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Anti-angiogenic activity of resveratrol, a natural compound from medicinal plants

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Resveratrol (3,4′,5-trihydroxy-*trans*-stilbene), a naturally occurring phytoalexin found in grapes and wine, possesses cancer-preventive activity. Angiogenesis is a crucial step in the growth and metastasis of cancers. We have investigated the effect of resveratrol on angiogenesis *in vitro* and *ex vivo*, and found that resveratrol directly inhibited human umbilical vein endothelial cell growth and decreased the gelatinolytic activities of matrix metalloproteinase-2. Tube formation was inhibited by treatment with resveratrol after plating endothelial cells on Matrigel. Resveratrol treatment also inhibited endothelial cell attachment to basement membrane components fibronectin and laminin, and displays similar behavior on cell chemotaxis. In addition, resveratrol has been found to be an angiogenesis inhibitor in the rat aorta matrix culture model. Therefore, inhibition of angiogenesis associated with cancer may be a novel mechanism for the anticancer activity of resveratrol.

Keywords: Resveratrol; Angiogenesis; Endothelial cells; Matrix metalloproteinase-2; Adhesion; Migration

1. Introduction

Angiogenesis, or neovascularization, is the formation of new capillaries from pre-existing blood vessels and is a fundamental process involved in several physiological and pathological processes [1,2]. The growth of solid tumors and their established metastases depend on the induction of an adequate blood supply [3]. Angiogenesis has become a very promising target for experimental therapies in cancer, and a wide variety of therapies directed at interfering with this process are in development [4].

Cancer chemopreventive agents inhibit cell growth, proliferation and induce apoptosis in various cancer cell lines, and the blocking of angiogenesis provides a novel therapeutic target against tumor spread. Several chemopreventive agents, such as genistein [5] and curcumin [6], inhibit angiogenesis in various *in vitro* and *in vivo* models. Thus, identification of chemopreventive agents with multiple biological activities that are non-toxic and effectively act at various steps of angiogenic cascade could be of great clinical significance [7].

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Resveratrol (3,4′,5-trihydroxy-*trans*-stilbene), a naturally occurring compound present in grapes and other plants, provides cancer chemopreventive effects in different systems based on its striking inhibition of diverse cellular events associated with tumor initiation, promotion, and progression [8]. Recently, resveratrol has been reported to be an angiogenesis inhibitor that is sufficiently potent to suppress FGF-2 and VEGF-induced neovascularization *in vivo* [9]. Igura *et al.* have shown that resveratrol directly inhibits bovine aorta endothelial cell proliferation, migration and tube formation *in vitro* [10].

The angiogenesis process involves a complex sequence of events. With angiogenic stimulation, the vascular endothelial cells increase their expression and secretion of matrix metalloproteinases (MMPs) to break down the extracellular and tissue matrix, increase endothelial cell motility and undergo cell proliferation to provide the necessary number of cells for the growing vessels [11].

In this study, we analyzed the effect of resveratrol on angiogenesis both *in vitro* and *ex vivo*. We found that resveratrol inhibits endothelial cell proliferation, migration and tube formation on Matrigel *in vitro*. Studies on proteinases demonstrated that resveratrol inhibited the secretion of MMP-2. Resveratrol treatment also decreased the number of capillary tubes in a rat aorta model of angiogenesis. These results show that resveratrol is one of many natural products that can act as an angiogenesis inhibitor.

2. Results and discussion

2.1 Resveratrol causes HUVECs growth inhibition

To determine whether resveratrol could inhibit endothelial cell growth, using human vein endothelial cells (HUVECs), we first evaluated the effect of resveratrol on the growth of cells by MTT assay. As shown in figure 1, resveratrol inhibited HUVECs in a dose-dependent manner. Resveratrol induced marked morphological changes at concentrations up to $80\,\mu\mathrm{mol\,L^{-1}}$ when examined by phase contrast microscopy. Control cells displayed the characteristic cobblestone-like growth patterns typical of these cells maintained in cultures.
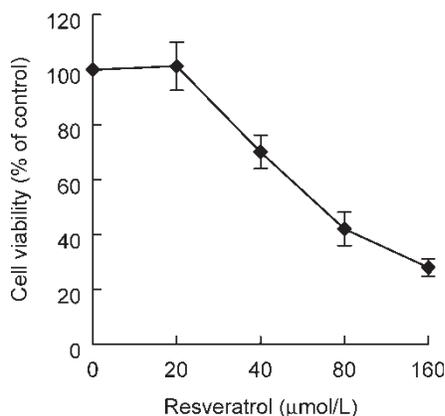

