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Hamamelitannin from *Hamamelis virginiana* inhibits the tumour necrosis factor-α (TNF)-induced endothelial cell death in vitro

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Abstract

The tumour necrosis factor-α (TNF) inhibitory activity of hamamelitannin from *Hamamelis virginiana* was investigated by assessing the TNF-mediated EAhy926 endothelial cell death and adhesiveness to monocytes. Treatment of this ells by TNF (25 ng/ml) and actinomycin D (0.1 ng/ml) resulted in significant DNA fragmentation (34 ± 0.6, $n = 4$) and cytotoxicity ($97 \pm 4.5\%$, $n = 6$) following treatment for 8 and 24 h, respectively. One to 100 μM concentrations of hamamelitannin inhibited the TNF-mediated endothelial cell death and DNA fragmentation in a dose-dependant manner. One hundred % protection against TNF-induced DNA fragmentation and cytotoxicity was obtained for hamamelitannin concentrations higher than 10 μM. The protective effect of hamamelitannin was comparable with that of a related compound epigallocatechin gallate while gallic acid was a weak protective agent (<40% protection). EAhy926 endothelial cells upregulated (by 4- to 7-fold) the surface expression of intercellular adhesion molecule-1 (ICAM-1) and adhesiveness to monocytic U937 cells after treatment with TNF (0.5 ng/ml) for 6 or 24 h. Concentrations (1–100 μM) of hamamelitannin that inhibited the TNF-mediated cell death and DNA fragmentation, however, failed to inhibit the TNF-induced ICAM-1 expression and EAhy926 cell adhesiveness to U937 cells. Thus, hamamelitannin inhibits the TNF-mediated endothelial cell death without altering the TNF-induced upregulation of endothelial adhesiveness. The observed anti-TNF activity of hamamelitannin may explain the antihamorrhaegic use of *H. virginiana* in traditional medicine and its claimed use as a protective agent for UV radiation.

Keywords: *Hamamelis virginiana*; Hamamelitannin; TNF; Endothelial cell death

1. Introduction

Tumour necrosis factor-α (TNF) is a 17-kDa cytokine that affect almost every type of cells in the body. As its name suggests, TNF causes haemorrhagic necrosis of certain tumours in experimental animals. TNF is also known to mediate the wasting condition cachexia, associated with chronic diseases such as late stage cancer and AIDS (Argiles et al., 1997; Garcia-Martinez et al., 1997).

Activation of endothelial cells by TNF results in upregulation of expression of several adhesion molecules such as endothelial-leucocyte adhesion molecule-1, intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (Bevilacqua et al., 1994). The regulated expression of these adhesion molecules leads to leucocyte-endothelial adhesion and the subsequent leucocyte migration to extravascular tissues. TNF also induces endothelial cell surface expression of tissue factor and the secretion of plasminogen activator inhibitor there by promoting coagulation (Karsan, 1998 and references therein). There is now overwhelming evidence that suggest that TNF mediates various inflammatory and autoimmune diseases (Vassalli, 1992; Tracy and Cerami, 1994).

In addition to its proinflammatory and antitumour activities, TNF also signal programmed cell death (apoptosis) and cytotoxicity in non-tumour/normal cells including endothelial cells (Polunovsky et al., 1994; Habtemariam, 2000). Inhibitors of the TNF-induced cytotoxicity are thus of therapeutic value to suppress the elevated apoptic decay in TNF-dependant diseases. During the past few years, research in this laboratory has resulted in the identification of several natural TNF modulators (Habtemariam, 1997a,b, 1998a,b). In the

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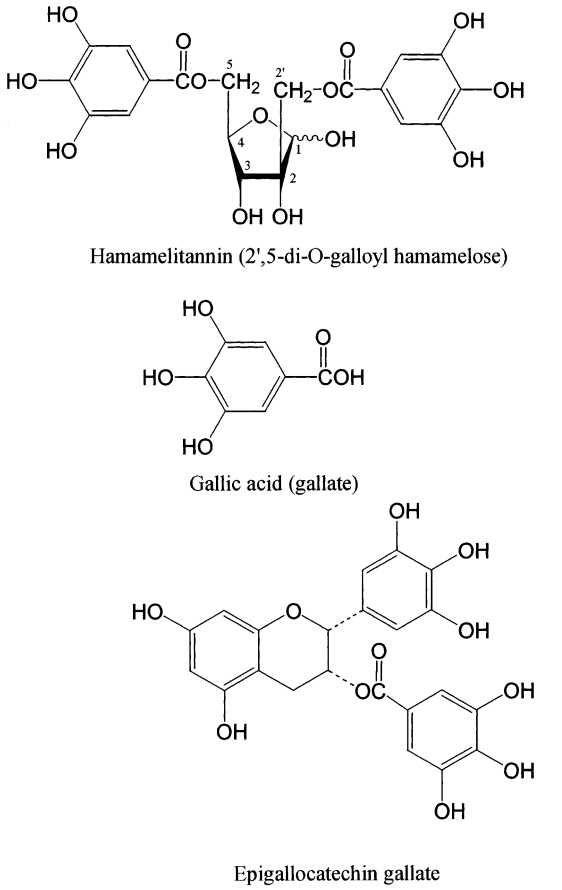

