J. Pharm. Pharmacol. 1996, 48: 951–954
Received January 22, 1996
Accepted February 27, 1996

Effects of the Anticancer Agent Vinorelbine on Endothelial Cell Permeability and Tissue-factor Production in Man

CORINE MOUCHARD-DELMAS, ISABELLE DEVIE-HUBERT AND JEAN DUFER*

*Laboratoire d'Hématologie et d'Hémostase, Institut Jean-Godinot, 1 rue Gal Koenig, and *Laboratoire de Physiologie Cellulaire, UFR de Pharmacie, 51 rue Cognacq-Jay, F-51100 Reims, France*

Abstract

Because antineoplastic drugs could increase endothelial blood barrier permeability and thrombotic diseases have been described as a complication of treatments with vinca alkaloids, the effect of a therapeutic dose (10^{-8} M) of vinorelbine on transendothelial permeability was analysed by measuring the movement of albumin across a monolayer of human venous endothelial cells.

Induction of procoagulant activity was assessed by evaluation of tissue-factor activity in cell lysates. Vinorelbine increased the permeability of endothelial cells after 3 h of culture, as observed with thrombin. In addition, thrombin induced strong tissue-factor activity, a phenomenon not observed after vinorelbine treatment.

These data suggest that vinorelbine could modulate endothelial barrier permeability. This effect is not linked to an increase in tissue-factor activity, suggesting that their induction could operate through separate pathways.

By virtue of its unique position at the interface between blood and tissues, the vascular endothelium plays an active role in critical homeostatic functions including the maintenance of a non-thrombogenic surface and the barrier function of the vessel. Perturbation of endothelium can induce procoagulant activity, enabling these cells to initiate and propagate an entire coagulation pathway leading to the deposition of fibrin. A major component of these procoagulant activities is tissue factor (or thromboplastin), a specific membrane-bound protein that is an essential cofactor in triggering the coagulation of blood via the extrinsic pathway.

Thromboses and thromboembolic events are well recognized complications of malignancies. Although a direct causal relationship between a given chemotherapeutic agent and thromboembolism is difficult to establish because of the many concurrent risk factors that are potentially involved, some drugs are becoming increasingly suspect and drug-associated syndromes of vascular toxicity have been described (for a review see Doll & Yarbro (1992)). Profound alterations in endothelial cell-barrier function might also be involved. From this aspect, observations suggest that the incidence of metastatic brain tumours was increased as chemotherapy was applied against primary extracranial neoplasms (Paterson et al 1982) and some experiments showed that antineoplastic drugs could increase blood-brain-barrier permeability (McDonnell et al 1978; Spigelman et al 1986). The mechanisms of these changes are not fully understood but the relevance of the endothelial cells, cytoskeleton in modulating the permeability of a layer of endothelial cells is indicated by several experiments performed in-vitro (Shasby et al 1982; Bussolino et al 1987; Camussi et al 1991).

Because brain metastases originate as blood-borne emboli we decided to investigate the effects of antineoplastic drugs on endothelial barrier permeability; the aim of the study was to characterize changes in endothelial cell functions in response to the direct action of a drug. Because vinorelbine, a recently available vinca alkaloid, is an inhibitor of microtubule polymerization, its effects on endothelial permeability were investigated. The effects of vinorelbine on the generation of procoagulant activity in endothelial cells were also analysed, because thrombotic microangiopathy has also been described as a complication of treatment with vinca alkaloids (Jackson et al 1984).

Correspondence: I. Devie-Hubert, Laboratoire d'Hématologie et d'Hémostase, Institut Jean-Godinot, 1 rue du Général Koenig, F-51100 Reims, France.

Materials and Methods

Cell culture

Human venous endothelial cells were isolated from umbilical cords by the method of Jaffe et al (1973). In brief, cells obtained by collagenase digestion were suspended in Dulbeco Minimal Essential Medium (DMEM; Gibco-BRL, Paisley, Scotland) containing 20% foetal calf serum (Gibco) and plated on 35-mm diameter culture dishes. After 24 h of culture the medium was replaced by fresh DMEM supplemented with 20% pooled human serum. When primary culture reached confluency, cells were trypsinized and passaged at a split ratio of 1 : 2.