Figure 1. Effect of resveratrol on HUVECs growth. Cells were incubated with various concentrations of resveratrol. The chromogenic MTT assay was performed after 72 h treatment with resveratrol. Values expressed in percentage of cell viability as compared with the control. Data are means ± SE of triplicate of each sample.
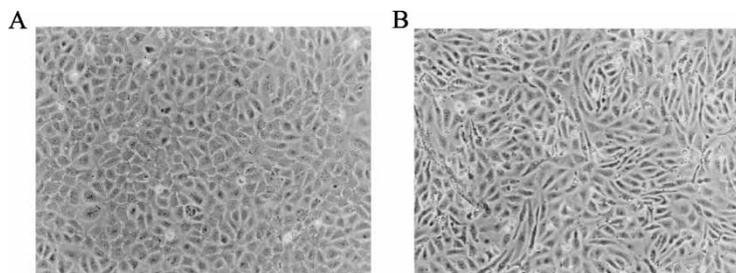
Figure 2. Effect of resveratrol on the morphology of HUVECs. (A) Control; (B) 80 μmol L^{-1} resveratrol; cells photographed after 72 h treatment with resveratrol. Original magnification, $\times$ 100.

In contrast, cells treated with 80–200 μmol L^{-1} resveratrol exhibited a long, spindle-shaped morphology in the tissue culture flasks (figure 2).

2.2 Inhibition of vascular tube formation on Matrigel

When plated on Matrigel, HUVECs underwent rapid reorganization (visible within 1–2 h) and subsequently formed capillary-like structures. In contrast, the addition of resveratrol in a culture medium for 12 h caused a dose-dependent inhibition of Matrigel-induced network formation (figure 3). There was slight inhibition at lower concentrations of resveratrol (40 μmol L^{-1}); however, tube formation was incomplete as compared with controls. Treatment with higher doses of resveratrol (80 μmol L^{-1}) resulted in a significant reduction in mean tube length; the tubes were less extensive, thinner, foreshortened. At 120 μmol L^{-1}, resveratrol inhibits the formation of vessel-like structures. These findings further confirmed that resveratrol can modify endothelial cell functions at indicated concentrations and
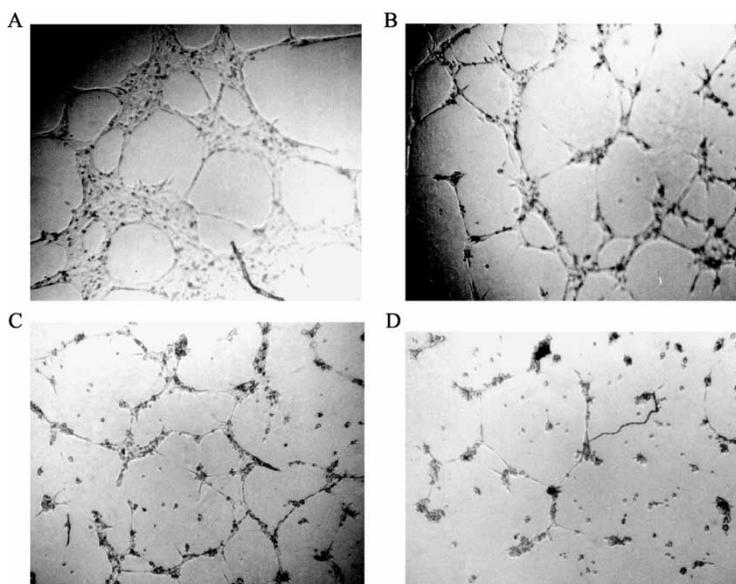