Fig. 1. Structures of hamamelitannin and its analogues, gallate and epigallocatechin gallate.

present report, the anti-TNF effects of hamamelitannin, the active principle of a popular medicinal plant, Witch Hazel (bark of *Hamamelis virginiana* L., Hamamelidaceae) has been investigated. In traditional medicine, Witch Hazel is used to treat several ailments, including internal and external haemorrhages (Newall et al., 1996).

Hamamelitannin (2'5-di-O-galloy hamamelose; Fig. 1) is the major constituent *of H. virginiana* and its molecular structure bears two gallate moieties and a sugar unit, hamamelose (Hartisch and Kolodziej, 1996 and references therein). Hamamelitannin has been shown to protect cells from ultra violet B (UVB) radiation-induced cell death; a process known to be mediated by reactive oxygen species (Masaki et al., 1995a). Hamamelitannin has also been demonstrated to display direct free radical (e.g. superoxide anion and hydroxyl radicals) scavenging activity (Masaki et al., 1994,1995b,c) and hence recommended as anti-ageing agent for the skin. So far, the effect on TNF-mediated cell death of neither hamamelitannin nor *H. virginiana* extracts has been reported.

2. Materials and methods

2.1. Extraction and isolation

One kg of powdered roots of *H. virginiana* (The Herbal Apothecary, Syston, Leicester, UK) was soaked with absolute ethanol (5 l) for a week. The resulting extract was taken and the plant residue further re-extracted twice with absolute ethanol. Extracts were combined, filtered and evaporated under a reduced pressure using rotary evaporator to yield 5 gm of the crude extract. The crude extract (4.5 g) was suspended in water (200 ml) and successively extracted with three times each (500 ml) of petroleum ether 40–60° (yield, 0.12 g), chloroform (yield, 0.13 g) and ethyl acetate (yield, 1. 5 g). The ethyl acetate fraction was further subjected to repetitive column chromatography over sephadix LH 20 (50 × 4 cm column, solvent—chloroform:-methanol, 1:1) to yield the pure major constituent, hamamelitannin (52 mg). The structure of this compound was established by means of ^{1}H and ^{13}C NMR and mass spectrometry which were all in perfect agreement with those published for hamamelitannin (Haberland and Kolodziej, 1994; Hartisch and Kolodziej, 1996 and references therein).

2.2. Cytoprotection assay

The established method of cytoprotection assay (Habtemariam, 1997a,b) was used to study the effect of compounds on TNF-mediated cytolysis. The human EAhy926 endothelial cell line (Habtemariam, 1998a) was maintained with Dulbeco's modified Eagle's medium (DMEM) supplemented with 10% foetal calf serum (FBS) and HAT (hypoxanthine, aminopterin and thymidine; Sigma–Aldrich Chemical Company, Poole, Dorset, UK). For the cytotoxicity assay, cells were harvested by trypsin-EDTA (Sigma) and established in 96 well plates (2×10^4 cells/well) by culturing overnight. Protective agents (hamamelitannin, gallate or epigallocatechin gallate) were added 30 min before TNF (Sigma; 25 ng/ml) and actinomycin D (Sigma; 0.1 ng/ml). In some experiments protective agents were added 30 or 60 min after TNF treatment. Plates were then incubated for 24 h at 37°C, 5% CO_2. At the end of incubation, cells were washed twice with DMEM and cell viability measured by using the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) microplate assay as described previously (Habtemariam, 1997a,b). Percent protection was calculated as: % protection $= (D - T) \times 100/(C - T)$, where D is the absorbance intensity of TNF-treated cells in the presence of drugs, T is the mean absorbance of TNF (alone) treated cells, and C is the mean absorbance from control cells (no-TNF).

