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SPECIAL REPORT

The effect of linomide on the migration and the proliferation of capillary endothelial cells elicited by vascular endothelial growth factor

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In order to assess the mechanism of action of the quinoline-3-carboxyamide linomide as an antiangiogenic drug, the effect of linomide was studied *in vitro* on postcapillary endothelial cells exposed to vascular endothelial growth factor (VEGF). Linomide did not block the spontaneous replication of endothelial cells, but significantly suppressed endothelial cell growth and migration elicited by VEGF. It is concluded that linomide appears to be an effective tool to inhibit VEGF-dependent angiogenesis.

Keywords: Vascular endothelial growth factor; linomide; capillary endothelial cells; migration; proliferation; antiangiogenesis

Introduction Antiangiogenic therapy represents a new therapeutic approach for the treatment of solid tumours and for the reduction of metastasis. Many angiogenic factors have been identified and characterized and their expression studied in correlation with tumour growth. Vascular endothelial growth factor (VEGF) appears to play a key role in physiological and tumour angiogenesis (Ferrara *et al.*, 1992). The quinoline-3-carboxyamide linomide has been demonstrated to have immunomodulator activity (Kalland *et al.*, 1985), secondarily leading to reduced tumour vascularization and antitumour effects. Blood flow and microvessel density of prostatic cancer were reduced by linomide treatment suggesting that part of the antitumour effect is mediated by antiangiogenic properties (Vukanovic *et al.*, 1993; Vukanovic & Isaacs, 1995a). However, so far, no evidence has been presented showing whether linomide inhibits angiogenesis by affecting specific angiogenic pathways. With the aim of assessing the mechanism of linomide as an antiangiogenic drug, we have investigated *in vitro* the ability of the drug to interfere with the angiogenic phenotype induced by VEGF on cultured endothelium isolated from postcapillary venules of bovine origin.

Methods *Cell line and culture conditions* Postcapillary endothelial cells (CVEC) were obtained by a bead-perfusion technique of the coronary sinus (Schelling *et al.*, 1988). Cells were maintained in culture in Dulbecco's modified Eagle's medium (DMEM) supplemented with antibiotics and 10% foetal calf serum (FCS) on gelatin coated dishes. The endothelial morphology of the venular cells was typical for microvascular endothelial cells, and their origin was confirmed by immunofluorescent staining for factor VIII-related antigen and uptake of acetylated low-density lipoproteins. Subclones growing unmodified for morphological appearance and biological response to the angiogenesis factors up to 28 passages, were selected and cells between passage 12 and 18 were used in these experiments.

DNA synthesis Cellular proliferation was quantified by DNA synthesis in 96 multiwell plates as the number of labelled nuclei counted following 24 h 5-bromo-2'-deoxyuridine (BrdU) uptake and immunocytochemical processing (Cell Proliferation Kit, Amersham) (Morbidelli *et al.*, 1996). Proliferation was evaluated after 48 h of exposure to test substances. To assess the effect of linomide on VEGF induced proliferation, the drug was added to the cells 24 h before VEGF. The number of cells following fixation with methanol and staining with Diff-Quik was counted in seven random fields of each well at 200× magnification with the aid of a 21 mm^2 ocular grid. Data are expressed as the mean number of total labelled nuclei counted in each well.

Migration Migration was assessed on adherent cells (Parenti *et al.*, 1996); 3.5×10^4 cells were seeded into a rectangular silicon gasket inside a six-well plate in 10% FCS medium. At confluence the silicon gasket was removed and the four sides of the rectangular cell monolayer were marked with a scalpel on the outside of the tissue culture dish to define the starting line of cell progression. Linomide (100 $\mu g\ ml^{-1}$) was added to the cells 24 h before the gaskets were removed. VEGF was added for the following 72 h. The cells fixed with methanol and stained with Diff-Quik were counted and the distance of migration was determined microscopically with the aid of an ocular squared grid (225 $\mu m \times 225\ \mu m$). Experiments were carried out in quadruplicate. Eighty cell counts were then performed on each.

Results The effect of linomide on the survival and the replication of capillary endothelial cells was assessed on semiconfluent and serum starved monolayers. DNA synthesis in CVEC was not reduced by exposure for 48 h to the drug at 50 and 100 $\mu g\ ml^{-1}$ (Table 1). A higher concentration of linomide (200 $\mu g\ ml^{-1}$) reduced cell replication by 30-36% (data not shown). In CVEC exposed to VEGF DNA synthesis was increased by 40 and 68% at 10 and 20 ng ml^{-1}, respectively. Linomide at 100 $\mu g\ ml^{-1}$ reduced the effect of the growth factor by 55 and 40% at 10 and 20 ng ml^{-1}, respectively (Table 1). At 50 $\mu g\ ml^{-1}$ linomide blocked the effect of VEGF at the concentration of 20 ng ml^{-1}, but did not substantially modify the effect of 10 ng ml^{-1} VEGF. Based on these observations the concentration of 100 $\mu g\ ml^{-1}$ was used in further experiments.