Figure 3. Inhibition of tube formation by resveratrol. HUVECs (2×10^4 cells well^{-1}) were plated on Matrigel pre-coated 96-well plates and treated with different concentrations of resveratrol for 12 h. (A) Control; and (B) 40, (C) 80, and (D) 120 μmol L^{-1} resveratrol. Original magnification, $\times$ 100.
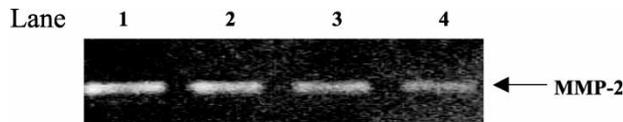
Y. Cao et al.

Figure 4. Zymographic analysis of MMP-2. The conditioned medium of HUVECs treated with different concentration of resveratrol was harvested after 24 h and electrophoresed on gelatin-containing non-reducing SDS-polyacrylamide gels. After processing, MMP-2 appears as a clear band of gelatinolytic activity at 72 kDa. Lanes: (1) control (2) 20, (3) 40, (4) 80 μmol L^{-1}.

exposure times at which cell proliferation is not affected and suggest that it might indeed prevent the process of angiogenesis.

2.3 Inhibition of MMP-2 gelatinolytic activity by resveratrol

Treatment of HUVECs for 24 h with resveratrol caused a dose-dependent reduction of the secreted 72 kDa MMP-2 gelatinolytic activity in the conditioned medium (figure 4). Densitometric analysis showed that 40 μmol L^{-1} resveratrol caused a 35% decrease in MMP-2. No gelatinolytic activity corresponding to MMP-9 was detectable in the conditioned medium of HUVECs.

2.4 Inhibition of adhesion and stimulated motility

Upon preincubation of ECV304 cells with resveratrol for 12 h, the adhesion of cells to basement membrane components, fibronectin and laminin was inhibited in a dose-dependent fashion (figure 5). This was statistically significant for laminin and fibronectin at 40 and 80 μmol L^{-1}, respectively. To determine whether resveratrol could inhibit endothelial cell migration, a chemotaxis assay was carried out using fibronectin as chemoattractant. At 80 μmol L^{-1}, resveratrol significantly inhibited the FN-induced endothelial cell migration (figure 6).
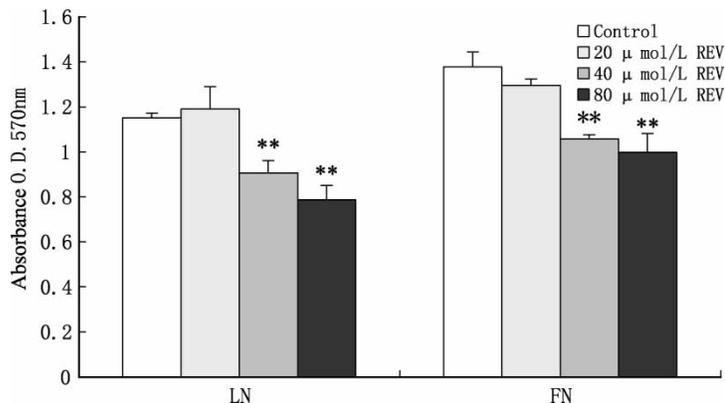

Figure 5.   Effect of resveratrol on ECV304 cells adhesion in response to basement membrane components laminin and fibronectin. **$P < 0.01$ *vs*. control group.
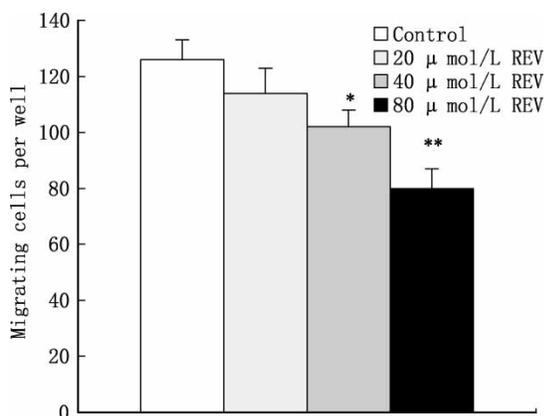
Figure 6. Effect of resveratrol on ECV304 cell migration. Cells were seeded in a transwell chamber precoated with fibronectin. The number of cells migrating through the filter was counted. Values represent means ± SEM ($n = $ **3**). *$P < $ **0**.**05**, **$P < $ **0.01** *vs.* control group.

