Biochemical and Biophysical Research Communications **258,** 113–118 (1999)
Article ID bbrc.1999.0596, available online at http://www.idealibrary.com on IDEAL®

Inhibition of the Expression of VE-Cadherin/Catenin Complex by Gamma Linolenic Acid in Human Vascular Endothelial Cells, and Its Impact on Angiogenesis

Jun Cai, Wen G. Jiang,[1] and Robert E. Mansel
Metastasis Research Group, University Department of Surgery, University of Wales College of Medicine, Cardiff CF4 4XN, United Kingdom

Received March 22, 1999

Gamma linolenic acid (GLA) has been recently shown to inhibit tumour-induced angiogenesis. The present study investigated the effects of GLA on the HUVEC-specific adhesion. After treatment with GLA, HUVECs decreased the amounts of Triton soluble and insoluble VE-cadherin and β-catenin and reduced tube formation in matrix in a concentration-dependent manner. An anti-VE-cadherin antibody dissociated HUVECs' colonies and exerted similar inhibitory effects on tube formation of HUVECs. These data indicate that the VE-cadherin/catenins complex is essential for formation and maintenance of new capillaries. It is concluded, therefore, that GLA inhibits tumour-induced angiogenesis partly via the decrease in the expression of VE-cadherin and β-catenin.

Gamma linolenic acid (GLA), is one member of the n-6 polyunsaturated fatty acid (PUFA) family. It has been extensively studied for the past decade because of its reported anti-cancer effect in cancer cells (1-11). Our recent work (12) has demonstrated that it also has an inhibitory effect towards tumour-induced angiogenesis.

Angiogenesis is a multistep process, in which there must be degradation of the extracellular matrix, migration and proliferation of vascular endothelial cells, and capillary formation with a new extracellular matrix (13). Although our study (12) has shown that the inhibitory effect of GLA is partly due to its reduction of motility and migration of vascular endothelial cells, the molecular mechanisms of the antiangiogenic effect of GLA are still not clear.

Vascular endothelial (VE)-cadherin (14), a member of the cadherin superfamily and also known as cadherin 5 (15), is specific for vascular endothelium and localises exclusively to the lateral junctions of intact endothelium. Its function is to maintain integrity of the endothelium and participate the creation of new capillaries (16-18). Like other members of cadherin family, VE-cadherin is involved in homophilic endothelial cell-to-cell adhesion via its extracellular domain while its cytoplasmic domains associates with the cytosolic proteins β- and α-catenins. These, in turn, link to actin cytoskeleton (19-21). Since VE-cadherin is important in mediating intercellular adhesion in endothelium, VE-cadherin has thus been implicated as having a role in regulating angiogenesis (22-24).

To better understand the molecular basis of inhibition of angogenesis by GLA, we have examined the effects of GLA on the VE-cadherin expression in human umbilical vascular endothelial cells (HUVECs). We report here that GLA was seen to reduce VE-cadherin expression in HUVECs. Therefore, we propose that the decrease in expression of VE-cadherin by GLA provides an explanation for the inhibition of tumour-induced angiogenesis by GLA.

MATERIALS AND METHODS

Materials. Gamma linolenic acid (GLA) was purchased from Sigma (Poole, Dorset, UK). GLA was initially dissolved in ethanol, stored in liquid nitrogen, and diluted in culture medium immediately before use (ethanol final concentration $<0.01\%$). Monoclonal antibodies to VE-cadherin were from Pharmingen (USA). Rabbit purified Ig to human β-catenin was from Sigma (Pool, Dorset, UK). Horseradish peroxidase-conjugated anti-mouse IgG, anti-rabbit IgG and TRITC-conjugated anti-mouse IgG were from Sigma (Pool, Dorset, UK). FITC-conjugated anti-rabbit IgG was from Santa Cruz biotechnology (Santa Cruz, CA, USA). All other materials were purchased from Sigma (Poole, Dorset, UK) unless otherwise stated.

Cell culture. Human umbilical vascular endothelial cells (HUVECs) were obtained from human umbilical vein and cultured in M199 with 20%-30% foetal calf serum (FCS) as previous described (25). The cells were always used within 3 passages.