2.3. Analysis of DNA fragmentation

A quantitative DNA fragmentation assay was used. EAhy926 endothelial cells (8×10^4 cells/well in 400 μl

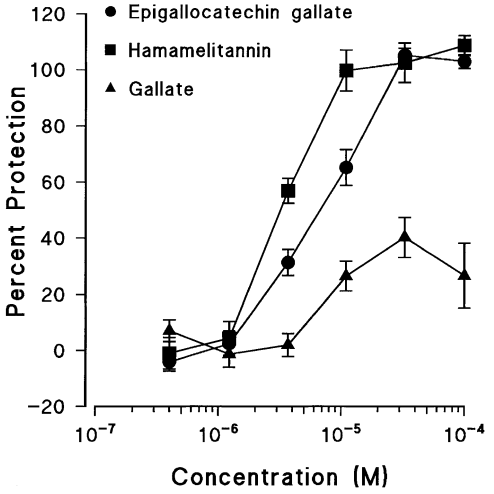

Fig. 2. Protection of EAhy926 endothelial cells against TNF-mediated cell death by hamamelitannin and analogues. Percent protection was calculated as described in the material and methods. Results are mean values ± SEM ($n = 6$).

volume) were established in 24-well plates and pulsed overnight with 0.5 μCi/ml [methyl-^{3}H]-thymidine (Amersham Life Sciences Ltd, Little Chalfont, Buckinghamshire, UK). After removing unincorporated thymidine by washing three times with DMEM, drugs, TNF and actinomycin D were added as described above. Plates were further incubated at 37°C, 5% CO_2 for 8 h. For analysis of released DNA fragments, cell supernatants were taken and centrifuged for 10 min at 13,000 *g*. The radioactivity of the pellet was quantified by using liquid scintillation spectrometry. In a separate experiment, DNA fragmentation was assessed by cytolysis. Briefly, equal volume of 2 × lysis buffer (20 mM Tris, pH 7.4 containing 4 mM EDTA and 4% triton X-100) was added to endothelial cell culture. After complete lysis (15 min at room temperature), cell lysates were taken and centrifuged at 1000 *g* for 10 min so that intact and fragmented DNA are separated. The radioactivity associated with the supernatant was then determined by scintillation spectrometry. The total ^{3}H-thymidine available for release was determined by using 20 mM Tris buffer, pH 7.4, containing 1% sodium dodecyl sulphate (SDS; Sigma). Percent fragmentation was calculated by using the equation: % fragmented DNA = (CPM in lysate supernatant or medium/CPM in SDS lysate) × 100.

2.4. Cell adhesion assay

The modified method of the previously described bioassay (Habtemariam 1998a,c) was used. Briefly endothelial monolayers were established in 96 well plates. TNF (0.5 ng/ml) was then added and plates incubated at 37°C, 5% CO_2 for either 6 or 24 h. For inhibition studies, drugs were added 30 min before TNF. Monocytic U937 cells (Habtemariam, 1998a), routinely maintained with RPMI 1640 medium (Sigma) supplemented with 10% FBS, were activated by PMA (200 ng/ml; Sigma) and labelled with a fluorescent indicator, BCECF-AM (2′,7′-bis(2-carboxyethyl)-5(6)-carboxyfluorescein acetoxymethyl ester; 5 μM; Sigma). Endothelial cell-monocyte adhesions were performed as described before (Habtemariam, 1998a,c) and quantified by measuring the fluorescence intensity using Fluoroskan Ascent FL fluorimeter (Labsystem).

2.5. Measurement of ICAM-1 expression

Confluent monolayers of endothelial cells were established in 96 well plates. Endothelial cells were then treated with TNF (0.5 ng/ml) in the presence or absence of drugs and incubated for either 6 or 24 h. ICAM-1 expression was quantified by ELISA, essentially as described previously (Habtemariam, 1998a).

3. Results and discussion

The human endothelial cells are resistant to TNF cytotoxicity as signalling by TNF leads to production of various apoptosis inhibitory proteins. These cytoprotective proteins include, manganous superoxide dismutase, the zinc finger protein A20, the Bcl-2 family member A1 and the recently characterised novel TNF inhibitory protein, TIP-B1 (Berleth et al., 2000 and references therein). Recent evidence also suggests that activation of NF-κB by TNF plays a central role in the transcription of genes involved in inhibition of the TNF-induced apoptic pathway (Zen et al., 1999). Not surprisingly then, TNF induces rapid endothelial cell death in vitro only when a protein syntheses inhibitor (e.g. actinomycin D or cyclohexamide) is present (Karsan, 1998 and references therein).