Linomide was tested on the migration of growth arrested endothelial cells. VEGF increased by 2 and 3 fold the total number of cells migrating at 10 and 20 ng ml^{-1}, respectively (Figure 1). In response to either concentration of the growth factor the distance travelled by the cells increased by approximately 45% compared to controls. Basic fibroblast

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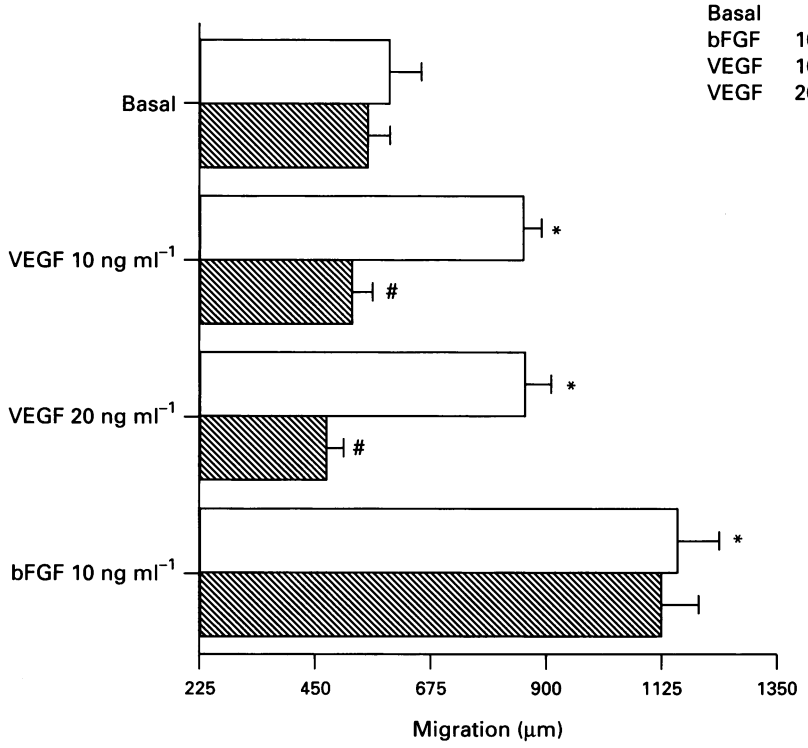

		Total cell number	
		Control	+ Linomide
Basal		373 ± 50	326 ± 90
bFGF	10 ng ml^{-1}	1412 ± 100	1685 ± 110
VEGF	10 ng ml^{-1}	803 ± 58	449 ± 90
VEGF	20 ng ml^{-1}	1007 ± 68	425 ± 8

Figure 1 Effect of linomide on adherent endothelial cell migration. Confluent and serum starved monolayers of postcapillary endothelial cells (CVEC) were pretreated with 100 μg ml^{-1} linomide for 24 h and then challenged for 48 h with the growth factors. Migration is expressed in μm. Mobilization of CVEC is shown in the presence of the growth factors in control condition (open columns) and after treatment with 100 μg ml^{-1} of linomide (hatched columns). In the table insert, the total number of migrated cells counted is presented. Data are mean ± s.e.mean of 3 experiments. *$P<0.001$ vs basal migration; #$P<0.001$ vs vascular endothelial growth (VEGF) alone (Student's t test for paired data).

Table 1 Effect of linomide on postcapillary endothelial cells (CVEC) proliferation in response to vascular endothelial growth factor (VEGF)

	Number of labelled nuclei		
	Control	*+ Linomide* (50 μg ml^{-1})	*+ Linomide* (100 μg ml^{-1})
Basal	9.5 ± 3	10.5 ± 2.1	14.3 ± 2
VEGF 10 ng ml^{-1}	13.3 ± 1.6	12 ± 3	6 ± 4**
VEGF 20 ng ml^{-1}	16 ± 0.5	11 ± 2.6*	9.5 ± 5**

Semiconfluent and synchronized CVEC were treated with linomide for 24 h and exposed to VEGF for 48 h. The values represent the number of labelled nuclei counted at 200× magnification in seven fields/well after 5-bromo-2′-deoxyuridine uptake and immunocytochemical processing. Data are mean ± s.e.mean from 3 experiments run in triplicate. *$P<0.05$, **$P<0.01$ vs VEGF alone (Student's t test for paired data).

growth factor (bFGF) increased by approximately 4 fold the number of migrating cells and by 2 fold the distance travelled compared to control unstimulated cells. As shown in the table insert of Figure 1, the total number of cells recovered after 96 h exposure to 100 μg ml^{-1} of linomide was not modified compared to control cultures. At the same concentration linomide completely blocked the effect of VEGF at 10 and 20 ng ml^{-1}, but was totally ineffective when cell migration was induced by bFGF.

Discussion In the present study we have shown that the acquisition of angiogenic phenotype, as measured by capillary endothelial cell proliferation and mobilization triggered by VEGF, is blocked by the quinoline-3-carboxyamide linomide. The effect of linomide appears to be specific since long term exposure to the drug does not block the spontaneous replication of normally quiescent capillary endothelium or impair its ability to respond when exposed to bFGF.

Mitogenic and migratory signals from growth factors are required to promote the switch to an angiogenic phenotype of the vascular endothelium. Among the various angiogenic factors identified to date VEGF is unique because it specifically acts on endothelial cells (Ferrara *et al.*, 1993).

In previous studies linomide has been shown to exert an antiangiogenic effect on prostatic cancer by affecting cytokine production by macrophages, which then causes apoptosis of tumour cells (Vukanovic & Isaacs, 1995a, b). In the present study it is shown that linomide targets the specific vascular mitogen VEGF. The drug does not interfere with the bFGF-induced migration of capillary endothelial cells, whereas it specifically inhibits the movement of cells stimulated by VEGF. Inhibition of angiogenesis is a promising therapeutic approach for a range of diseases characteried by the switch of vascular behaviour into an angiogenic phenotype (Folkman, 1995). Our data suggest that linomide can be of use in those pathological conditions in which angiogenesis is predominantly governed by VEGF overexpression.

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