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Induction of endothelial cell differentiation into capillary-like structures by substance P

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Abstract

Angiogenesis is an important process in inflammatory diseases and wound healing. We observed that the proinflammatory neuropeptide, substance P, stimulated angiogenesis in an in vitro model using human umbilical cord vein endothelial cells cultured on a basement membrane (Matrigel) substrate. Substance P stimulated endothelial cell differentiation into capillary-like structures in a dose-dependent manner. Stimulation of endothelial cell differentiation is a newly recognized biological function of substance P. The increased levels of substance P found in chronic inflammatory conditions may play an important role in tissue repair by promoting the development of new vessels and thus achieving compensation for ischemia.

Keywords: Angiogenesis; Basement membrane; Neurogenic inflammation; Tachykinin; Wound healing

1. Introduction

Substance P, which may be released in vascular tissue from primary afferent nerve endings and is found in arterial endothelial cells (Hökfelt et al., 1975; Loesch and Burnstock, 1988), potently affects various aspects of vascular wall functions. Substance P, via direct and indirect effects on endothelial cells, induces vasodilatation and plasma extravasation in vivo (Lembeck and Holzer, 1979). Migration and proliferation of endothelial cells (Ziche et al., 1990a, b), as well as proliferation of smooth muscle cells is induced by substance P in vitro (Payan, 1985; Nilson et al., 1985). Stimulation of the adherence of leukocytes to endothelial cells by substance P appears to be mediated by induction of endothelial leukocyte adhesion molecules in endothelial cells (Zimmerman et al., 1992; Matis et al., 1990). Most substance P effects on endothelial cells are mediated by tachykinin NK_1 receptors (Ziche et al., 1990a), and their expression has been demonstrated in human umbilical cord vein endothelial cells in culture (Greeno et al., 1993), and vascular sinuses and high endothelial venules of lymphoid tissues in situ (Tang et al., 1993).

More recent in vivo studies on the vascular effects of substance P suggest that it may induce neovascularization (Ziche et al., 1990a; Fun et al., 1993). This process is known to be under the regulatory control of various angiogenic and angiostatic growth factors. Normally, angiogenic growth factors are released by thrombocytes and are produced locally (Folkman and Klagsbrun, 1987). The mechanisms of the neurogenic effects of substance P on angiogenesis, however, are unknown. Substance P may act indirectly in new vessel development, e.g. via induction of angiogenic growth factor release by activation of thrombocyte degranulation (Folkman and Klagsbrun, 1987). In vitro evidence for a more direct action of substance P in angiogenesis has been obtained by observations of its chemotactic and mitogenic effects on endothelial cells (Ziche et al., 1990a, b). However, the in vivo relevance of in vitro stimulation of endothelial cell chemotaxis and proliferation by angiogenic factors is not clear (Sholley et al., 1984).

In order to substantiate the assumption of direct angiogenic actions of substance P on endothelial cells, we investigated the effect of substance P on differentiation of endothelial cells in vitro, i.e. formation of capillary-like

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structures, induced by basement membrane complexes (Kleinman et al., 1986).

2. Materials and methods

2.1. Test substances

Lyophilized substance P (Neosystems, Strassbourg, France) was reconstituted to stock solutions of 10 mM in 0.02 N acetic acid, stored at −80°C, and diluted in assay medium to final concentrations prior to use. Human recombinant interleukin-8, which served as positive control angiogenic factor (Koch et al., 1992), was purchased from Boehringer (Mannheim, Germany; specific activity $> 10^4$ U/mg protein).

2.2. Endothelial cell culture

Human umbilical cord vein endothelial cells from fresh placental cords were isolated by previously described methods (Jaffe et al., 1973) and grown until confluence at 37°C in 5% CO_2. The growth medium consisted of Medium-199 (Gibco Laboratories, Grand Island, NY, USA) supplemented with 20% fetal calf serum (Biological Industries, Beth Haemek, Israel), 200 μg/ml endothelial cell growth supplement (Collaborative Research, Bedford, MA), 100 U/ml penicillin-streptomycin, 50 μg/ml gentamycin, 2 mM glutamine (Gibco Laboratories, Grand Island, NY), and 50 μg/ml sodium heparin (Sigma Chemical Corporation, St. Louis, MO, USA). Cells used for experiments were from passage 4 to 8.