Cells were identified and characterized as endothelial cells by their typical 'cobblestone' morphology and their positivity for von Willebrand factor (Jaffe et al 1974) using anti-vWF monoclonal antibody and the alkaline phosphatase anti-alkaline phosphatase revelation method (Dako, Glostrup, Denmark; Cordell et al 1984).

Permeability studies

For diffusion experiments, human venous endothelial cells in the second passage were grown to confluence on polycarbonate filters (pore size 0·4 μm) of Transwell chamber assemblies (Costar, Dutscher, Paris, France) coated with fibronectin (Sigma, St Louis, MO, USA; Camussi et al 1991).

Cells were plated on to filters at a density of $3{\cdot}5 \times 10^5$ cells mL^{-1} and cultured for up to 7 days (Schaeffer et al 1992).

The permeability of the endothelial cell monolayers was measured by diffusion of bovine serum albumin. Before the experiment, DMEM was replaced by protein-free Hanks' solution. Fluid volumes were selected to avoid any hydrostatic pressure gradient across monolayers. The upper chamber was filled with 1·5 mL of Hanks' medium containing 200 μM albumin (Sigma). Albumin concentration was determined in the lower chamber by the bicinchoninic acid protein assay (Pierce, Interchim, France) and measurement of absorbance at 570 nm (Wiechelman et al 1988).

Cells were cultured for up to 3 h in Hanks' solution alone or containing 10^{-8} M vinorelbine, 0·1 int. units mL^{-1} thrombin, or both. Three separate experiments were performed on cells obtained from three different umbilical cords. Transport of albumin across monolayers was determined by taking samples at different times during the incubation period.

Results are expressed according to Shasby et al (1985) as a percent change in albumin transfer calculated as follows:

OD_{570} after stimulus $- OD_{570}$ control/OD_{570} control $\times$ 100.

Incubation and coagulation assays

Experiments were performed exclusively with confluent monolayers at the second passage. Cells were washed twice with serum-free medium and incubated for 6 h in medium alone or containing 10^{-8} M vinorelbine, 0·1 int. unit mL^{-1} thrombin, or both.

At the end of the culture period, treated and control cells were washed twice in saline. Cellular extracts were prepared by solubilization with 16 mM octyl-β-D-glucopyranoside (Sigma) in HEPES-buffered saline for 10 min at 37°C, after three freeze-thaw cycles (Tsao et al 1984).

Tissue-factor activity was assayed in a one-stage clotting system by measuring the ability of cell homogenates to shorten the recalcification time of a human-platelet-poor citrated plasma. The times were converted into units of tissue-factor activity by reference to a standard curve derived from a rabbit-brain thromboplastin standard (Difco, Detroit, MI, USA) which was reconstituted in saline at 37·5 mg mL^{-1} and assigned a value of 10^5 m units mL^{-1} as described by Levy & Edgington (1980). Serial dilutions were used to produce a log-log plot. Protein content in each sample was determined by the bicinchoninic acid protein assay. Tissue-factor activity was expressed in m units mg^{-1} cell protein.

Statistical analysis

Because the transfer of albumin across endothelial monolayers and tissue-factor expression vary from umbilical cord vein to umbilical cord vein, each experiment was performed with its own internal control. Results are presented as the mean ± s.e.m., and statistical analysis was performed by using the Student's *t*-test for paired data.

Results

Permeability studies

The action of vinorelbine on tubuline could lead to cytoskeletal arrangements and changes in endothelial barrier function. To test this directly, human venous endothelial cells were cultured on microporous (0·4 μm) polycarbonate membranes. Endothelial cell-confluent monolayers formed on these membranes are able to restrict diffusional passage of albumin as compared with polycarbonate membrane alone (Fig. 1).