VE-cadherin neutralisation. HUVECs were plated at 1×10^4 cells/well in 24-well plates and incubated to form colonies. The anti-VE-

[1] To whom correspondence should be addressed. Fax: +44 1222 761623. E-mail: Jiangw@cardiff ac.uk.

0006-291X/99 $30.00

cadherin antibody (2.5 μg/ml) and GLA (50 μM) were separately added into some of wells for 24 hr. The resultant colonies were then observed under a light microscope (Leica Ltd, Cambridge, UK) and photographed.

Tube formation. Tube formation of endothelial cells was assessed as previously reported by Kanayasu *et al* (26). HUVECs were seeded at 5×10^4 cells/well onto 0.5 ml of Matrigel (Collaborative Research, Bedford, USA) in 24-well plates for 24 hr. The anti-VE-cadherin (2.5 μg/ml) and GLA (50 μM) were added into some of wells separately or in combination for further 24 hr The medium was then aspirated and 0.5 ml of Matrigel was overlaid and the resultant cultures incubated for 24 hr. The following day, the cultures were observed under a phase contrast microscope and photographed.

VE-cadherin immunofluorescent staining. HUVECs were cultured to reach confluence in 16 well chamber slides. Then GLA was added into some of wells for 24 hr. The cells were fixed with fresh 4% formaldehyde, 5% sucrose for 20 min and permeabilized with 0.05% Triton X-100 at room temperature for 5 min. After blocking with 10% milk, the cells were incubated with VE-cadherin antibody (1:125) at room temperature for 1 hr. These were followed by TRITC-conjugated secondary antibody (1:1500) at room temperature for 1 hr. Coverslips were mounted in 80% glycerol. The staining was examined with microscope equipped with epifluorescence (Leica Cambride Ltd, UK) and photogrophed (Elite II, 400; Kodak Ltd., England).

Western blotting. The HUVECs were plated at the same density of 5×10^4/ml in 25 cm^2 flasks (Nunclon Inc., USA). The confluent HUVEC monolayers were treated with GLA (0-50 μM) for 24 hr. At the end of culture, cells were subject to two extractions, in order to obtain Triton X-100-soluble (TX-sol) and insoluble (TX-insol) fractions, as previously described by Lampugnani *et al* (19). HUVEC monolayers were first extracted with 1 ml Triton lysis buffer [25 mM Tris, 150 mM NaCl (TBS), 1 mM PMSF, 0.5% Sodium Deoxychate, 0.5% Sodium azide, 10 mM sodium orthovanadate (TBS with protease inhibitors), 1% Igepal CA-630 and 1% Triton X-100] at 4°C for 30 min with gently agitation. The total cell lysate was then collected and centrifuged at 13,000 rpm for 5 min. The supernatant was collected and referred to as the TX-sol fraction. The cell debris were further extracted with 1 ml SDS lysis buffer (the same as the Triton lysis buffer except that it contained 1% SDS and 1% Igepal CA-630, and omitted Triton X100), at 4°C for 20 min. The extract buffer was vigorously pipetted, collected and centrifuged at 13000 rpm for 5 min. The supernatant was collected and defined as TX-insol fraction. Both TX-sol and TX-insol fraction were mixed with equal volume of electrophoresis sample buffer (0.22 M Tris-HCl pH 6.7, 17% glycerol, 5.2% SDS, 8% mercaptoethanol and 0.1% bromophenol blue), respectively and boiled at 100°C for 5 min. These were either used immediately or stored at −20°C until required. These proteins were loaded at approximate 15 μg of TX-sol or 4 μg of TX-insol into each lane on 8% SDS-PAGE gels, transferred onto nitrocellulose membrane. These membranes were then blocked with TBS-T buffer (25 mM Tris-HCl pH 7.5, 150 mM NaCl and 0.1% Tween-20) containing 10% skimmed milk at room temperature for 30 min and probed with antibody to VE-cadherin (1:125), or to β-catenin (1:4000) in 3% milk for 2 hr at room temperature. Then the membranes were incubated with horseradish peroxidase conjugated anti-mouse IgG (1:1500) or anti-rabbit IgG (1:2000), followed by detection with enhanced chemiluminescence (ECL) system (KPL, USA).

