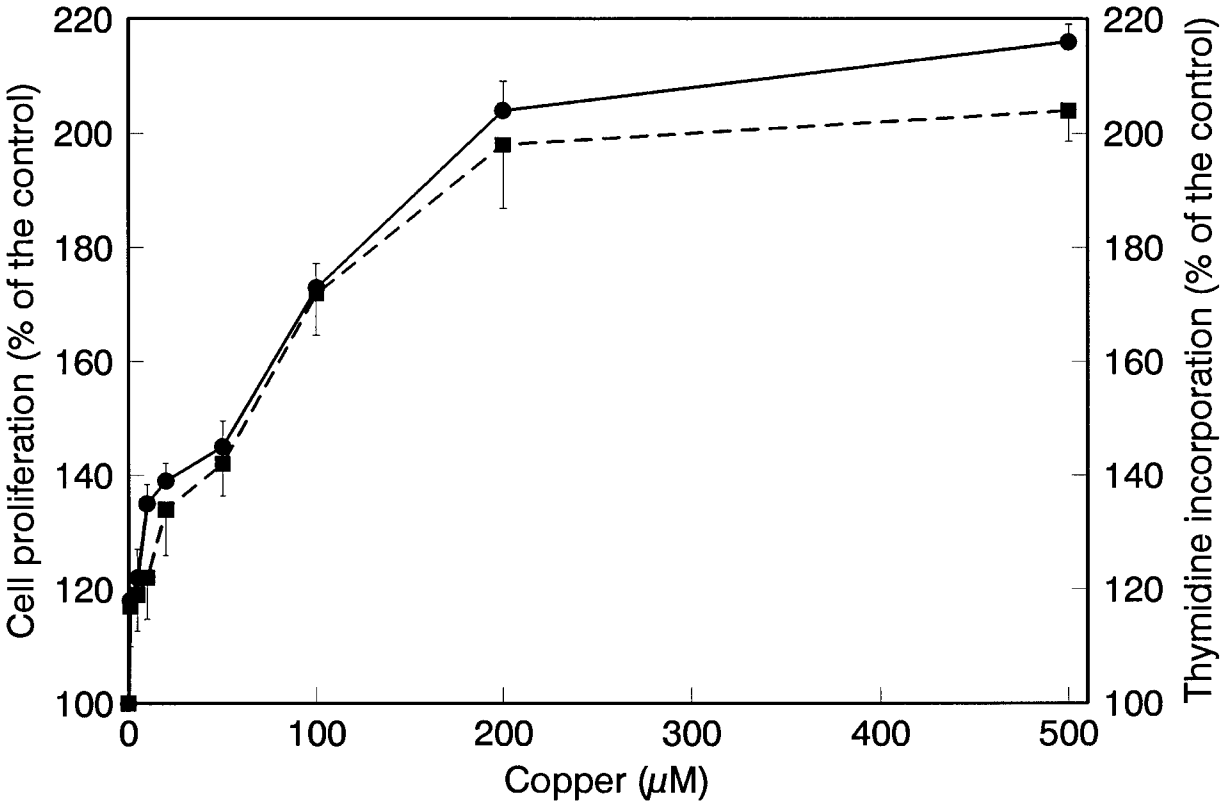

Fig. 1. Dose-dependent stimulation of cell proliferation and ^{3}H-thymidine incorporation of HUVE cells by copper. HUVE cells were seeded at 5×10^3 cells/cm^2 on attachment factor–coated dishes and stimulated by $CuSO_4$ for 48 h. Cell numbers were determined, and the percent increases over the control were calculated (solid line). For ^{3}H-thymidine incorporation assay (dashed line), cells were stimulated with $CuSO_4$ in the presence of 1 µCi ^{3}H-thymidine for 14 h as described in Materials and Methods.