2.5 Effect of resveratrol on rat aortic angiogenesis

In the aortic angiogenesis experiment, migrating cells appeared after 2–4 days, followed by microvascular sprouts, and vascular growth continued for 1 to 2 weeks after the beginning of the experiment. We treated aortic fibrin gel culture with resveratrol for 5–7 days and counted the number of tubes periodically. Resveratrol treatment decreased the number of capillary tubes, and cultures treated with $40\,\mu\mathrm{mol\,L^{-1}}$ resveratrol produced only a few short sprouts that underwent early regression (figures 7 and 8).

3. Discussion

Tumor-induced angiogenesis, the formation of neovessels from preexisting ones, is critical for supporting tumor growth and progression not only by providing the necessary blood supply but also by allowing metastatic cells into the circulation [12]. Angiogenesis is an exciting target for novel anticancer therapies because of the many advantages that it may offer, including accessibility to tumors, independence of tumor cell resistance mechanisms and broad applicability to many tumor types [4]. The potential use of natural and synthetic angiogenesis inhibitors is currently being studied intensively by many laboratories [13,14].
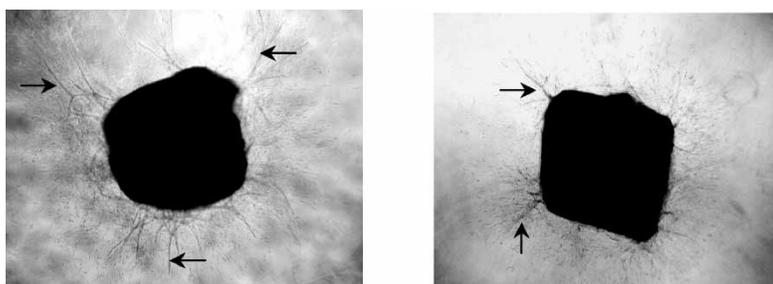

Figure 7. Effect of resveratrol on serum-free fibrin gel cultures of rat aorta rings; microvessels indicated by arrows. (A) Control; (B) $40\,\mu\mathrm{mol\,L^{-1}}$ resveratrol. Original magnification, ×20.
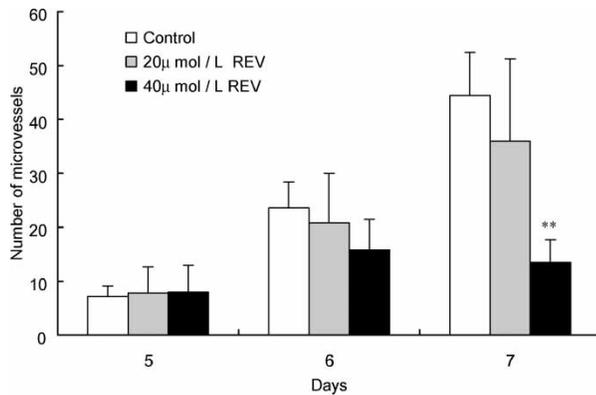
Figure 8.   Effect of resveratrol on microvessel growth in serum-free fibrin gel culture of rat aorta. Aortic gel culture was treated with resveratrol during days 5–7. Data expressed as means ± SEM ($n = 5$). **$P < 0.01$ *vs.* control group.

Inhibitors of angiogenesis block any of the steps in the angiogenic cascade, including proliferation and attachment of endothelial cells to the extracellular matrix proteins, migration and invasion through the matrix, which is required for the capillary sprouting and morphogenesis in a thin tube meshwork and differentiation and stabilization [15–17].

Nontoxic doses of resveratrol that did not affect cell viability significantly inhibited angiogenic differentiation of HUVECs in a dose-dependent manner on Matrigel. The assay of tube formation on Matrigel mimics the final events during angiogenesis when endothelial cells become organized in a three-dimensional network of capillaries.