RESULTS

Inhibition of vascular endothelial tube formation by GLA and VE-cadherin antibody. HUVECs, when cultured between the Matrigel layers, were seen to form typical tube-like structurs after 24 h. Figure 1A shows that the lumen was composed of several endothelial cells linked to each other. The effect of GLA and VE-cadherin antibody on tube formation were then tested in this system. Figures 1B and 1C show that when the cells were treated with GLA (50 μM) (1B) or VE-cadherin antibody (2.5 μg/ml) (1C), the tube formation decreased in comparison with control.

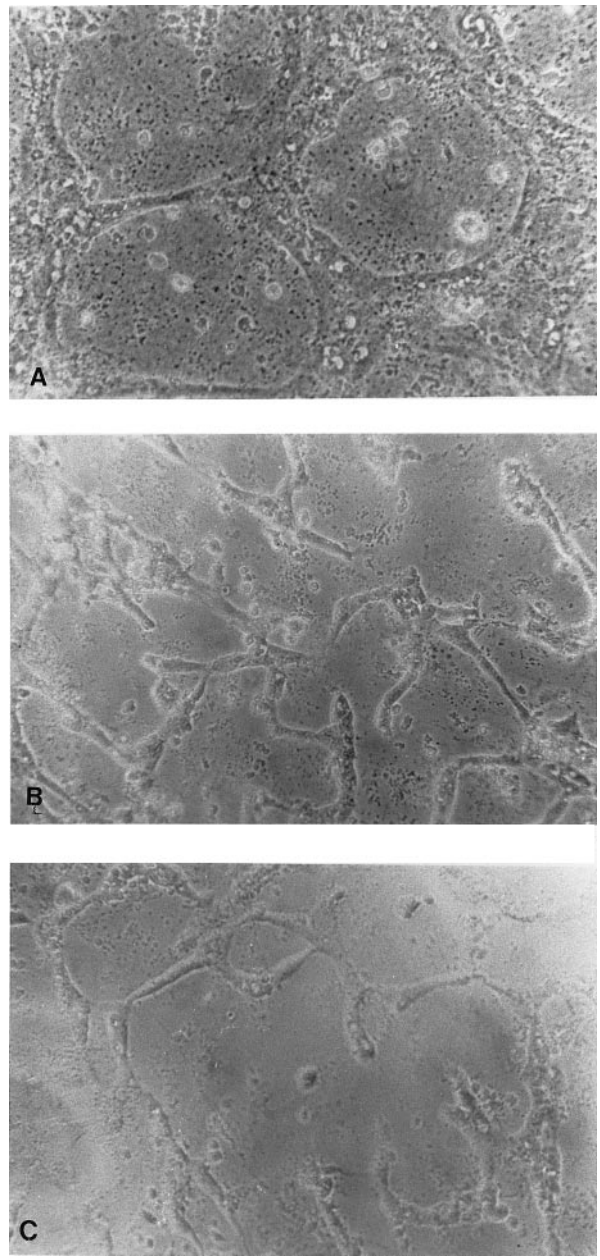

FIG. 1. Tube formation of HUVECs between Matrigels after 24 h at 37°C. (A) Control (normal); (B) HUVECs pretreated with GLA (50 μM) for 24 h; (C) HUVECs pretreated with antibody to VE-cadherin (2.5 μg/ml) for 24 h.

Dissociation of HUVEC colonies by GLA and VE-cadherin antibody. Figures 2A and 2C show photomicrographs of HUVECs exhibiting very tight colonies. Following cultured with VE-cadherin antibody (2.5 μg/

