PHYTOTHERAPY RESEARCH, VOL. **12**, 37–40 (1998)

Andrographolide Inhibits the Tumour Necrosis Factor-α-induced Upregulation of ICAM-1 Expression and Endothelial-monocyte Adhesion

Solomon Habtemariam

Department of Physiology and Pharmacology, University of Strathclyde, Glasgow G1 1XW, UK

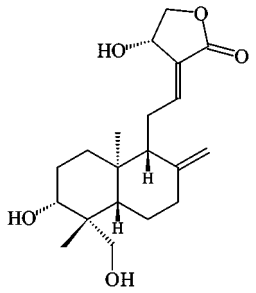
Andrographolide, a diterpene isolated from *Andrographis paniculata*, has been shown to have several biological activities including analgesic, antipyretic and antiinflammatory effects. Since the upregulation of adhesion molecules expression and endothelial-leucocytes adhesion are key steps in the development of inflammation, the present study examines whether andrographolide modulates these biological processes *in vitro*. Incubation of endothelial cells with non-toxic concentrations (0.16–16.7 μg/mL) of andrographolide attenuated the tumour necrosis factor-α (TNF)-induced intercellular adhesion molecule-1 (ICAM-1) expression. Similar concentration ranges of andrographolide also inhibited the TNF-induced endothelial-monocyte adhesion in a concentration-dependent manner. These effects of andrographolide may account for its reported *in vivo* antiinflammatory activity. © 1997 John Wiley & Sons, Ltd.

Phytother. Res. **12**, 37–40 (1998)

Keywords: andrographolide; *Andrographis paniculata*; TNF; ICAM-1; adhesion.

INTRODUCTION

Andrographolide, a diterpene lactone (see structure) isolated from *Andrographis paniculata* (Acanthaceae), has been shown to possess hepatoprotective activity against hepatotoxins including carbon tetrachloride (Handa and Sharma, 1990a; Kapil *et al.*, 1993), galactosamine and paracetamol (Handa and Sharma, 1990b; Visen *et al.*, 1993) and ethanol (Pramyothin *et al.*, 1994). Andrographolide has also been shown to have antidiarrhoeal (Gupta *et al.*, 1990), choleretic (Tripathi and Tripathi, 1991; Shukla *et al.*, 1992) and analgesic, antipyretic and antiulcerogenic effects (Madav *et al.*, 1995).



Andrographolide

Madav *et al.* (1996) have recently reported the *in vivo* antiinflammatory activity of andrographolide. In their report, andrographolide effectively reduced the carrageenin-, kaolon- and nystatin-induced paw oedema as well as adjuvant-induced arthritis in rats. Although a high dose (300 mg/kg) of andrographolide was reported to inhibit dye leakage in acetic acid-induced vascular permeability (Madav *et al.*, 1996), the mechanism whereby it inhibits inflammation has not yet been established. It is now well known that upregulation of adhesion molecules expression and/or increased leucocyte trafficking by inflammatory mediators are key steps in the development of various inflammatory diseases (Springer, 1990; Carlos and Harlan, 1994). Of the several inflammatory mediators known to date, tumour necrosis factor-α (TNF), is one of by far the most potent and characterized cytokines and has been shown to enhance endothelial-leucocyte adhesion through upregulation of expression of endothelial cell adhesion molecules (mainly ICAM-1, ELAM-1 and VCAM-1) (Nawroth and Stern, 1986; Beutler and Cerami, 1987; Sherry and Cerami, 1988; Springer, 1990). Since TNF also mediates the carrageenin- and other experimental agent-induced animal models of inflammation (Utsunomiya *et al.*, 1994; Romano *et al.*, 1997), it is selected in this study to test whether andrographolide inhibits the upregulation of adhesion molecules expression and endothelial adhesiveness to leucocytes.

MATERIALS AND METHODS

Cell lines. The human myelomonocytic cell line, U937, was purchased from the European Collection of Animal Cell Culture (Porton Down, Salisbury, UK) while the human endothelial cell line, EAhy 926, was obtained from Dr R. Plevin (Department of Physiology and Pharmacology, University of Strathclyde). Media (RPMI 1640 (Gibco; Life Technologies, Paisley, UK) for U937 and Dulbecco's Modified Eagle Medium (Gibco) for EAhy 926) were supplemented with 2 mM glutamine, 100 μ/mL penicillin, 100 μg/mL streptomycin, and 10% heat inactivated fetal bovine serum (FBS, Gibco). A HAT (Gibco) supplement was used for the EAhy 926 cells.