TABLE I. Effect of Serum on Copper-Induced Proliferation of HUVE Cells*

$CuSO_4$ (μM)	0% FBS		2% FBS		10% FBS	
	Cell number	%	Cell number	%	Cell number	%
0	46,400 ± 1,500	100	52,000 ± 1,300	100	66,000 ± 800	100
5	55,700 ± 2,100	120	63,400 ± 1,000	122	79,200 ± 3,000	120
10	58,900 ± 1,900	127	66,600 ± 1,800	128	81,000 ± 2,800	123
100	80,700 ± 2,400	174	87,900 ± 2,000	169	102,300 ± 3,200	155
500	95,100 ± 1,700	205	108,700 ± 2,900	209	151,100 ± 2,200	229

*HUVE cells were plated at 5×10^3 cells/cm^2 in attachment factor–coated dishes in HE-SFM containing 0, 2, and 10% FBS, respectively. $CuSO_4$ was added immediately after the cells were seeded and incubated with the cells for 48 h. Cell numbers were determined by Coulter counter. Data shown are cell numbers and percent relative to the corresponding controls in a representative experiment.

TABLE II. Effect of Metal Ions on HUVE Cell Proliferation*

μM	Cell number (%)					
	$CuSO_4$	$CuBr_2$	CuBr	$ZnSO_4$	$Fe(NH_4)_2(SO_4)_2$	$Fe_2(SO_4)_3.6H_2O$
0	63,300 ± 1,300 (100)	63,300 ± 1,300 (100)	63,300 ± 1,300 (100)	63,300 ± 1,300 (100)	63,300 ± 1,300 (100)	63,300 ± 1,300 (100)
10	85,500 ± 600 (135)	93,700 ± 1,000 (148)	84,800 ± 400 (134)	65,000 ± 300 (103)	71,500 ± 1,000 (123)	64,200 ± 800 (100)
100	110,100 ± 1,700 (174)	125,300 ± 1,500 (198)	96,200 ± 1,300 (152)	60,100 ± 500 (95)	72,200 ± 1,000 (115)	17,300 ± 400 (27)
500	136,700 ± 1,800 (216)	131,700 ± 500 (208)	119,600 ± 1,600 (189)	54,400 ± 1,200 (86)	40,300 ± 700 (64)	6,400 ± 500 (10)

*HUVE cells, seeded at $7 \times 10^3/cm^2$, were stimulated with freshly prepared $CuSO_4$, $CuBr_2$, CuBr, $ZnSO_4$, $Fe(NH_4)_2(SO_4)_2$, and $Fe_2(SO_4)_3$ for 48 h. Numbers shown are cell numbers in a representative experiment. Number in parentheses is the percent of cell number relative to the control.

HUVE cells was not inhibited by FBS. The percent increase in cell number induced by various concentrations of Cu(II) in the presence of 2 or 10% FBS is very close to that induced in the absence of serum.

Specificity of Copper-Induced Cell Proliferation

Both cupric and cuprous and ferric and ferrous as well as zinc ions have been tested for proliferative activity toward HUVE cells. $CuSO_4$ and $CuBr_2$ exhibited a similar concentration-dependent proliferation of HUVE cells (Table II), suggesting that it is the copper ion rather than the counter ion which induces cell proliferation. CuBr had an activity comparable to that of $CuSO_4$ and $CuBr_2$, indicating that induction of cell proliferation by copper is valence-independent. Zinc sulfate had no effect on the growth of HUVE cells, whereas the effect of iron was valence dependent. Thus, Fe(II) had moderate activity at low concentration but became cytotoxic at high concentration. After 48 h culture in the presence of 10, 100 and 500 μM ferrous ammonium sulfate, cell numbers were 123, 115, and 64% of the control, respectively. Fe(III) had no activity toward HUVE cells at 10 μM but caused massive cell death at a 100 μM (27% of the original seeded cells survived). At 500 μM, Fe(III) was lethal to HUVE cells; only 10% of the cells survived. The results that both Cu(II) and Cu(I) are active, that Fe(II) is only slightly active, and that Fe(III) is cytotoxic suggest that oxidation and reduction of copper within the cells is probably not involved in copper-induced cell proliferation.