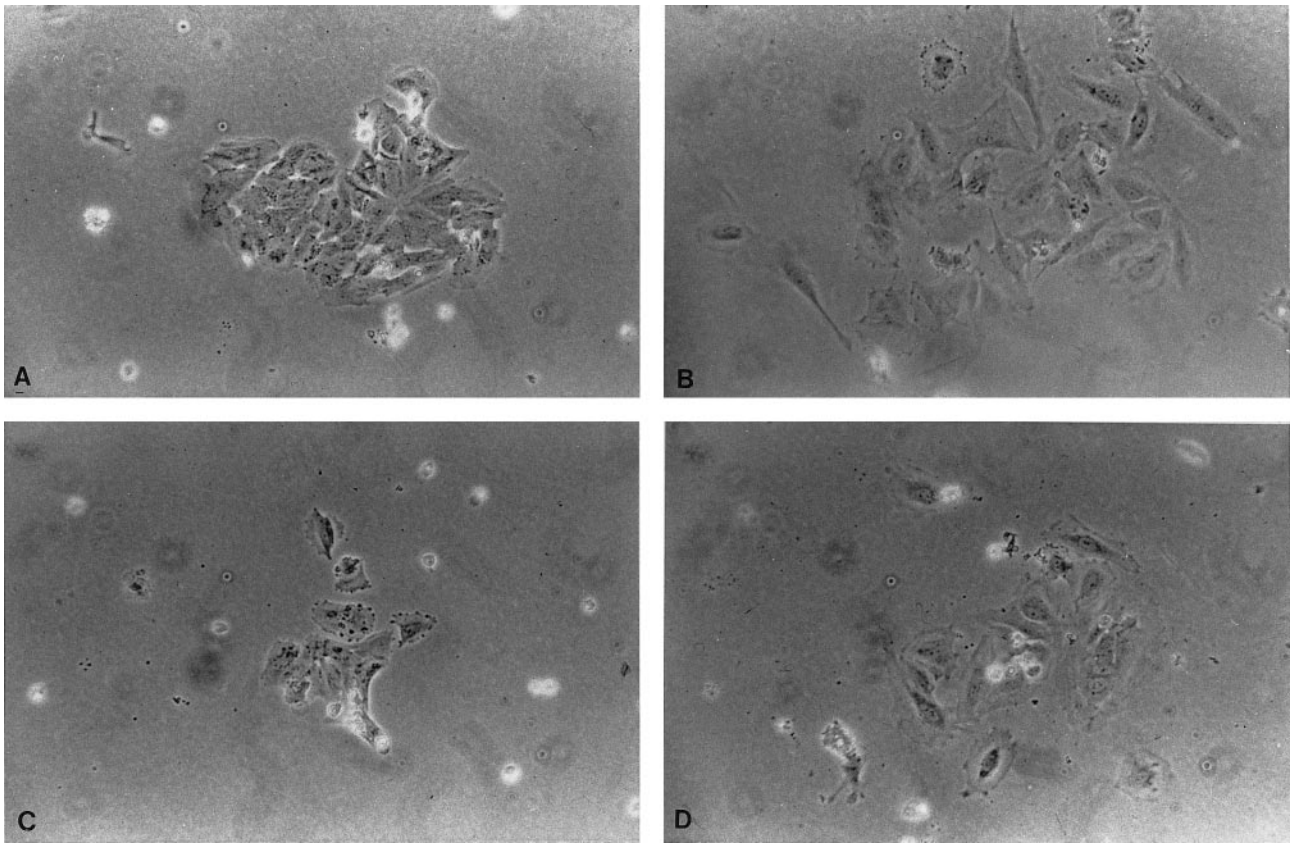

FIG. 2. Colony formation of HUVECs. HUVECs (A and C) were cultured to form colonies in complete medium as controls. Then these cells were treated with (B) GLA 50 μM or (D) antibody to VE-cadherin (2.5 μg/ml) for 24 h. Both GLA and antibody to VE-cadherin induced a dissociation of colonies.

ml) (Figure 2B) or GLA (50 μM) (Figure 2D) for 24 hr, the resultant cell colonies were observed to both expand in their area and loose the cell-to-cell contacts. The effect of GLA appeared to be slightly weaker than the antibody to VE-cadherin.

Decrease in immunofluorescent staining of VE-cadherin by GLA. Immunofluorescent labelling demonstrated that VE-cadherin was localised at regions of vascular endothelial cell-to-cell contact appearing as uninterrupted fluorescent intercellular lines in the confluent HUVEC monolayers (Figure 3A). Figure 3B shows that following GLA treatment (50 μM) for 24 hr, VE-cadherin fluorescent staining was seen to be markedly reduced from the vascular endothelial cell-to-cell contacts.