This involves endothelial cell attachment, migration, and the production of enzymes capable of modifying the extracellular process. Furthermore, resveratrol inhibited endothelial cell adhesion and migration in response to basement membrane components to which they are exposed *in vivo* during neoangiogenesis.

Matrix metalloproteinases (MMPs), a family of zinc-binding, calcium-dependent endopeptidases that degrade all of the proteins in the extracellular matrix, play an important role in angiogenesis, specifically the gelatinases (MMP-2 and MMP-9), owing to their ability to degrade components of the basement membrane such as type IV collagen and fibronectin [18]. The crucial role of MMP-2 in angiogenesis has been well documented [19–22]. Exposure of endothelial cells to resveratrol led to a concentration-dependent reduction of MMP-2 gelatinolytic activity. The result may partly explain our observation of marked inhibition of tube formation by resveratrol on Matrigel.

The *ex vivo* angiogenic response of the rat aorta is akin to the *in vivo* neovascular responses of injured tissues during wound healing and represents a model of physiologic angiogenesis [23]. We have used this model to investigate the potential activity of resveratrol on microvessel formation and disintegration. Our study demonstrates that the angiogenic response of aortic explants cultured under serum-free conditions is inhibited by resveratrol. At 40 μmol L^{-1}, resveratrol treatment significantly decreased the number of capillary tubes as compared with the control group.

In conclusion, this study has provided evidence of antiangiogenesis activities of resveratrol *in vitro* and *ex vivo*. The antiangiogenic activity may be a novel mechanism that contributes to the cancer chemopreventive activity of resveratrol. Further studies are necessary to elucidate the mechanisms of antiangiogenic action of resveratrol.

4. Experimental

4.1 Drugs and reagents

Resveratrol was kindly provided by Professor Lin Mao, Institute of Materia Medica, Chinese Academy of Medical Sciences. MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide], ε-aminocaproic acid and fibrin were from Sigma. Endothelial cell growth factor (ECGF) was from Roche. Matrigel was from BD Biosciences. Fibronectin was from Chemicon and MCDB131 came from Gibco.

4.2 Cell culture

Human umbilical vein endothelial cells (HUVECs) were routinely isolated from umbilical cord veins by collagenase digestion as described in the literature [24]. HUVECs were grown on 1% gelatin-coated flasks in M199 supplemented with 20% (v/v) heat-inactivated fetal bovine serum and $50 \, \mu g \, ml^{-1}$ ECGF, $100 \, U \, ml^{-1}$ penicillin, $100 \, \mu g \, ml^{-1}$ streptomycin and $50 \, U \, ml^{-1}$ heparin. To maintain uniform conditions all experiments were carried out between cell passages 2 and 4. Immortalized human endothelial cells ECV304 were cultured in RPMI 1640 supplemented with 10% fetal bovine serum. Both cells are maintained at 37°C in a humidified incubator containing 5% CO_2.

4.3 Endothelial cell proliferation assay

We performed MTT assays to determine the effect of resveratrol on HUVECs growth and viability. Briefly, cells were plated in 96-well tissue culture plates (5000 cells per well) in a final volume of $100 \, \mu l$ of M199. After 24 h incubation at 37°C in an incubator (5% CO_2), resveratrol was added to the wells and the cells were further cultured for 72 h. After completion of the treatment, cells were washed and MTT labeling reagent was added to the cells and incubated for 4 h at 37°C. Cells were then lysed and the reduced intracellular formazan product was dissolved in DMSO. The absorbance was recorded at 570 nm and the percentage of cell viability was calculated against untreated cells.

4.4 In vitro angiogenesis assay

Ninety-six-well culture plates were coated with $60 \, \mu l$ of Matrigel (11.1 mg-protein ml^{-1}), which was then allowed to solidify at 37°C for 1 h. HUVECs were trypsinized using trypsin−EDTA, washed, suspended in appropriate media, and added to Matrigel-coated wells (20,000 cells per well in $200 \, \mu l$ media). Cells were subsequently treated with various concentrations of resveratrol and were then incubated for 12 h at 37°C in a 5% CO_2 humidified atmosphere. Tube formation was observed periodically over time under a phase-contrast microscope and photographed with an Olympus camera. At 24 h after seeding, MTT was added to test the metabolic viability of resveratrol-exposed HUVECs. Each dose of control or test compound was assayed in triplicate.