Thrombin, a known permeability modulator which acts by rearranging cytoskeletal actin molecules, was chosen as a positive stimulus. The time-course of the effect of the addition of thrombin and vinorelbine on the permeability of the cultured endothelial cell monolayers is shown in Fig. 2. Incubation of endothelium with thrombin (0·1 int. units mL^{-1}) increases albumin transfer. This increase is detectable after 2 h of incubation ($P < 0{\cdot}05$). The permeability of the monolayer continues to increase progressively up to 3 h and the percent of transfer is then 65·3 ± 17·3%. Treatment with 10^{-8} M vinorelbine induced enhanced albumin permeability that was detectable after 2 h and increased up to 3 h ($P < 0{\cdot}05$). The percent transfer was 41·7 ± 6% at 3 h ($P < 0{\cdot}05$ compared with control cells) (Fig. 2). When compared with thrombin, the increase in permeability was never significantly different, suggesting that vinorelbine and thrombin were able to enhance albumin flux in the same way. The vinorelbine-induced increase in permeability could, however, be further enhanced by thrombin because thrombin-vinorelbine association enhances endothelial monolayer permeability compared with human venous endothelial cells treated with vinorelbine alone ($P < 0{\cdot}05$ at 3 h) but not with those treated with thrombin only.

Coagulation experiments

Five experiments were performed with endothelial cells obtained from five different umbilical cords. Tissue-factor activity was very low in the unstimulated endothelial cells but was strongly increased by thrombin ($P < 0{\cdot}05$; Fig. 3), as expected from literature data (Galdal et al 1985). In contrast, stimulation with vinorelbine did not induce any significant variation in procoagulant activity when compared with control cells. Similarly, the vinorelbine-thrombin association did not seem to induce greater tissue-factor expression in the endothelial cells than treatment with thrombin only.

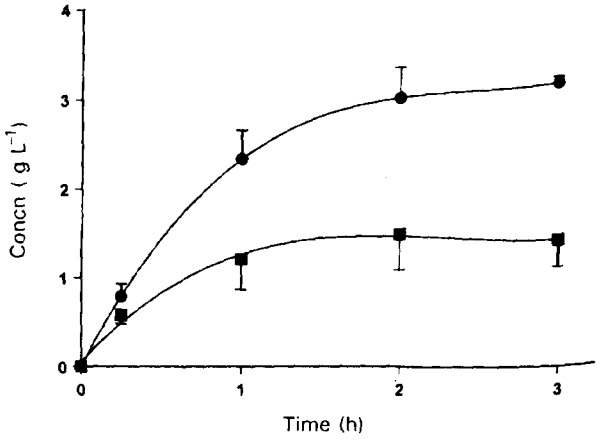

FIG. 1. Assessment of endothelial cell barrier function on albumin transfer. ● Polycarbonate membrane only; ■ confluent monolayer of endothelial cells. Albumin concentration was evaluated in the lower compartment of the diffusion chamber by bicinchoninic acid protein assay. All data shown are mean ± s.e.m. of 3 observations.

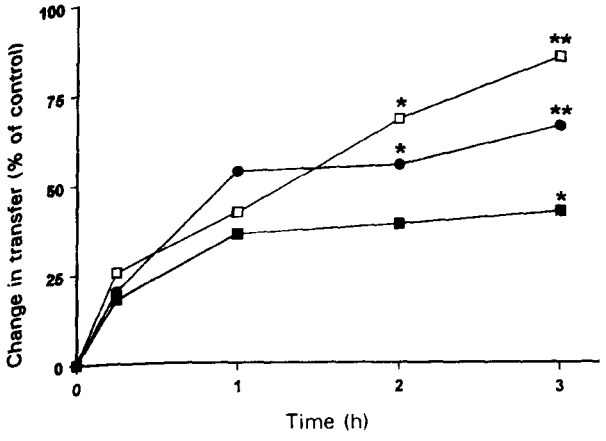

FIG. 2. Modulation of endothelial cell-barrier function by vinorelbine or thrombin. Cells were incubated for 0 to 3 h in the presence of 10^{-8} M vinorelbine (■) or 0·1 int. units mL^{-1} thrombin (●), or both drugs (□) and albumin transfer results were compared with data from cells incubated in medium alone. $*P < 0.05$, $**P < 0.01$ compared with control cells.