2.3. In vitro angiogenesis model

The basement membrane, Matrigel (Becton Dickinson, Bedford, MA, USA), was extracted from the murine Engelbreth-Holm-Swarm tumor, reconstituted, sterilized with chloroform and dialyzed against Medium-199 (Kleinman et al., 1986). For complete differentiation of human umbilical cord vein endothelial cells into capillary-like structures, 24-well plates (Costar Corporation, Cambridge, MA, USA) were coated with 300 μl of Matrigel per well, which was allowed to polymerize at 37°C for 30 min. For suboptimal stimulation of human umbilical cord vein endothelial cell differentiation, 300 μl per well of diluted Matrigel (1 : 2 with Medium-199) was kept at 4°C overnight for slow polymerization, followed by 37°C for 30 min before use. After polymerization, the remaining Medium-199 was removed from the wells by gentle aspiration. These suboptimal conditions were selected in order to detect factors that promote tube formation. Human umbilical cord vein endothelial cells (40 000 per well) suspended in Medium-199 were plated on diluted Matrigel and either different concentrations of substance P or 1 nmol/l interleukin-8 in a final volume of 1 ml were added. After 6 h of incubation at 37°C in 5% CO_2, the capillary-like structures were semiquantitatively evaluated by inverted microscopy. Representative microscopic fields were photographed for grading of degrees of differentiation by an independent examiner.

The following grades of differentiation were defined: (IV) – complete network of capillary-like structures; (III) – widespread networks of capillary-like structures with some fragments of unconnected tubes; (II) – widespread fragments of unconnected tubes with some networks of

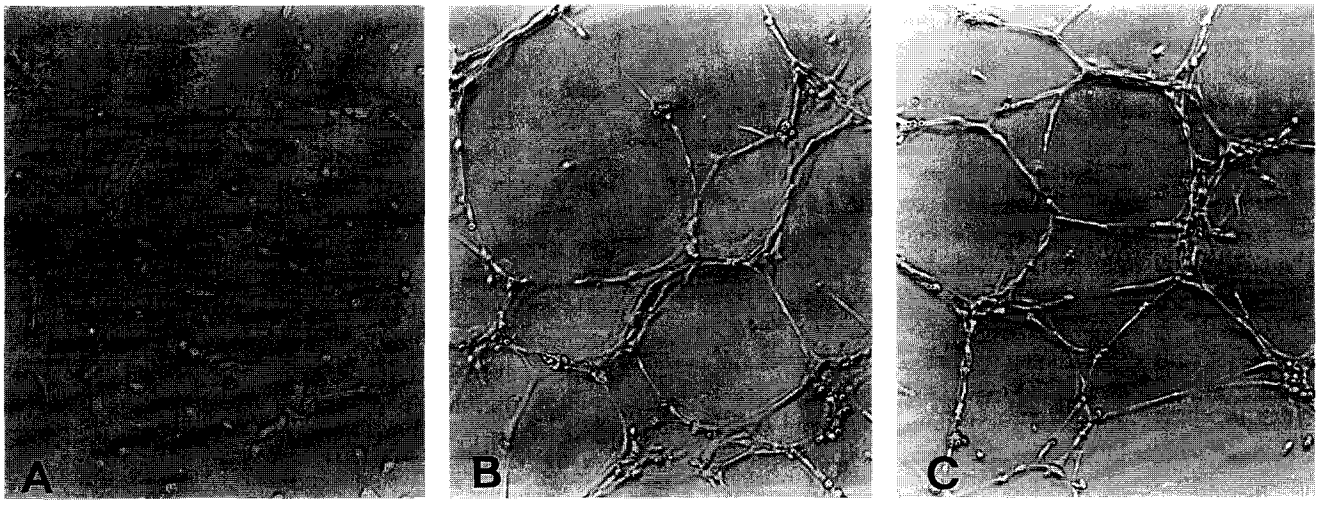

Fig. 1. Endothelial cell differentiation on Matrigel. At a low concentration of basement membrane complexes, interleukin-8 (10 nmol/l) and substance P (1 nmol/l) strongly stimulate endothelial cell differentiation into capillary-like structures. (A) Only fragments of unconnected tubes appear with Medium-199. (B) Complete network induced by interleukin-8 (10 nmol/l). (C) Complete network induced by substance P (1 nmol/l). Magnification ×100.

capillary-like structures; (I) – only fragments of unconnected tubes; (0) – neither networks of capillary-like structures nor fragments of unconnected tubes.

2.4. Statistical analysis

The effects of different concentrations of substance P on the differentiation of human umbilical cord vein endothelial cells on suboptimal Matrigel were analyzed by the Pearson Chi-Square test. Two-group comparisons were analyzed by Mann-Whitney Rank-Sum test.

3. Results

In the absence of additional angiogenic stimuli, suboptimal Matrigel assay conditions failed to support differentiation of human umbilical cord vein endothelial cells into networks of capillary-like structures or fragments of unconnected tubes (grade 0) in 16 out of 37 independent tests (43.3%); 14 tests (37.8%) gave fragments of unconnected tubes (grade I); and 7 tests (18.9%) allowed for differentiation of human umbilical cord vein endothelial cells into fragments of unconnected tubes with some networks of capillary-like structures (grade II). No differentiation of higher grades (III or IV) was seen. Addition of 10 nmol/l of interleukin-8, which was used as positive angiogenic control, however, induced differentiation of human umbilical cord vein endothelial cells into a complete network of tubes (grade IV) in 4 out of 4 assays (100%).