The proliferative activity of copper is specific to endothelial cells. Copper stimulates the proliferation of HUAE cells in much the same way as it does HUVE cells (Fig. 2). However, Cu(II) in a range of concentration from 1–500 μM was inactive toward human dermal fibroblasts or arterial smooth muscle cells cultured under the same conditions.

Effect of Antiangiogenic and Growth Factors Antibody on Copper-Induced Cell Proliferation

The proliferative activity of copper toward HUVE cells was not blocked by the presence of antibodies to a number of angiogenic, growth, and chemotactic factors. $CuSO_4$, 50 μM, stimu-

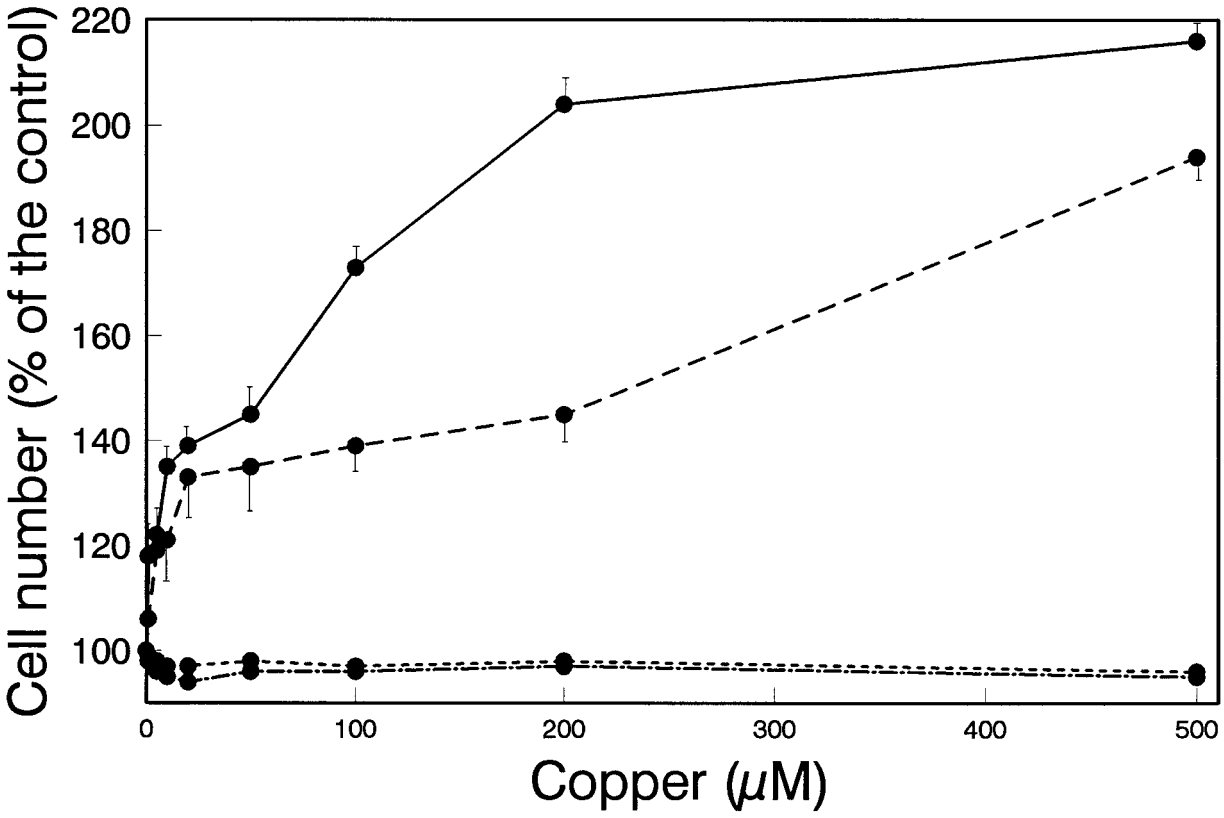

Fig. 2. Effect of copper on proliferation of human endothelial cells, smooth muscle cells, and fibroblasts. HUVE (solid line), HUAE (dashed line), human arterial smooth muscle cells (dotted line) and dermal fibroblasts (chained line) were seeded at 5×10^3 cells/cm^2 and stimulated with $CuSO_4$ for 48 h in HE-SFM on attachment factor–coated dishes. Cell numbers after stimulation with 20 ng/ml bFGF in the same experiments were 248, 221,144, and 196% of the controls for HUVE, HUAE, smooth muscle cells, and fibroblasts, respectively.