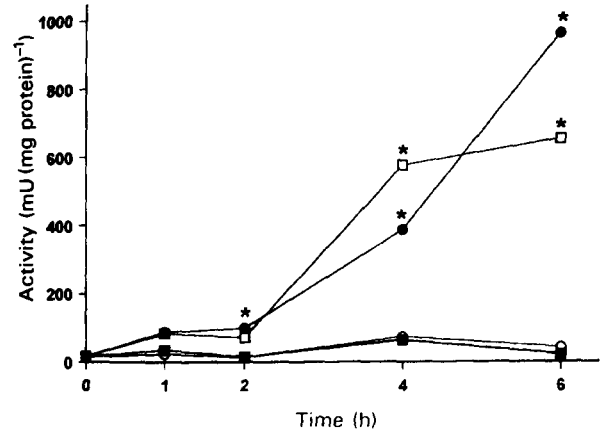

FIG. 3. Modulation of endothelial cell procoagulant activity by vinorelbine or thrombin. Cells were incubated for 0 to 6 h in medium alone (○), or in the presence of 10^{-8} M vinorelbine (■), or 0·1 int. units mL^{-1} thrombin (●), or both drugs (□). $*P < 0.05$ compared with vinorelbine alone.

Discussion

The results shown in this paper indicate that, as observed with thrombin, vinorelbine increases the permeability of a confluent cultured human venous endothelial cells monolayer.

Thrombin is a powerful physiological cell agonist which is derived from prothrombin, its circulating precursor, as a result of activation of the blood cascade. An important endothelial cell function mediated by thrombin is its ability to enhance tissue-factor expression on the cell surface (Galdal et al 1985). Thrombin is also known as a permeability enhancer (Killackey et al 1986) which acts by modulation of the rearrangement of cytoskeletal actin molecules (Malik & Fenton 1992). Vinorelbine concentrations in patients vary from 10^{-7} M after injection to 10^{-9} M at the terminal phase (Krikorian et al 1989; Jehl et al 1991). All the experiments were performed with a dose of 10^{-8} M, an effective blood concentration for patients.

The use of an in-vitro endothelial cell permeability model, as used in this study, has a number of advantages, including direct access to luminal and abluminal fluid for analysis, limitation to a single cell type, and that the experimental medium can be defined in terms of its chemical composition (Downie et al 1992).

Localized changes in blood-barrier permeability have been reported after the administration of antineoplastic drugs (McDonnell et al 1978; Spigelman et al 1986). Such changes might be relevant to the development of secondary intracranial tumours after antineoplastic chemotherapy. Indeed, it appeared that there has been a modest but significant increase in the incidence of brain metastasis as site of first recurrence in patients receiving adjuvant chemotherapy (Paterson et al 1982). The biochemical and molecular mechanisms whereby cytotoxic drugs increase endothelial permeability is poorly understood but has been partially elucidated for different bioactive mediators, including thrombin (Malik & Fenton 1992), TNF-α (Camussi et al 1991), interleukin 1 (Campbell et al 1992), and interleukin 2 (Downie et al 1992). In particular, the morphological machinery present in endothelial cells has proven able to produce a functional contractile response (De Clerck et al 1981). Microtubules and filaments containing contractile proteins have been demonstrated in endothelial cells (Shasby et al 1982) and such a cytoskeletal reorganization has been implicated as a final common pathway for changes in endothelial barrier function although albumin, and other macromolecules, could be transported across endothelium by receptor-mediated or pinocytotic processes, or both (Eaton et al 1991). The antineoplastic activity of vinorelbine is caused by its ability to inhibit microtubule polymerization by binding tubuline (Fellous et al 1989) which results in inhibition of cellular replication. The action of vinorelbine on tubuline could, therefore, lead to cytoskeletal rearrangements inducing changes in the shape of the endothelial cells and in endothelial barrier function. It is conceivable that separation of junctions between endothelial cells would provide a paracellular pathway for macromolecule movement similar to that described for TNF-α (Camussi et al 1991) or thrombin (Garcia et al 1986).