Endothelial-monocyte adhesion assay. For adhesion experiments, U937 cells were labelled with 1 μCi/mL

* Correspondence to: S. Habtemariam, Department of Physiology and Pharmacology, University of Strathclyde, Glasgow G1 1XW, UK.

CCC 0951–418X/98/010037–04 $17.50

Accepted 22 July 1997

[methyl-^{3}H]thymidine (Amersham International plc, Little Chalfont, UK) for 24 h. Monocyte integrins were then activated by incubating cells with 200 ng/mL phorbol 12-myristate 13-acetate (PMA; Sigma-Aldrich Company, Poole, Dorset, UK) for 1.5 h.

Confluent monolayers of EAhy 926 endothelial cells were established in 96 well plates. TNF (R & D Systems, Abingdon, Oxon, UK) was then added in the presence and absence of andrographolide (Sigma) and incubated for 18 h. At the end of incubation, endothelial cell monolayers were washed twice with the assay buffer (RPMI-1% FBS). 2×10^5 [^{3}H]-thymidine-labelled U937 cells in a 50 μL volume were then added to the wells and incubated for 45 min under static conditions at 37°C, 5% CO_2. The nonadherent U937 cells were removed by plate inversion followed by gentle centrifugation (250x**g**, 5 min). Cells were harvested using a Dynatech Automash 2000 and radioactivity counted using liquid scintillation spectrometry. Results (DPM values) of each treatment were expressed as the percentage of total (radioactivity associated with 2×10^5 U939 cells) added.

Measurement of ICAM-1 expression. Confluent monolayers of EAhy 926 cells were incubated for 18 h with TNF in the presence and absence of andrographolide (Sigma). At the end of incubation, cells were washed three times with Dulbecco's phosphate buffer saline (PBS; Gibco), fixed with 0.5% (in PBS) glutaraldehyde and non-specific adhesion was blocked by incubation with 2% bovine serum albumen (Sigma) for 1 h. Monoclonal mouse anti-human ICAM-1 (clone BBIG-Il; R & D Systems) was then added to the wells and incubated for 2 h at 37 °C. The wells were washed and peroxidase conjugated goat anti-mouse antiserum (1/1000; Sigma) was added for a further 2 h. Wells were then washed and incubated with *o*-phenylenediamine substrate (Sigma) for 30 min and the absorbance at 450 nm was read using a Dynatech MR5000 ELISA reader. Wells containing tissue culture medium and those treated only with the second antibody and substrate served as control.

Endothelial cell viability assays. The viability of endothelial cells following treatment with andrographolide was determined based on their ability to reduce MTT and tosynthesize DNA. For the DNA synthesis experiments, endothelial cells monolayers in 96 well plates were exposed for 18 h to different concentrations of andrographolide. The cells were then labelled with [methyl-^{3}H]thymidine (0.5 μCi/well; Amersham) during the last 6 h of incubation. After removing unincorporated thymidine by washing, cells were harvested and radioactivity counted using liquid scintillation spectrometry. The MTT reduction assay was performed as described previously (Habtemariam, 1995).

RESULTS AND DISCUSSION

Treatment of cultured human endothelial cells, including the EAhy 926 cells, with TNF enhances their adhesiveness to leucocytes (Gamble *et al.*, 1985; Luscinskas *et al.*, 1989; Brown *et al.*, 1993; BurkeGaffney and Hellewell, 1996b). As with the EAhy 926 endothelial cells, the human monocyte like cell line, U937 cells, is one of the few cell lines routinely used for endothelial-leucocyte adhesion experiments and its interaction with TNF-activated endothelial cells has been well characterized (Cavender *et al.*, 1991; Sung *et al.*, 1991; Ikewaki and Inoko, 1996). In the present study, exposure of the EAhy 926 endothelial cells for 18 h with 0.5 ng/mL of TNF resulted in a 15-fold increase (over the basal level) of the endothelial-U937 cell adhesion (Fig. 1). The addition of andrographolide to endothelial culture together with TNF resulted in a concentration-dependent reduction of the TNF-induced enhancement of endothelial-monocyte adhesion (Fig. 1). As a positive control, the effect of a monoclonal anti-human ICAM-1 antibody was also examined. Incubation of the TNF-activated (18 h) endothelial cells for 30 min with 1 μg/mL of this antibody significantly ($p<0.001$ versus control, unpaired *t*-test) reduced the endothelial-monocyte adhesion (Fig. 1). It appears from this result that the TNF-induced EAhy 926 endothelial-U937 cell adhesion is mediated predominantly through ICAM-1.