TABLE III. Effect of Copper on Angiogenin-Induced Proliferation of HUVE Cells*

	Control	Angiogenin	$CuSO_4$	Angiogenin + $CuSO_4$
Cell number	43,200 ± 900	59,800 ± 800	53,100 ± 300	71,300 ± 1,100
%	100	138	123	165

*HUVE cells, at 5×10^4 per 35 mm dish, were stimulated with 50 μM $CuSO_4$, 1 μg/ml human angiogenin, or a mixture of two in HE-SFM for 48 h. Data shown are from a representative experiment of quadruplicates of each sample.

lated a 38% increase in cell number in a 48 h culture of HUVE cells in HE-SFM. In the presence of 50 μg/ml of antibodies to angiogenin, aFGF, bFGF, EGF, TNF-α, TGF-β, PDGF, MCAF, and MIP-1α, the same amount of $CuSO_4$ increased cell number by 47, 41, 53, 44, 48, 51, 54, 39, 47, and 55%, respectively. Thus, it seems that the growth-stimulating activity of copper is not mediated through the production of any of these endogenous growth factors acting in an autocrine fashion.

Copper Does Not Modulate the Mitogenic Activity of Angiogenin

Copper has been reported to increase specific binding of angiogenin to endothelial cells [Badet et al., 1989; Chamoux et al., 1991] with an unknown mechanism and unknown consequences. Recently, a 170 kDa receptor on the surface of human endothelial cells has been shown to mediate angiogenin-stimulated ^{3}H-thymidine incorporation and cell proliferation [Hu et al., 1997]. We therefore investigated whether copper enhances angiogenin-induced proliferation of HUVE cells. Table III shows that Cu(II) neither enhances nor antagonizes the proliferative activity of angiogenin toward HUVE cells. Angiogenin at 1 μg/ml and $CuSO_4$ at 50 μM stimulated a 23 and 38% increase of cell number, respectively. In the presence of both angiogenin and Cu(II), there was an increase of 65% in cell number, which corre-

sponds to a 19 and 34% increase over that induced by Cu(II) and angiogenin individually. Thus, the effects of copper and angiogenin are additive. Apparently, they induce HUVE cell proliferation by different mechanisms that neither overlap nor interfere with each other. Although Cu(II) increases specific binding of ^{125}I-angiogenin to endothelial cells, it does not enhance its angiogenic potency, presumably because it does not affect binding to the 170 kDa receptor which is apparently responsible for angiogenin-induced cell proliferation. This was established by chemical cross-linking experiments which showed that binding of ^{125}I-angiogenin to the 170 kDa receptor was not affected by the presence of 100 μM Cu(II) (Fig. 3). The copper-induced increase in binding of ^{125}I-angiogenin to endothelial cell surface is coincidental and is therefore fortuitous or unproductive in regard to cell proliferation.

DISCUSSION

Copper is a potent inducer of cell proliferation for human endothelial cells. Its optimal proliferative activity toward HUVE cells is equivalent to that of bFGF, one of the best endothelial cell growth factors. It does not induce any morphological changes when cells are cultured in its presence at concentrations ranging from 1–500 μM. HUVE cells can be subcultured for more than five successive passages in serum-free medium supplemented with copper as the only growth stimulator. Cells that have been cultured in the presence of 100 μg/ml Cu(II) for five passages retain their ability to respond to exogenous growth factors such as bFGF and EGF in both ^{3}H-thymidine incorporation and cell proliferation assays (data not shown). The mechanism and signal transduction pathways for copper-induced cell proliferation are unknown. However, copper does not seem to change the general metabolic behavior of the cells. They did not develop copper dependence or any hypertrophic and hypotrophic phenomena that are usually associated with altered cellular metabolic pathways.

