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Inhibition of angiogenic differentiation of human umbilical vein endothelial cells by diallyl disulfide (DADS)

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Abstract

Angiogenesis is a crucial step in the growth and metastasis of cancers. The activation of endothelial cells and their further behaviour are very critical during angiogenesis. We analyzed the effect of diallyl disulfide (DADS) on angiogenesis in *in vitro* models using human umbilical vein endothelial cells (HUVECs). DADS significantly inhibited endothelial cell migration, invasion and tube formation. ^{3}H-thymidine proliferation assay clearly showed the inhibitory effect of DADS on the proliferation of HUVECs *in vitro*. The role of metalloproteinases has been shown to be important in angiogenesis; therefore, zymography was performed to determine whether DADS affected protease activity. Gelatin zymographic analysis showed the inhibitory effect of DADS on the activation of matrix metalloproteinases-MMP-2 and MMP-9. These findings suggest that DADS acts as an angiogenesis inhibitor by inhibiting the activation of matrix metalloproteinases during endothelial morphogenesis.

Keywords: Angiogenesis; Diallyl disulfide; Human umbilical vein endothelial cells; Invasion; Matrix metalloproteinases; Motility; Proliferation; Tube formation

Introduction

Angiogenesis, the process of generating new blood vessels from pre-existing vessels, is a crucial process in normal physiology and plays an essential role in embryonic development, wound healing and the normal menstrual cycle (Risau, 1997; Griffioen and Molema, 2000; Ferrara and Kerbel, 2005). Uncontrolled angiogenesis is pathological and is often associated with several diseases such as atherosclerosis, arthritis, diabetic retinopathy and psoriasis (Coultas et al., 2005; Griffioen and Molema, 2000; Folkman, 1990). The relationship of pathological angiogenesis and its role in tumour biology has been one of the major areas of active research in recent years. It has been shown that solid tumours are dependent on the process of angiogenesis for growth beyond 2–3 mm in diameter and that increased tumour diameter required a corresponding increase in vascularization (Nyberg et al., 2005). The angiogenic phenotype depends on the balance of proangiogenic growth factors such as VEGF and inhibitors, as well as interactions with extra cellular matrix, allowing for endothelial cell migration (Murphy and Docherty, 1992; Thaloor et al., 1998). Matrix metalloproteinases (MMPs) are a family of Zn dependent endopeptidases that are able to degrade the extra cellular matrix and allow angiogenesis and tumour invasion with each MMP acting on a different substrate (Stamenkovic, 2003; Matrisian, 1992). The MMP family includes collagenases, gelatinases, and stromlysins and their activities are regulated by mechanisms including their secretion in the zymogen form requiring activation and formation of complex between TIMP and activated or latent enzyme (Liotta et al., 1991). MMP-2 and MMP-9 are both type IV collagenases that have been shown to be important in tumour invasion *in vitro* because of their ability to break down basement membrane, in particular degrading collagen IV (Schnaper et al., 1993). It has been shown that a reduction in tube formation by endothelial cells was associated with a decrease in gelatinolytic activities of both MMP-2 and MMP-9, whereas an enhancement of activity increased tube formation (Egeblad and Werb, 2002). The importance of MMPs in cancer, in particular the contribution of MMP-2 and MMP-9 to cancer metastasis and angiogenesis promoted the development of synthetic inhibitors capable of targeting gelatinase activity in tumours (Zucker et al., 2000).

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Unfortunately, clinical trials in cancer patients with synthetic MMP inhibitors showed lack of therapeutic efficacy and unacceptable side effects (Coussens et al., 2002).