The involvement of ICAM-1 and other adhesion molecules in leucocyte adherence to and migration across endothelium has been well documented (Springer, 1990; Carlos and Harlan, 1994). Since TNF treated EAhy 926 cells has been shown to display a high level of cell surface ICAM-1 (Brown *et al.*, 1993; BurkeGaffney and Hellewell, 1996a, 1996b) and anti-ICAM-1 antibody inhibited endothelial-monocyte adhesion, the effect of andrographolide on the TNF-induced ICAM-1 expression was also studied. Exposure of the EAhy 926 endothelial cells for 18 h to 0.5 ng/mL of TNF increased the cell surface expression of ICAM-1 by 10 fold (Fig. 2). As shown in Fig. 2, this upregulation of ICAM-1 expression by TNF was attenuated by andrographolide in a concentration-dependent manner. The inhibition of cell adhesion and ICAM-1 expression by andrographolide is unlikely to be due to direct cytotoxicity as the ability of endothelial cells to reduce MTT and to synthesize DNA was not altered (Fig. 3) by all concentrations shown in Fig. 1 and 2. Andrographolide, however, exhibits structural similarity to many cytotoxic diterpenes (Habtemariam, 1995) and showed direct endothelial cytotoxicity at higher concentrations (Fig. 3). The results

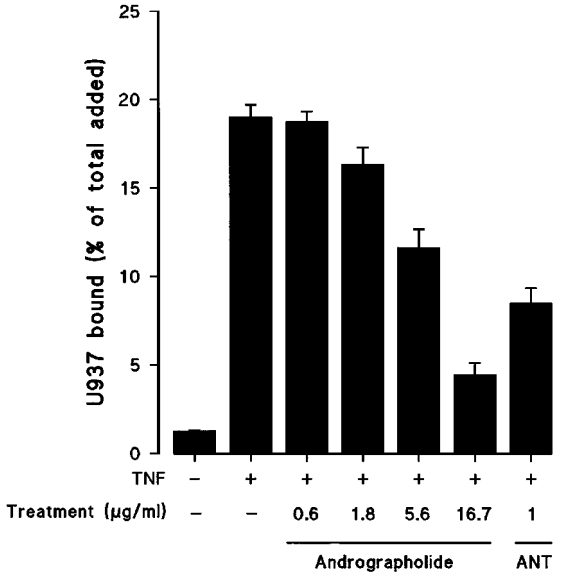


Figure 1. Effect of andrographolide on the TNF-induced endothelial adhesiveness to monocytes. Increasing concentrations of andrographolide were incubated with the EAhy 926 cells monolayers in the presence (0.5 ng/mL) or absence of TNF. U937 cell adhesion was measured as described in Materials and Methods. The effect of the monoclonal anti-human ICAM-1 antibody (ANT) added only 30 min before the adhesion assay is also shown. Data points are mean values ± SEM($n=4$).

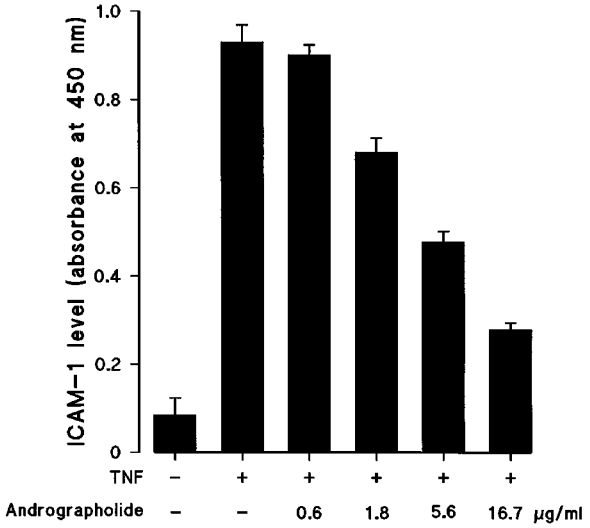


Figure 2. Effect of andrographolide on the TNF-induced endothelial cell surface expression of ICAM-1. Increasing concentrations of andrographolide were incubated with the EAhy 926 cells monolayers in the presence (0.5 ng/mL) or abence of TNF. ICAM-1 expression was measured as described in Materials and Methods. Data points are mean absorbance values ± SEM (n=6).