Another important endothelial cell function mediated by thrombin is the enhancement of tissue-factor expression on the cell surface (Galdal et al 1985). Tissue-factor, a transmembrane protein, is the main initiator of blood clotting in-vivo (Andree & Nemerson 1995). The presence of clotting abnormalities might facilitate metastatic spread of tumours through the formation of tumour emboli which can readily arrest in capillary beds (Murray 1991). Because thrombotic microangiopathy has been described as a complication of vinca alkaloids (Jackson et al 1984), the effects of vinorelbine on the generation of procoagulant activity were also analysed and compared with thrombin effects. Because vinorelbine was ineffective on tissue-factor expression, its action on endothelial cell permeability appears not linked to induction of this tissue-factor activity, suggesting that stimulation of the two activities could operate through separate pathways.

In conclusion, these data indicate that vinorelbine enhances endothelial permeability without induction of any procoagulant effect. The ability of vinorelbine to increase the movement of macromolecules through vascular endothelium might have in-vivo relevance and suggests that the entry of vinorelbine and other cytotoxic drugs or cytokines into tumour tissue could be

facilitated. This also could explain the large tissue diffusion of vinorelbine despite its high molecular weight (Krikorian et al 1989).

Acknowledgements

We wish to thank Mrs Lucile Minardi for technical help, and the staff of the Reims University Maternity Hospital for umbilical cord samples. This work was supported by grants from the Comité Départemental des Ardennes de la Ligue Française contre le Cancer.

References

Andree, H. A. M., Nemerson, Y. (1995) Tissue factor: regulation of activity by flow and phospholipid surfaces. Blood Coag. Fibrinol. 6: 189–197

Bussolino, F., Camussi, G., Aglietta, M., Braquet, P., Bosia, A., Pescarmona, G., Sanavio, F., D'Urso, N., Marchisio, P. C. (1987) Human endothelial cells are target for platelet-activating factor. J. Immunol. 139: 2439–2446

Campbell, W. N., Ding, X., Goldblum, S. E. (1992) Interleukin-1α and -β augment pulmonary artery transendothelial albumin flux in-vitro. Am. J. Physiol. 236: L128–L136

Camussi, G., Turello, E., Bussolino, F., Baglioni, C. (1991) Tumor necrosis factor alters cytoskeletal organization and barrier function of endothelial cells. Int. Arch. Allergy Appl. Immunol. 96: 84–91

Cordell, J. L., Falini, B., Erber, W. N., Ghosh, A. K., Abdulaziz, Z., MacDonald, S., Pulford, K. A. F., Stein, H., Mason, D. (1984) Immunoenzymatic labeling of monoclonal antibodies using immune complexes of alkaline phosphatase and monoclonal anti-alkaline phosphatase (APAAP complexes). J. Histochem. Cytochem. 32: 219–229

De Clerck, F., De Brabander, M., Neels, H., Van de Velde, V. (1981) Direct evidence for the contractile capacity of endothelial cells. Thromb. Res. 23: 505–520

Doll, D. C., Yarbro, J. W. (1992) Vascular toxicity associated with antineoplastic agents. Semin. Oncol. 19: 580–596

Downie, G. H., Ryan, U. S., Hayes, B. A., Friedman, M. (1992) Interleukin-2 directly increases albumin permeability of bovine and human vascular endothelium in vitro. Am. J. Respir. Cell. Mol. Biol. 7: 58–65

Eaton, B. M., Toothill, V. J., Davies, H. A., Pearson, J. D., Mann, G. E. (1991) Permeability of human venous endothelial cell monolayers perfused in microcarrier cultures: effects of flow rate, thrombin, and cytochalasin D. J. Cell. Physiol. 149: 88–99

Fellous, A., Ohayon, R., Vacassin, T., Binet, S., Lataste, H., Krikorian, A., Couzinier, J. P., Meiniger, V. (1989) Biochemical effects of navelbine on tubulin and associated proteins. Semin. Oncol. 16: 9–14

Galdal, K. S., Lyberg, T., Evensen, S. A., Nilsen, E., Prydz, H. (1985) Thrombin induces thromboplastin synthesis in cultured vascular endothelial cells. Thromb. Haemost. 54: 373–376