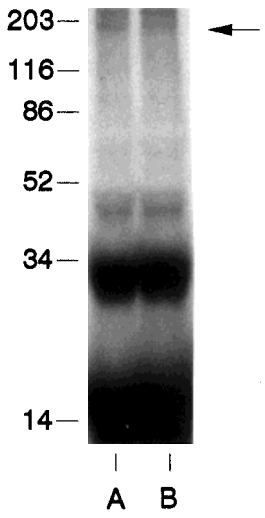

Fig. 3. Effect of copper on binding and cross-linking of angiogenin to the 170 kDa angiogenin receptor. Binding and cross-linking of ^{125}I-angiogenin to the 170 kDa angiogenin receptor in the absence **(lane A)** or presence of 100 μM $CuSO_4$ **(lane B)** were carried out as described in Materials and Methods. The arrow indicates the location of angiogenin-receptor complex.

Cu(II)-induced proliferation seems to be limited to endothelial cells. The metal has no effect on human fibroblasts or smooth muscle cells under the same conditions. It is unknown whether there is a specific receptor for Cu(II) on the surface of endothelial cells or whether there is a specific intracellular target for Cu(II) which is present only in endothelial cells. In any event, the specific activity of copper on endothelial cell proliferation may have physiological significance and may relate to its long recognized angiogenic activity [Hoffman et al., 1976; Gullino, 1992; Gullino et al., 1990; McAuslan and Gole, 1980; McAuslan and Reilly, 1980; McAuslan et al., 1983; Raju et al., 1982; Ziche et al., 1982]. Endothelial cells are the primary cells responding to angiogenic stimuli, whereas smooth muscle cells, which are recruited to support the endothelium only after endothelial tubes are formed [Vikkula et al., 1996], react secondarily.

Cu(II) is the only tissue ion whose concentration increases in rabbit cornea undergoing angiogenic stimulation [Raju et al., 1982; Ziche et al., 1982], and it is the only transition metal with angiogenic activity [McAuslan et al., 1983]. Consistent with these results, we found that copper is the only metal to have significant stimulatory activity in HUVE cell proliferation. Zn(II) neither stimulates nor inhibits the growth of HUVE cells in a range between 10 and 500 μM. Fe(II) at concentrations between 10 and 100 μM had a moderate stimulatory activity, considerably lower than that of Cu(II). Fe(III), however, was severely cytotoxic to the cells, probably owing to the oxidative stress that Fe(III) can generate at these concentrations. It is interesting that Cu(II) and Cu(I) are equally

active, indicating that redox regulation within the cell is not involved in the process of copper-induced cell proliferation. Moreover, serum had no effect on the proliferative activity of copper, indicating that the copper-binding proteins such as ceruloplasmin, transferrin, and albumin are also not involved. These results also excluded the possibility that copper-induced cell proliferation is mediated by increased cellular uptake of Fe(III). Iron is a metal necessary for cellular functions and is taken up by transferrin only as the ferric salt. Expression of ceruloplasmin is induced by copper [Davis, 1980], and since it has a ferrous oxidase activity it would oxidize Fe(II) to Fe(III) and thus increase cellular uptake of iron. The fact that both Cu(II) and Cu(I) are potent inducers of cell proliferation and that Fe(II) shows mild activity while Fe(III) is cytotoxic indicates that copper-induced proliferation does not involve iron uptake.

Although no measurable free copper exists in plasma and interstitial fluids [Halliwell and Gutteridge, 1990], serum is a rich source of protein-bound copper. Culture medium containing 10% FBS has an approximate copper concentration of 15 μM [Shapcott et al., 1985]. Protein-bound copper may contribute to the basal proliferative activity of serum to endothelial cells. Copper-binding proteins also exist in high concentration in serum. In aerated media supplemented with FBS, copper is probably complexed to those scavengers in its higher valence state. However, serum had no effect on copper-induced cell proliferation, indicating that either copper enters cells uncomplexed to any extracellular ligand, as has been reported [Campbell et al., 1981], or that free copper and/or copper complexes with low molecular weight components present in the medium, such as amino acids, are equally capable of binding to a copper receptor on the surface of endothelial cells.