The time course and concentration-dependent apoptosis and cell death response of TNF and actinomycin D-stimulated EAhy926 endothelial cells have previously been established (Polunovsky et al., 1994). In the present study, exposure of EAhy926 cells to TNF (25 ng/ml) or actinomycin D (0.1 ng/ml) alone for 24 h resulted in a limited cytolysis, 15 ± 2 and 18 + 2.5% (n = 6) cell death respectively. Co-treatment of endothelial cells with TNF and actinomycin D has resulted in an accelerated cytotoxicity leading to 97 ± 4.5% ($n = 6$) cell death after incubation for 24 h.

The concentration-dependant cytoprotective effect of hamamelitannin on TNF treated EAhy926 endothelial cells is shown in Fig. 2. For comparison purposes, the effects of gallate and epigallocatechin gallate (for structures, see Fig. 1) are also shown. Apart form the highest concentration of gallate (100 μM) which produced 75 ± 4.8% ($n = 6$) cell death, all concentrations of drugs did not display direct cytotoxicity to endothelial cells. The order of potency in inhibiting the TNF-mediated cytotoxicity was:

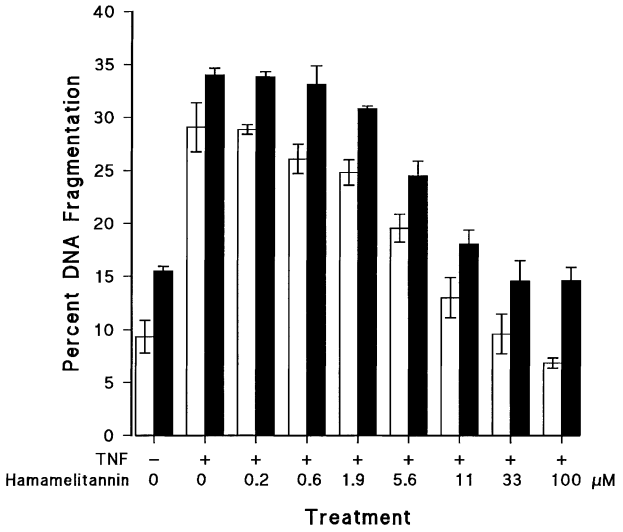

Fig. 3. Inhibition of TNF-mediated DNA fragmentation by hamamelitannin. Endothelial cells were treated with TNF and actinomycin D in the presence or absence of hamamelitannin as described in the Materials and methods Section. As assessed by DNA leakage to the medium (open bars) and DNA fragmentation quantified from cell lysates (solid bars), hamamelitannin inhibited the TNF-mediated DNA fragmentation in a concentration-dependant manner. Results are mean values ± SEM from four separate experiments.

hamamelitannin > epigallocatechin gallate ⩾ gallate. These data are in agreement with previous reports (Habtemariam, 1997a,b) which revealed that compounds with the catecholic functional moiety could inhibit the TNF-mediated apoptosis and cytotoxicity in the murine fibroblast, L929 cells. Thus hamamelitannin and epigallocatechin gallate, which bear two-gallate moieties in their structure, were more potent than gallate. In agreement with these data, partial protection of the TNF-mediated endothelial cell death has been noted for the gallate derivative, propyl-gallate (Kaiser et al., 1997).

As with many other cells, endothelial cell death induced by co-treatment with TNF and actinomycin D is preceded by DNA fragmentation (Polunovsky et al., 1994). In this study, a quantitative DNA fragmentation assay was used to study the effect of hamamelitannin on earlier events of the TNF-induced endothelial cell death. At 8 h following treatment by TNF, there appears to be no significant losses of cells (morphological observation) but as shown in Fig. 3, TNF causes extensive leakage of DNA to the medium. Analysis of DNA fragmentation by cytolysis is likely to provide a more accurate picture of DNA fragmentation as it can quantify fragmented DNA which are not released to the extracellular medium. Hence, more TNF-induced DNA fragmentation was quantified when a cell lysate was used than cell supernatant/culture medium (Fig. 3). In parallel with the cytotoxicity results, hamamelitannin inhibited the TNF-induced DNA fragmentation in a concentration-dependant manner (Fig. 3).