4.5 Zymogram analysis

HUVECs were seeded to 24-well plates at a density of 7.5×10^4 cells per well. After reaching $>90\%$ confluence in 3 days, cells were washed twice with phosphate-buffered saline (PBS) to remove spent medium, and incubated with fresh serum-free M199 containing various concentrations of resveratrol at 37°C. After 24 h, the conditioned medium was collected and the hydrolytic activities of metalloproteinase measured by gelatin zymography as previously described [25]. Equal protein from each sample was mixed with $5 \times$ sample buffer (0.4 mol L^{-1} Tris–HCl, pH 6.8, 5% SDS, 20% glycerol, 0.1% bromophenol blue) and were applied to a 10% SDS-PAGE containing 0.1% gelatin. After electrophoresis, gels were incubated in 2.5% Triton X-100 for 1 h and incubated in buffer (0.05 mol L^{-1} Tris–HCl, pH 7.5, 10 mmol L^{-1} CaCl$_2$, 0.2 mol L^{-1} NaCl and 1 μmol L^{-1} ZnCl$_2$) for 16 h at 37°C. The gels were then stained with Coomassie brilliant blue 250 in a mixture of methanol–acetic-acid–water (4:1:5) for 1 h and destained in the same solution without dye. Proteolytic areas appeared as clear bands against a blue background after intensive destaining. The molecular weights (M_r) of the proteolytic bands were determined in relation to the reference marker proteins, which were simultaneously loaded in the gel.

4.6 Adhesion assay

Adhesion of control and resveratrol-treated ECV304 cells was tested by placement of 50 μl aliquots of 2.0 μg fibronectin and laminin into wells of 96-well plates. The plate was incubated at 37°C for 2 h, air dried overnight, and then blocked with 50 μl RPMI 1640 containing 2% bovine serum albumin. Some 80,000 cells pretreated with resveratrol for 12 h were added in each well. After incubation at 37°C for 1 h, non-adherent cells were removed with gentle washing. MTT (100 μl, 0.4 mg ml^{-1}) was then added to each well and incubated for 4 h, after which the absorbance was measured at 570 nm.

4.7 Cell migration assay

The migration assay of the ECV304 cells was performed *in vitro* using a transwell chamber system with 8.0 μm-pore polycarbonate filter inserts. The lower side of the filter was coated with fibronectin (10 μl, 0.5 mg mL^{-1}) as chemoattractant. ECV304 cells (2×10^5 cells) were then placed in the upper part of the filters and treated with resveratrol at 37°C for 5 h. Subsequently, membranes of the inserts were fixed with methanol and stained with hematoxylin and eosin. Five high-power fields per triplicate well were then counted under a microscope.

4.8 Rat aorta model of angiogenesis

The rat aorta matrix culture assay was performed by the method described by Nicosia *et al.* [26]. Thoracic aortas were obtained from 8 to 12-week-old male Wistar rats. The fibroadipose tissue around the aorta was carefully removed and rinsed with Hank's solution. The aortas were cut into small fragments (about 2×2 mm) and cultured in fibrin gels that were formed by addition of thrombin to the same medium containing fibrinogen in a 48-well plate. After gel formation, MCDB131 medium supplemented with 300 μg ml^{-1}

ε-aminocaproic acid, 10 mM L-glutamine, 100 U ml^{-1} penicillin and 100 μg ml^{-1} streptomycin was added on top of the gel. The gel cultures were grown in an atmosphere of 5% CO_2 at 37°C for 7 days, and the medium was changed every two days. Resveratrol was added to the medium at day 5, towards the end of the experiment. The angiogenic response of the aortic culture was determined by counting the number of microvessels in the living cultures, according to the published criteria [26].