Copper is important to cells. It is an essential component of many proteins such as cytochrome oxidase and superoxide dismutase [Gralla and Kosman, 1992], which are involved in sustained cellular respiration and detoxification of oxygen free radicals, respectively. On the other hand, excess copper is toxic. The cytotoxicity of copper has been attributed to the production of free hydroxyl radicals during oxidation of Cu(I) to Cu(II) [Lunec et al., 1982], which can inactivate essential enzymes and participate in lipid peroxidation causing the disruption of membranes and organelles [Stacey and Klaassen, 1987]. It is known that Cu(II) is reduced to Cu(I) by ascorbate [Lam et al., 1984], providing a continuous supply of Cu(I) for copper autooxidation. Ascorbate is present in medium as an antioxidant and is also present at 0.6–1.5 mM in the normal aqueous humor of most mammalian species [Cole, 1984]. Medium containing 10% FBS will have an ascorbate concentration of about 100 μM. Another proposed mechanism for copper cytotoxicity is the direct inactivation of essential enzymes. For example, copper binds to the sulfhydryl groups of lipoamide dehydrogenase [Nakamura and Yamazaki, 1972] and promotes the formation of cystine cross-links in the presence of oxygen.

Copper has been reported to inhibit the proliferation of mouse zygotes [Brinster and Cross, 1972] and bovine lens epithelial cells [Wolff et al., 1987]. Indeed, it has also been reported that the proliferation of bovine corneal endothelial cells was significantly inhibited in a serum-free medium containing 30 μg/ml copper [Yu et al., 1990]. We did not observe any cytotoxicity at copper concentrations up to 500 μM with both HUVE and HUAE cells. Presumably under our conditions no free radicals were produced or they were not harmful. It is also interesting to note that $CuSO_4$ showed no cytotoxicity to human dermal fibroblasts or human arterial smooth muscle cells under the conditions used in these studies.

Copper is not only nontoxic at the concentration used; in fact, it stimulates proliferation of human endothelial cells in both serum-free and serum-supplemented media. The mechanism by which this copper-stimulated cell proliferation occurs is not clear. Endothelial cells are known to secrete growth factors such as bFGF and aFGF that act in an autocrine or paracrine manner to stimulate their own proliferation [Folkman et al., 1988]. Neutralization of a variety of endogenous angiogenic, growth, and chemotactic factors including angiogenin, aFGF, bFGF, EGF, TNF-α, TGF-β, PDGF, MCAF, and MIP-1α by their monoclonal or polyclonal antibodies did not affect the proliferative activity of Cu(II) on HUVE cells. Thus, Cu(II)-induced proliferation is not mediated by stimulated secretion of these endogenous growth factors for endothelial cells or by factors released from an intracellular pool as a result of limited copper-induced cell death. In this regard, our results differ from the report that Zn(II)-stimulated

proliferation of bovine aorta endothelial cells is mediated by endogenous bFGF [Kaji et al., 1994].

The additivity of the effects of Cu(II) and angiogenin on HUVE cell proliferation suggests that Cu(II) and angiogenin stimulate cell growth by different mechanisms. It is possible that they use different signal systems within the cell that do not overlap or interfere with each other. Despite the fact that Cu(II) increases total specific binding of angiogenin to endothelial cells, the mitogenic activity of angiogenin is not altered. Whether or not the Cu(II) enhancement of specific binding correlates with other cellular activities of angiogenin such as migration and invasion [Hu et al., 1994], differentiation [Jimi et al., 1995], adhesion [Soncin, 1992], or nuclear translocation [Moroianu and Riordan, 1994] is unknown at present.

Copper has been reported to induce endothelial cell migration [McAuslan and Reilly, 1980]. This, together with the results that copper stimulates endothelial cell proliferation, may provide further understanding of how copper is involved in the process of angiogenesis.

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