Establishing the mechanism of action of hamamelitannin is beyond the scope of this communication and hence awaits further research. The protective effect of hamamelitannin (10 μM) added 30 (94 ± 4.2%, $n = 6$) or 60 min (91 ± 5.5%, $n = 6$) after TNF was not significantly different (unpaired t-test, $P = 0.05$) from that added 30 min prior (98 ± 5.3%, $n = 6$) to TNF. As shown for catechol and flavonoid compounds (Habtemmariam, 1997a,b), this suggests a post receptor mechanism of action; i.e. an action mediated neither through direct interaction with TNF nor with TNF receptors. Gallates have been extensively studied and shown to display ubiquitous biological activities including those associated with enzyme inhibition and free radical scavenging. Direct free radical scavenging activity by its own right may account for the inhibitory effects of gallates as TNF signalling in endothelial cells has been shown to lead to the generation of free radicals (Wang et al., 1997; Dimmeler and Zeiher, 2000). The anti-TNF effects of antioxidants in many cell systems are, however, shown to be independent of direct free radical scavenging activity (Oddonnel et al., 1995). Other possible targets are lipoxygenase enzymes, which are shown to play a role in the TNF-mediated cytolysis in murine fibroblast cells (Oddonnel et al., 1995; Habtemariam, 1997a,b). Hence, a number of lipoxygenase enzyme inhibitors, including iron chelating catecholic compounds, have been shown to inhibit the TNF cytotoxicity in murine L929 cells (Habtemariam, 1997a,b). Interestingly, in vitro inhibition of 5-lipoxygenase activity by hamamelitannin has been reported (Hartisch et al., 1997). Signalling of endothelial cell death following activation of TNF receptor(s) further involves several docking proteins and caspase and kinase enzymes (Karsan, 1998) which could all be targets for hamamelitannin and analogues.

The other major effect of TNF on endothelial cells is the

upregulation of adhesion molecules and the subsequent enhancement of endothelial cell adhesiveness to leucocytes. The ICAM-1-dependent TNF-mediated EAhy926 endothelial cell adhesiveness to PMA-activated monocytic U937 cells has previously been established (Habtemariam, 1998a,b,c). In the present study, the concentration of TNF (0.5 ng/ml) that produced 75% of the maximum TNF response on endothelial adhesiveness (data not shown) was used. At this concentration, TNF increased the adhesiveness of EAhy926 endothelial cells by 4 ± 0.2 and 5 ± 0.4-fold ($n = 5$) following treatment for 6 and 24 h, respectively. Since differential regulation of adhesion molecules expression in endothelial cells has been noted following exposure of EAhy926 cells to TNF for 6 and 24 h (Burke-Gaffney and Hellwell, 1996), the effects of hamamelitannin was assessed at these two different time periods. Hamamelitannin added 30 min before TNF, failed to modify (data not significantly different at the level of $P = 0.05$ from the TNF alone treated group, unpaired *t*-test) the TNF effect on endothelial adhesiveness to activated U937 cells (data not shown). While the anti-ICAM-1 monoclonal antibody (R & D Systems, Oxon, UK; 10 μg/ml) added during the adhesion assay inhibited endothelial cell adhesion to U937 cells by $70 \pm 4\%$ ($n = 5$), hamamelitannin did not have any effect up to the concentration of 100 μM (data not shown).

The time-course and TNF concentration-dependent expression of the major adhesion molecule, ICAM-1 on EAhy926 endothelial cells were previously characterised (Habtemariam, 1998a). In the present study, TNF (0.5 ng/ml) enhanced ICAM-1 expression by 5.9 ± 0.2 and 6.7 ± 0.2-fold ($n = 6$) following exposure of endothelial cells for 6 and 24 h, respectively. As with the adhesion assay, the addition of hamamelitannin, did not modify (data not significantly different at the level of $P = 0.05$ vs. TNF control; unpaired *t*-test) the TNF-mediated ICAM-1 expression (data not shown). While some gallic acid esters (e.g. ethyl gallate) have been shown to inhibit the TNF-induced NF-κB translocation and the subsequent ICAM-1 expression on endothelial cells (Murase et al., 1999), neither gallic acid nor epigallocatechingallate altered the TNF-mediated ICAM-1 expression and EAhy929 cells adhesiveness to U937 cells (data not shown). It is worth to note that the failure of hamamelitannin and related compounds to inhibit the TNF-mediated cell–cell adhesion and ICAM-1 expression further rule out the possible non-specific action of the compounds towards the TNF-mediated cell death.

In summary, the present study demonstrated that hamamelitannin inhibits the cytotoxic effects of TNF without altering its effect on endothelial adhesiveness. Since TNF is widely known to cause haemorrhagic disorders, the anti-TNF activity displayed by hamamelitannin may explain some of the traditional uses of *H. virginiana,*in particular as antihaemorrhagic agent. It is also interesting to note that in addition to reactive oxygen species, TNF has been shown to play a role in UVB mediated cell damage (Duthie et al., 2000; Strickland et al., 1997; Zhuang et al., 1999). The anti-TNF activity of hamamelitannin in the present study thus further shed some light on the mechanism of its protective effect against UVB-induced cell damage (Masaki et al., 1995a). The data further supports the different signal transduction pathways involved in the TNF-induced apoptosis and adhesion molecules expression.

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