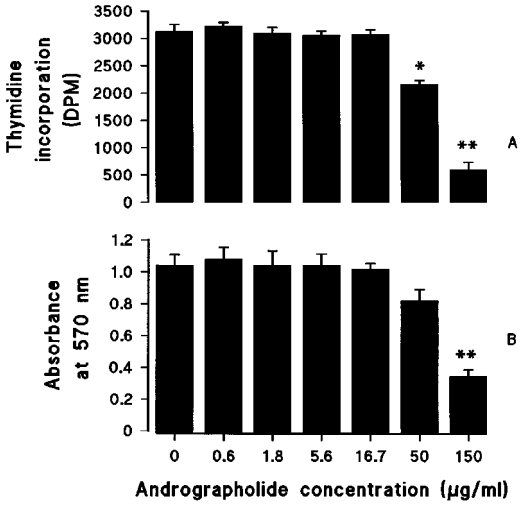


Figure 3. Effect of andrographolide on endothelial cell survival. Endothelial cells were exposed to various concentrations of andrographolide and cell viability was measured using the thymidine incorporation (A) or the MTT assay (B) as described in Materials and Methods. Results are mean values ± SEM (n=5). * $p<0.01$, ** $p<0.001$ versus control (unpaired t-test).

obtained from both the MTT and thymidine incorporation assays revealed that a 150 μg/mL concentration of andrographolide is overtly cytotoxic to endothelial cell monolayers. As evidenced by the thymidine incorporation studies (Fig. 3), andrographolide also showed significant inhibition of DNA synthesis at a concentration of 50 μg/mL.

In conclusion, the present *in vitro* study supports those by Madav *et al.* (1996) which reported the *in vivo* anti-inflammatory activity of andrographolide. At least part of its antiinflammatory activity is likely to be through inhibition of adhesion molecules expression and endothelial-leucocyte adhesion. The direct cytotoxicity of andrographolide to endothelial cells may indicate a possible dose limiting toxicity that will associate with the use of the compound as an antiinflammatory drug.

REFERENCES

Beutler, B., and Cerami, A. (1987). Cachectin: more than a tumor necrosis factor. *N. Engl. J. Med.* **316**, 379–385.

Brown, K. A., Vora, A., Biggerstaff, J. *et al.* (1993). Application of an immortalized human endothelial cell line to the leucocyte:endothelial assay. *J. Immunol. Meth.* **163**, 13–22.

BurkeGaffney, A., and Hellewell, P. G. (1996a). Tumour necrosis factor-α-induced ICAM-1 expression in human vascular endothelial and lung epithelial cells: Modulation by tyrosine kinase inhibitors. *Br. J. Pharmacol.* **119**, 1149–1158.

BurkeGaffney, A., and Hellewell, P. G. (1996b). Regulation of ICAM-1 by dexamethasone in a human vascular endothelial cell line EAHY926. *Am. J. Physiol.-Cell Physiol.* **270**, C552-C561.

Carlos, T. M., and Harlan, J. M. (1994). Leukocyte-endothelial adhesion molecules. *Blood* **84**, 2068–2101.

Cavender, D. E., Edelbaum, D., and Welkovich, L. (1991). Effects of inflammatory cytokines and phorbol esters on the adhesion of U937 cells, a human monocyte-like cell line, to endothelial cell monolayers and extracellular matrix proteins. *J. Leukocyte Biol.* **49**, 566–578.

Gamble, J. R., Harlan, J. M., Klebanoff, S. J., and Vadas, M. A. (1985). Stimulation of the adherence of neutrophils to umbilical vein endothelium by human recombinant tumor necrosis factor. *Proc. Natl Acad. Sci. USA* **82**, 8667–8671.

Gupta, S., Choudhry, M. A., Yadava, J. N. S., Srivastava, V., and Tanndon, J. S. (1990). Antidiarrhoeal activity of diterpenes of *Andrographis paniculata* (kal-Megh) against *Escherichia coli* enterotoxin in *in vivo* models. *Int. J. Crude Drug Res.* **28**, 273–283.