Natural products are rich source of pharmacologically active compounds in which plant materials deserves an important position. Naturally occurring organosulphur compounds from garlic and onion mediate significant chemopreventive activities against the initiation stage of carcinogenesis induced by various chemical carcinogens (Reddy et al., 1993; Mignard et al., 1996; Arunkumar et al., 2006). The detoxifying effects of these organosulphur compounds were thought to be related to their ability to inhibit phase I enzymes, induce phase II enzymes or bind to exogenous toxins through sulfhydryl groups (Dausch and Nixon, 1990). Diallyl disulfide (DADS) is an oil soluble organosulphur compound present in garlic. Since DADS is reported to contribute about 60% of garlic's essential oil (Kwon et al., 2002), it may be a major factor in the observed relationship between garlic intakes and decreased human cancer incidence. The nutritionally achievable dose of DADS was found to be 550 μg, which was equivalent to average intake of garlic powder per day per rat when a garlic powder based diet was administered. The concentration of garlic powder in the diet was 800 mg/kg (Oi et al., 1995). Our earlier in vivo experiments clearly showed that DADS was very effective even at a much lower concentration of 250 μg/kg body wt/animal (Kuttan and Kuttan, 2003). DADS have been shown to inhibit the proliferation of human colon, lung and skin cancer cells (Kwon et al., 2002; Yang et al., 2006). Earlier studies in our laboratory clearly demonstrated the inhibitory effect of DADS on B16F-10 melanoma induced lung metastasis in C57BL/6 mice (Kuttan and Kuttan, 2003). On the basis of these potential anticancer activities of DADS, in this study, we analyzed its effect on angiogenesis in an *in vitro* model using human umbilical vein endothelial cells (HUVECs).

Materials and methods

Cells

Human umbilical vein endothelial cells (HUVECs) were isolated from human umbilical cord vein by collagenase treatment as described by Jaffe et al. (1973). The cells were grown in medium 199, supplemented with 20% fetal calf serum (FCS), 100 units/ml penicillin, 100 μg/ml streptomycin and 2 ng/ml VEGF and FGF at 37 °C in 5% CO_2 atmosphere.

Table 1
Effect of DADS towards HUVECs viability in culture

Concentration (μg/ml) of DADS	Percentage of viable cells
1	99.15
2	98.73
5	96.18
10	93.70
20	84.26
25	65.54
50	53.62

HUVECS were incubated with different concentrations (1–50 μg/ml) of DADS. Percentage of cell viability was determined using MTT assay.

Table 2
Effect of DADS on proliferation of HUVECs

Treatment	Radioactive count/minute (cpm)	% Inhibition
Untreated control	4463.33±112.61	–
DADS (μg/ml)		
1	3655.33±49.54***	18.1
2	2783.66±35.72***	37.63
5	1723.66±58.56***	61.38

HUVECs (5×10^3 cells/ well) were grown in 96 well flat bottom plate. After 24 h, various concentrations of DADS (1, 2 and 5 μg/ml) were added and incubation was continued for 48 h. After incubation, ^{3}H-thymidine was added to each well (1 μCi/well) and incubation was continued for 18 h. Cells were lysed and radioactivity was counted by using a Rack Beta liquid scintillation counter. Values are mean±S.D.
***$p<0.001$.

Chemicals

Diallyl disulfide (DADS) was purchased from Sigma chemicals, St. Louis, USA. Standard MMP-2 and MMP-9 were purchased from Chemicon International Inc, USA.

Determination of cell viability by MTT assay

The viability of cultured cells was determined by assaying for the reduction of MTT to formazan (Cole, 1986; Campling et al., 1991). HUVECs were seeded (5000 cells/well) in 96 well flat bottomed titre plate and incubated for 24 h at 37 °C in 5% CO_2 atmosphere. Different concentrations of DADS (1 μg–50 μg/ml) were added and incubated further for 48 h. Before 4 h completion of incubation, 20 μl MTT (5 mg/ml) was added. The formazan crystals were dissolved in dimethyl sulfoxide (100 μl) and absorbance was measured at 570 nm using an ELISA plate reader.

Determination of the effect of DADS on endothelial cell proliferation (^{3}H-thymidine incorporation assay)

HUVECs (5000 cells/well) were plated in a 96-well culture plate and incubated at 37 °C in 5% CO_2 atmosphere. After 24 h, various concentrations of DADS (1, 2 & 5 μg/ml) were added along with 2 ng/ml VEGF and further incubated for 48 h. ^{3}H-thymidine was added to each well (1 μCi/well) and incubation was continued for additional 18 h. After completing incubation, the plates were centrifuged and the culture supernatant was removed, the cells were washed three times with PBS and then treated with ice cold PCA for 15 min. The resulting precipitate was dissolved in 0.5 N NaOH and was added to the scintillation fluid and the radioactivity was counted using a Rack Beta liquid scintillation counter.

Determination of the effect of DADS on endothelial cell migration/motility

HUVECS were seeded into wells of collagen coated 96-well plates at a density of 2×10^5 cells/well and incubated for 24 h at 37 °C in 5% CO_2 atmosphere. A clear area was scraped in the