Inhibition of VE-cadherin and β-catenin expression in HUVECs by GLA. Our immunofluorescent result indicated that GLA induced a loss of VE-cadherin from vascular endothelial lateral junctions. To further examine whether this effect of GLA on VE-cadherin and β-catenin was the result of either an alteration in expression or relocation of these molecules to the cytoskeletal portion, we extracted both Triton X-100 soluble (TX-sol) and SDS soluble (Triton insoluble) (TX-insol) proteins. HUVECs were incubated with GLA at range of concentrations (0-50 μM) for 24 hr. Both TX-sol and -insol of HUVECs protein extractions were then separated on SDS-PAGE gels and immunoblotted with either VE-cadherin or β-catenin antibody. Figure 4 shows that the level of VE-cadherin gradually decreased in both TX-sol and insol fractions as the GLA concentration increased. β-catenin showed a similar pattern of reduction in both fractions, as seen with VE-cadherin (Figure 5).

DISCUSSION

This study shows that gamma linolenic acid, an n-6 polyunsaturated fatty acid, inhibited vascular endothelial cell tube formation, an effect that is in part attributed to the inhibition of VE-cadherin expression in vascular endothelial cells.

During angiogenesis, vascular endothelial cells form capillaries-a continuous endothelium lumen (13, 27-29). In recent years it has been identified that vascular endothelial adherens junctions, the formation of which is mediated VE-cadherin/catenins complex, play an im-