Garcia, J. G. N., Siflinger-Birnboim, A., Bizios, R., Del Vecchio, P. J., Fenton, J. W., Malik, A. B. (1986) Thrombin-induced increase in albumin permeability across the endothelium. J. Cell. Physiol. 128: 96–104

Jackson, A. M., Rose, B. D., Graff, L. G., Jacobs, J. B., Schwartz, J. H., Strauss, G. M., Yang, J. P. S., Rudnick, M. R., Elfenbein, B. I., Narins, R. G. (1984) Thrombotic microangiopathy and renal failure associated with antineoplastic chemotherapy. Ann. Intern. Med. 101: 41–44

Jaffe, E. A., Nachman, R. L., Becker, C. G., Minick, C. R. (1973) Culture of human endothelial cells derived from umbilical veins. J. Clin. Invest. 52: 2745–2756

Jaffe, E. A., Hoyer, L. W., Nachman, R. L. (1974) Synthesis of von Willebrand factor by cultured human endothelial cells. Proc. Natl. Acad. Sci. USA 71: 1906–1909

Jehl, F., Quoix, E., Leveque, D., Pauli, G., Breillout, F., Krikorian, A., Monteil, H. (1991) Pharmacokinetic and preliminary metabolic fate of navelbine in humans as determined by high performance liquid chromatography. Cancer Res. 51: 2073–2076

Killackey, J. J. F., Johnston, M. G., Movat, H. Z. (1986) Increased permeability of microcarrier-cultured endothelial monolayers in response to histamine and thrombin. A model for the in-vitro study of increased vasopermeability. Am. J. Pathol. 122: 50–61

Krikorian, A., Rhamani, R., Bromet, M., Bore, P., Cano, J. P. (1989) Pharmacokinetics and metabolism of navelbine. Semin. Oncol. 16: 21–25

Levy, G. A., Edgington, T. S. (1980) Lymphocyte cooperation is required for amplification of macrophage procoagulant activity. J. Exp. Med. 151: 1232–1244

Malik, A. B., Fenton, J. W. (1992) Thrombin-mediated increase in vascular endothelial permeability. Semin. Thromb. Haemost. 18: 193–199

McDonnell, L. A., Potter, P. E., Leslie, R. A. (1978) Localized changes in blood-brain barrier permeability following the administration of antineoplastic drugs. Cancer Res. 38: 2930–2934

Murray, J. C. (1991) Coagulation and cancer. Br. J. Cancer 64: 422–424

Paterson, A. H. G., Agarwal, M., Lees, A., Hanson, J., Szafran, O. (1982) Brain metastases in breast cancer patients receiving adjuvant chemotherapy. Cancer 49: 651–654

Schaeffer, R. C., Gong, F., Bitrick, M. S. (1992) Restricted diffusion of macromolecules by endothelial monolayers and small-pore filters. Am. J. Physiol. 263: L27–L36

Shasby, D. M., Shasby, S. S., Sullivan, J. M., Peach, M. J. (1982) Role of endothelial cell cytoskeleton in control of endothelial permeability. Circ. Res. 51: 657–661

Shasby, D. M., Lind, S. E., Shasby, S. S., Goldsmith, J. C., Hunninghake, G. W. (1985) Reversible oxidant-induced increases in albumin transfer across cultured endothelium : alterations in cell shape and calcium homeostasis. Blood 65: 605–614

Spigelman, M. K., Zappulla, R. A., Strauchen, J. A., Feuer, E. J., Johnson, J., Goldsmith, S. J., Malis, L. I., Holland, J. F. (1986) Etoposide-induced blood-brain barrier disruption in rats: duration of opening and histological sequelae. Cancer Res. 46: 1453–1457

Tsao, B. P., Fair D. S., Curtiss, L. K., Edgington, T. S. (1984) Monocytes can be induced by lipopolysaccharide-triggered T lymphocytes to express functional factor VII/VIIa protease activity. J. Exp. Med. 159: 1042–1057

Wiechelman, K. J., Braun, R. D., Fitzpatrick, J. D. (1988) Investigation of the bicinchonic acid protein assay: identification of the groups responsible for color formation. Anal. Biochem. 175: 231–237