At a concentration of 1 nmol/l, substance P stimulated differentiation of human umbilical cord vein endothelial cells to a similar extent as interleukin-8 (Fig. 1). Detailed results of substance P experiments at concentrations ranging from 1 fmol/l to 1 μmol/l (Fig. 2) indicate that significant stimulation of differentiation of human umbilical cord vein endothelial cells occurred (Pearson Chi Square test: value 90.33645; df 16; $P < 0.0001$) at 1 nmol/l and 1 pmol/l of substance P (Mann-Whitney Rank-Sum test: $Z = -6.0074$, 2-tailed $P = 0.0000$; and $Z = -6.1655$, 2-tailed $P = 0.0000$, respectively). Some effect was seen at higher (1 μmol/l) and no effect observed at lower (1 fmol/l) doses (Mann-Whitney Rank-Sum test: $Z = -2.2499$, 2-tailed $P = 0.0245$; and $Z = -0.4567$, 2-tailed $P = 0.6479$, respectively). No difference was obtained between the two stimulating doses, 1 nmol/l and 1 pmol/l, of substance P (Mann-Whitney Rank-Sum test: $Z = -0.1057$, 2-tailed $P = 0.9158$).

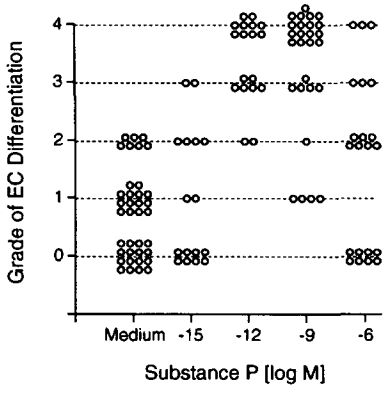

Fig. 2. Angiogenic activity of substance P at different concentrations (37 controls, 21 at 1 μmol/l, 27 at 1 nmol/l, 18 at 1 pmol/l, and 16 at 1 fmol/l). The dots indicate the grade of differentiation. The level of differentiation is classified according to five grades: (IV) – complete network of capillary-like structures; (III) – widespread networks of capillary-like structures with some fragments of unconnected tubes; (II) – widespread fragments of unconnected tubes with some networks of capillary-like structures; (I) – only fragments of unconnected tubes; (0) – neither networks of capillary-like structures nor fragments of unconnected tubes.

4. Discussion

The present study demonstrates that the proinflammatory neuropeptide substance P from primary afferent nerve fibers possesses angiogenic properties, since it stimulates endothelial cell differentiation into capillary-like structures.

Previous investigations of the proinflammatory effects of substance P led to the observation that substance P has neovascularization-inducing effects in vivo (Ziche et al., 1990b; Fun et al., 1993). However, such a stimulation of angiogenesis by substance P may have been indirect and achieved through the known property of substance P of activating thrombocytes, which have the potential for releasing angiogenic factors (Öhlen et al., 1989). Moreover, substance P stimulates the synthesis and release of angiogenic cytokines in monocytes (Lotz et al., 1988), which may contribute to the in vivo angiogenic effects. A direct angiogenic action of substance P on endothelial cells was suggested by the chemotactic and mitogenic effects of substance P on endothelial cells in vitro (Ziche et al., 1990a, b). Additional evidence, however, is required in order to establish that the angiogenic effect of substance P is by direct actions on endothelial cells, since factors known to be chemotactic and mitogenic for endothelial cells in vitro are not necessarily angiogenic in vivo.

Endothelial cells grow as a monolayer on plastic, but when put on a reconstituted basement membrane (Matrigel), they cease to proliferate and differentiate into a complete network of capillary-like structures within less than 18 h (Kubota et al., 1988; Grant et al., 1989). Matrigel can also be injected subcutaneously in mice and used as a vehicle to assess the angiogenic activity of different compounds (Passaniti et al., 1992; Kibbey et al., 1992). To date, the factors tested in vitro and in vivo Matrigel assays show similar activities to those observed in the chick chorioallantoic membrane (Sakamoto et al., 1991; Passaniti et al., 1992; Kibbey et al., 1992). Therefore, an in vitro Matrigel assay was used to test the

angiogenic properties of substance P. Substance P stimulated endothelial cell differentiation into capillary-like structures, and similar potencies of substance P and interleukin-8 were seen. The effect of substance P was dose-dependent and significant in the nano- and femtomolar range which are physiological concentrations.

Inflammatory processes often lead to the occlusion or stenosis of the vascular lumen either by thrombus formation or by myointimal proliferation and fibrosis, resulting in ischemia and tissue necrosis. The release of substance P from primary afferents by various stimuli in inflammatory processes as well as other situations including wound healing, may play an important role in tissue repair, as substance P may directly compensate for ischemia by promoting the development of new vessels.

In summary, our studies demonstrate that substance P stimulates angiogenesis in an in vitro model. Substance P elevation in chronic inflammation and/or ischemic conditions may be important for tissue repair and for the promotion of neovascularization.

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