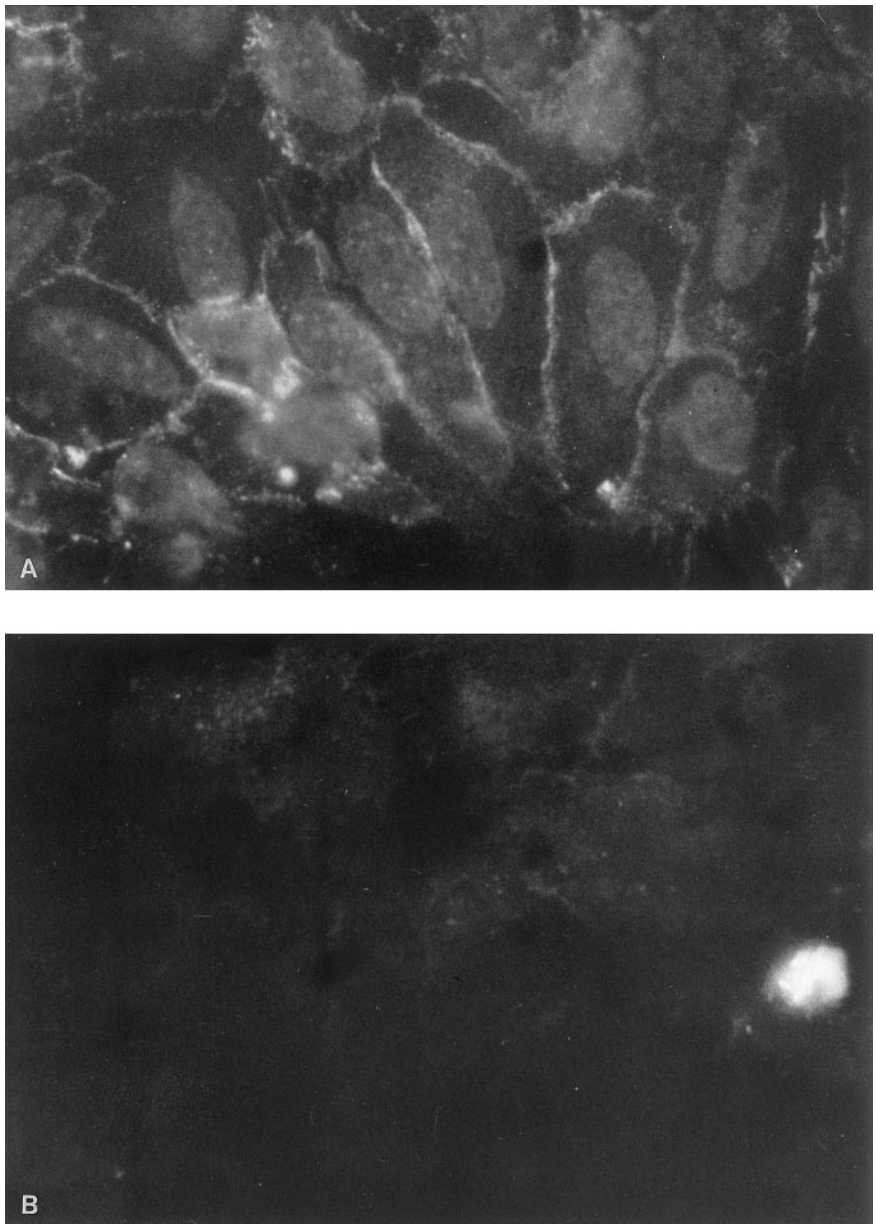

FIG. 3. Immunofluorescent staining of VE-cadherin. (A) Localization of VE-cadherin at cell-to-cell contacts of HUVECs. (B) Reduction of VE-cadherin staining after GLA treatment at 50 μM for 24 h.

portant role in capillary tube formation (30-33). Bach et al (34) have demonstrated that antibodies to VE-cadherin not only inhibit new capillary formation, but also induce disorganisation within the formed capillaries. These studies indicated that VE-cadherin is essential for the re-establishment and maintenance of vascular endothelial cell-to-cell contacts. In our current study, we also confirm that by neutralising VE-cadherin function using anti-VE-cad antibodies, HUVEC tube formation is inhibited and vascular endothelial cell-to-cell contacts is disorganised. The study further demonstrate the importance of VE-cadherin in the lumen formation from the endothelial cells.

We have recently reported that GLA inhibits angiogenic factor- and tumour-induced angiogenesis, which is partly due to its inhibitory effect on motility of vascular endothelial cells (9). In this study, we found that GLA not only inhibits HUVEC tube formation, but also loosen endothelial cell-to-cell contacts, indicative of weakened cell-cell adhesion mechanism. Based on the knowledge of the angiogenic process (13), this study set to further investigate the effects of GLA on VE-cadherin/catenin complex within endothelial cells. As reported previously (35, 36), GLA up-regulates cadherins at a time- and concentration-dependent manner in certain cancer cells. It is unexpected that in this

study our immunocytochemistry shows a decrease in VE-cadherin staining at interendothelial cell-to-cell contacts by GLA treatment. In consistent with the immunofluorescent results, Western blotting showed that levels of VE-cadherin and β-catenin were inhibited by GLA in concentration-dependent manner.

Although the exact mechanisms by which GLA mediates different expression of cadherins between cancer cells and normal endothelial cells is not known and needs to be further investigated, several studies (37-40) using GLA have shown that cancer cells and the normal counterpartners respond to GLA differently. For example, GLA is toxic to cancer cells but not to the normal cells. It has been shown that cancer cells have deficiency to convert GLA to down stream metabolites (41), which may be part of the mechanisms of the different responses to GLA in cancer and normal cells. This is the first report showing that the expression of cell-cell adhesion molecules of the cadherin family are differentially regulated by GLA in a cell type-dependent manner, i.e. VE-cadherin in normal cells decrease with GLA (as reported here) but E-cadherin in cancer cells increase (35, 36). Our study further demonstrated that the reduction of VE-cadherin and β-catenin is not the result of relocation into the cytoskeletal fraction, as both the Triton soluble and insoluble protein extractions revealed a similar reduction of both molecules.

In summary, this study shows that GLA inhibits angiogenesis by its inhibition on VE-cadherin/catenin complex expression of vascular endothelial cells. Together with previous results referring GLA inhibitory effect on endothelial motility, the present data suggest that GLA inhibition on tumour-induced angiogenesis was at least attributed to its inhibitory effects on endothelial cell motility and VE-cadherin/catenin complex expression.

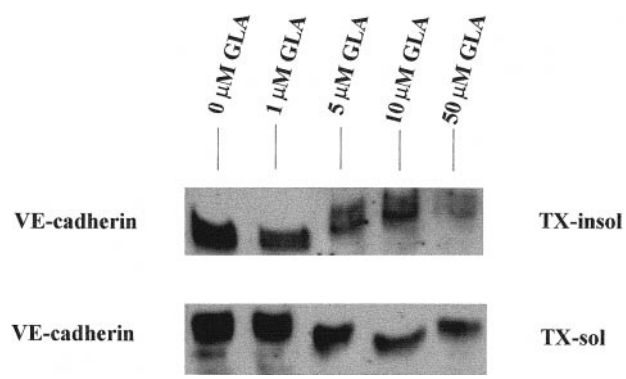

FIG. 4. Effect of GLA on the level of VE-cadherin in both Triton X100 soluble (TX-sol) and insoluble (SDS soluble) (TX-insol) fractions from the HUVECs. VE-cadherin levels were seen to decrease in both fractions following GLA treatment (0-50 μM) in a concentration-dependent manner.