Habtemariam, S. (1995). Cytotoxicity of diterpenes from *Premna schimperi* and *Premna oligotricha. Planta Med.* **61**, 368–369.

Handa, S. S., and Sharma, A. (1990a). Hepatoprotective activity of andrographolide against carbon tetrachloride. *Indian J. Med. Res.-B.* **92**, 276–283.

Handa, S. S., and Sharma, A. (1990b). Hepatoprotective activity of andrographolide against galactosamine and paracetamol intoxication in rats. *Indian J. Med. Res-B* **92**, 284–292.

Ikewaki, N., and Inoko, H. (1996). Development and characterization of a novel monoclonal antibody (mNI-11) that induces cell adhesion of the LPS-stimulated human monocyte-like cell line U937. *J. Leukocyte Biol.* **59**, 697–708.

Kapil, A., Koul, I. B., Banerjee, S. K., and Gupta, B. D. (1993). Antihepatotoxic effects of major diterpenoid constituents of *Andrographis paniculata. Biochem. Pharmacol.* **46**, 182–185.

Luscinskas, F. W., Brock, A. F., Arnaout, M. A., and Gimbrone, M. A. (1989). Endothelial-leukocyte adhesion molecule-1 dependent and leukocyte (CD11/CD18)-dependent mechanisms contribute to polymorphonuclear leukocyte adhesion to cytokine-activated human vascular endothelium. *J. Immunol.* **142**, 2257–2263.

Madav, S., Tandan, S. K., Lal, J., and Tripathi, H. C. (1996). Anti-inflammatory activity of andrographolide. *Fitoterapia* **67**, 452–458.

Madav, S., Tripathi, H. C., Tandan, S. K., and Mishra, S. K. (1995). Analgesic, antipyretic and antiulcerogenic effects of andrographolide. *Indian J. Pharm. Sci.* **57**, 121–125.

Nawroth, P. P., and Stern, J. (1986). Modulation of endothelial cell homostatic properties by tumour necrosis factor. *J. Exp. Med.* **163**, 740–745.

Pramyothin, P., Udomuksorn, W., Poungshompoo, S., and Chaichantipyuth, C. (1994). Hepatoprotective effect of *Andrographis paniculata* and its constituent, andrographolide, on ethanol hepatotoxicity in rats. *Asia Pac. J. Pharmacol.* **9**, 73–78.

Romano, M., Faggioni, R., Sironi, M. *et al.* (1997). Carrageenan-induced acute inflammation in the mouse air pouch synovial model. Role of tumour necrosis factor. *Mediators*

Inflam. **6**, 32–38.
Saraswat, B., Visen, P. K. S., Patnaik, G. K., and Dhawan, B. N. (1995). Effect of andrographolide against galactosamine-induced hepatotoxicity. *Fitoterapia* **66**, 415–420.
Sherry, B., and Cerami, A. (1988). Cachectin/tumor necrosis factor exerts endocrine, paracrine and autocrine control of inflammatory responses. *J. Cell Biol.* **107**, 1269–1277.
Shukla, B., Visen, P. K. S., Patnaik, G. K., and Dhawan, B. N. (1992). Choleretic effect of andrographolide in rats and guinea pigs. *Planta Med.* **58**, 146–149.
Springer, T. A. (1990). Adhesion receptors of the immune system. *Nature* **346**, 425–434.
Sung, C. P., Strorer, B., Arleth, A., Stadel, J., and Feuerstein, G. (1991). Evidence for the involvement of carbohydrate moieties in the adhesion of U937 cells to tumor necrosis factor-α (TNFα)-stimulated vascular endothelium *in vitro. Agents Actions* **34**, 205–207.
Tripathi, G. S., and Tripathi, Y. B. (1991). Choleretic action of andrographolide obtained from *Andrographis paniculata* in rats. *Phytother. Res.* **5**, 176–178.
Visen, P. K. S., Shukla, N., Patnaik, G. K., and Dhawan, B. N. (1993). Andrographolide protects rat hepatocytes against paracetamol-induced damage. *J. Ethnopharmacol.* **40**, 131–136.
Utsunomiya, I., Nagai, S., and Ohishi, S., (1994). Differential effects of indomethacin and dexamethasone on cytokine production in carrageenin-induced rat pleurisy. *Eur. J. Pharmacol.* **252**, 213–218.