References

[1] P. Carmeliet, R.K. Jain. *Nature*, **407**, 249–257 (2000).
[2] D. Hanahan, J. Folkman. *Cell*, **86**, 353–364 (1996).
[3] R. Auerbach, W. Auerbach, I. Polakowski. *Pharmacol. Ther.*, **51**, 1–11 (1991).
[4] F.A. Scappaticci. *J. Clin. Oncol.*, **20**, 3906–3927 (2002).
[5] F.H. Sarkar, Y. Li. *Cancer Metastasis Rev.*, **21**, 265–280 (2002).
[6] D. Thaloor, A.K. Singh, G.S. Sidhu, P.V. Prasad, H.K. Kleinman, R.K. Maheshwari. *Cell Growth Differ.*, **9**, 305–312 (1998).
[7] A.K. Singh, P. Seth, P. Anthony, M.M. Husain, S. Madhavan, H. Mukhtar, R.K. Maheshwari. *Arch. Biochem. Biophys.*, **401**, 29–37 (2002).
[8] M. Jang, L. Cai, G.O. Udeani, K.V. Slowing, C.F. Thomas, C.W.W. Beecher, H.H.S. Fong, N.R. Farnsworth, A.D. Kinghorn, R.G. Mehta, R.C. Moon, J.M. Pezzuto. *Science*, **275**, 218–220 (1997).
[9] E. Brakenhielm, R. Cao, Y. Cao. *FASEB J.*, **15**, 1798–1800 (2001).
[10] K. Igura, T. Ohta, Y. Kuroda, K. Kaji. *Cancer Lett.*, **171**, 11–16 (2001).
[11] J. Folkman. *Cancer Res.*, **46**, 467–473 (1986).
[12] D. Belotti, V. Vergani, T. Drudis, P. Borsotti, M.R. Pitelli, G. Viale, R. Giavazzi, G. Taraboletti. *Clin. Cancer Res.*, **2**, 1843–1849 (1996).
[13] K. Minamiguchi, H. Kumagai, T. Masuda, M. Kawada, M. Ishizuka, T. Takeuchitin. *Int. J. Cancer*, **93**, 307–316 (2001).
[14] R. Mohan, J. Sivak, P. Ashton, L.A. Russo, B.Q. Pharm, N. Kasahara, N.B. Raizman, M.E. Fini. *J. Biol. Chem.*, **275**, 10405–10412 (2002).
[15] J. Folkman. *Semin. Cancer Biol.*, **3**, 65–71 (1992).
[16] J. Folkman, M. Klagsbrun. *Science*, **235**, 442–447 (1987).
[17] J. Folkman. *Nat. Med.*, **1**, 27–31 (1995).
[18] M.A. Lafleur, M.D. Hollenberg, S.J. Atkinson, V. Knauper, G. Murphy, D.R. Edwards. *Biochem. J.*, **357**, 107–115 (2001).
[19] L.M. Coussens, Z. Werb. *Chem. Biol.*, **3**, 895–904 (1996).
[20] E.I. Deryugina, M.A. Bourdon, R.A. Reisfeld, A. Strongin. *Cancer Res.*, **58**, 3743–3750 (1998).
[21] N. Hiraoka, E. Allen, U. Apel, M.R. Gyetko, S.J. Weiss. *Cell*, **95**, 365–377 (1998).
[22] T. Itoh, M. Tanioka, H. Yoshida, T. Yoshioka, H. Nishimoto, S. Itohara. *Cancer Res.*, **58**, 1048–1051 (1998).
[23] W.H. Zhu, X. Guo, S. Villaschi, R.F. Nicosia. *Lab. Investig.*, **80**, 545–555 (2000).
[24] E.A. Jaffe, R.L. Nachman, C.G. Becker, C.R. Minick. *J. Clin. Investig.*, **52**, 2745–2756 (1973).
[25] S. Zucker, H. Mirza, C.E. Conner, A.F. Lorenz, M.H. Drews, W.F. Bahou, J. Jesty. *Int. J. Cancer*, **75**, 780–786 (1998).
[26] R.F. Nicosia, A. Ottinetti. *Lab. Investig.*, **63**, 115–122 (1990).