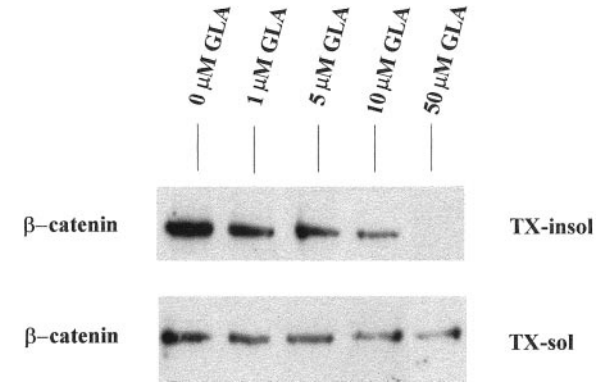

FIG. 5. Effect of GLA on the level of β-catenin in both Triton X100 soluble (TX-sol) and insoluble (SDS soluble) (TX-insol) fractions from the HUVECs. Treatment of HUVEC cells with GLA (0-50 μM) decreased β-catenin level in both fractions.

ACKNOWLEDGMENTS

The authors wish to thank Scotia Pharmaceuticals Ltd (UK) and the World Cancer Research Fund (WCRF) for supporting this study.

REFERENCES

1. Gardiner, N. S., and Duncan, J. R. (1991) *Prostagland. Leuk. Essent. Fatty* **42,** 149–153.
2. Rose, D. P., Connolly, J. M., and Liu, X. H. (1997) *Advances Exp. Med. Biol.* **422,** 47–55.
3. Pritchard, G. A., Jones, D. L., and Mansel, R. E. (1989) *Br. J. Surg.* **76,** 1069–1073.
4. Ramchurren, N., and Karmali, R. (1995) *Prostagland. Leuk. Essent. Fatty* **53,** 95–101.
5. Jiang, W. G., Hiscox, S., Puntis, M. C. A., Bryce, R. P., Horrobin, D. F., Mansel, R. E., and Hallett, M. B. (1996) *Int. J. Oncol.* **9,** 279–284.
6. Nicholson, M. L., Neoptolemos, J. P., Clayton, H. A., Talbot, I. C., and Bell P. R. F. (1990) *Carcinogenesis* **11,** 2191–2197.
7. Jiang, W. G., Bryce, R. P., and Horrobin, D. F. (1998) *Crit. Rev. Oncol. Hematology* **27,** 179–209.
8. Vartak, S., Robbins, M., E., C., and Spector, A. A. (1997) *Lipid* **32,** 283–292.
9. Jiang, W. G., Hiscox, S., Hallett, M. B., Scott, C., Horrobin, D. F., and Punits, M. C. A. (1995) *Br. J. Cancer* **71,** 744–752.
10. Begin, M. E., Ells, G., Das, U. N., and Horrobin, D. F. (1986) *J. Natl. Cancer Inst.* **77,** 1053–1062.
11. DeKock, M., Lottering, M. L., Grobler, C. J. S., Vijoen, T. C., LeRoux, M., and Seegers, J. C. (1996) *Prostagland. Leuk. Essent. Fatty* **55,** 403–411.
12. Cai, J., Jiang, W. G., and Mansel, R. E. (1999) *Prostagland. Leuk. Essent. Fatty* (in press).
13. Ausprunk, D. H., and Folkman, J. (1977) *Microvasc. Res.* **14,** 53.
14. Lampugnani, M. G., Resnati, M., Raiteri, M., Pigott, R., Pisancane, A., Huoen, G., Ruco, L. P., and Dejana, E (1992) *J. Cell Biol.* **118,** 1511–1522.
15. Kemler, R. (1993) *Trends Genet.* **9,** 317–321.
16. Dejana E. (1996) *J. Clin. Invest.* **98,** 1949–1953.
17. Gulino, D., Delachanal, E., Concord, E., Genoux, Y., Morand, B.,

Valiron, M. O., Sulpice, E., Scaife, R., Alemany, M., and Vernet, T. (1998) *J. Biol. Chem.* **273,** 29786-29793.

18. Navarro, P., Ruco, L., and Dejana, E. (1998) *J. Cell Biol.* **140,** 1475–1484.
19. Lampungnani, M. G., Corada, M., Caveda, L., Breviario, F., Ayalon, O., Geiger, B., and Dejana, E. (1995) *J. Cell Biol.* **129,** 203–217.
20. Navarro, P., Caveda, L., Breviario, F., Mandoteanu, I., Lampugnani, M. G., and Dejana, E. (1995) *J. Biol. Chem.* **270,** 309965–30972.
21. Ott, I., Fischer, E. G., Miyagi, Y., Mueller, B. M., and Ruf, W. (1998) *J. Cell Biol.* **140,** 1241–1253.
22. Ali, J., Liao, F., Martens, E., and Muller, W. A. (1997) *Microcirculation-London* **4,** 267–277.
23. Bach, T. L., Barsigian, C., Yaen, C. H., and Martinez, C. (1998) *J. Biol. Chem.* **273:** 30719–30728.
24. Vitolo, D., Paradiso, P., Uccini, S., Ruco, L. P., and Baroni, C. D. (1996) *Histopathology* **28,** 521–528.
25. Gimbrone, M. A., Cotroan, J. R. S., and Folkman, J. (1974) *J. Cell Biol.* **60,** 673–684.
26. Kanayasu, T., Morita, I., Nakao-Hayashi, J., Asuwa, N., Fuijsawa, C., Ishii, T., Ito, H., and Murota, S. I. (1991) *Glyconjugate J.* **14,** 577–584.
27. Hashimoto, H., Ishikawa, H., and Kusakabe, M. (1998) *Microvasc. Res.* **55,** 179–183.
28. Beck, L., and D Amore, P. A. (1997) *FASEB J.* **11,** 365.
29. Baish, J. W., and Jain, R. K. (1998) *Nature Med.* **4,** 984.
30. Matsuyoshi, N., Toda, K., Horiguchi, Y., Tanaka, T., Nakagawa, S., Tekeichi, M., and Imamura, S. (1997) *Proc. Associ. Am. Physi.* **109,** 362–371.
31. Nagashima, H., Okada, M., Hidai, C., Hosoda, S., Kasanuki, H., and Kawana, M. (1997) *Heart Vessels* **12,** 110–112.
32. Matsumura, T., Wolff, K., and Petzelbaue, P. (1997) *J. Immunol.* **158,** 3408–3416.
33. Kowalczyk, A. P., Navarro, P., Dejana, E., Bornslaeger, E. A., Green, K. J., Kopp, D. S., and Borgward, J. E. (1998) *J. Cell Sci.* **111,** 3045–3057.
34. Bach, T. L., Barsigin, C., Chalupowiz, D. G., Busler, D., Yaen, C. H., Grant, D., S., and Martinez, J. (1998) *Exp. Cell Res.* **238,** 324–334.
35. Jiang , W. G., Hiscox, S., Hallett, M. B., Horrobin, D. F., Mansel, R. E., and Puntis, M. C. A. (1995) *Cancer Res.* **55,** 5043–5048.
36. Jiang, W. G., Hiscox, S., Hallett, M. B., Scott, C., Horrobin, D. F., and Puntis, M. C. A. (1995) *Br. J. Cancer* **71,** 744–752.
37. Vartak, S., McCaw, R., Davis, C. S., Robbins, M. E. C., and Spector, A. A. (1998) *Br. J. Cancer* **77,** 1612–1620.
38. Tsai, W. S., Nagawa, H., and Muto, T. (1997) *Int. J. Cancer* **70,** 357–361.
39. Begin, M. E., Ells, G., and Horrobin, D. F. (1988) *J. Natl. Cancer Inst.* **80,** 188–184.
40. Begin, M. E., Das, U. N., and Ells, G. (1986) *Prog. Lipid. Res.* **25,** 573–576.
41. Hrelia, S., Bordoni, A., Biagi, P., Rossi, C. A., Bernardi, L., Horrobin, D. F., and Pession, A. (1996) *Biochem. Biophys. Res. Comm.* **225,** 441–447.