Oxysterols induce membrane procoagulant activity in monocytic THP-1 cells

Karine AUPEIX*, Florence TOTI†, Nathalie SATTA†, Pierre BISCHOFF* and Jean-Marie FREYSSINET†‡

*Laboratoire de Recherche en Immunologie, and †Groupe 'Thrombose et Autoimmunité' et Unité 143 INSERM, Institut d'Hématologie et d'Immunologie, Faculté de Médecine, Université Louis Pasteur, 4, rue Kirschleger, F-67085 Strasbourg, France

Oxidized cholesterol compounds or oxysterols are thought to be potent membrane-destabilizing agents. Anionic phospholipids, chiefly phosphatidylserine, have a procoagulant potential due to their ability to favour the membrane assembly of the characteristic clotting enzyme complexes including the tissue factor-dependent initiating complex. However, in resting cells, phosphatidylserine is sequestered in the inner leaflet of the plasma membrane. When THP-1 monocytic cells were cultured in the presence of 7β-hydroxycholesterol (7β-OH) or 25-hydroxycholesterol (25-OH), prothrombinase, which reflects anionic phospholipid exposure and tissue factor (TF) procoagulant activities, increased in a time- and dose-dependent manner. 7β-OH appeared 1.5- to 2-fold more potent than 25-OH. Interestingly, no effect of cholesterol itself could be detected on procoagulant activities. Nevertheless, no difference in TF activity could be detected between oxysterol-treated and control cells after disruption. TF antigen expression was the same in oxysterol-treated and control cells as shown by flow cytometry. In contrast, the use of labelled annexin V, a protein probe of anionic phospholipids, revealed an elevated number of cells with exposed phosphatidylserine. Because the latter also constitutes a signal for phagocyte recognition of apoptotic cells and fragments, and a proportion of cells displayed altered morphology with condensed chromatin and membrane blebs, analysis of DNA was performed and indicated apoptosis in oxysterol-treated cells. Hence, oxysterol-induced phosphatidylserine exposure and enhanced TF activity may result from apoptosis. These results suggest relationships between oxysterol and the amplification of coagulation reactions by monocytic cells resulting from induced phosphatidylserine exposure.

INTRODUCTION

7β-Hydroxycholesterol (7β-OH) and 25-hydroxycholesterol (25-OH) are different hydroxylation products of cholesterol and constitute major representatives of the oxysterol (OS) derivative compounds. 7β-OH is hydroxylated on the sterol nucleus and 25-OH on the C-17 acyl chain. Each compound has its own potential in two major mechanisms thought to mediate OS effects [1], i.e. inhibition of 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase, and interaction with cell plasma membrane. OS compounds are synthesized in various organs [2,3], but are also found at increased concentrations in the atheromatous plaque [4] and appear more atherogenic than cholesterol itself. Interestingly, recent studies demonstrate that toxicity of oxidized low-density lipoproteins (ox-LDLs) arises from OS oxygenated at carbon 7 of the cholesterol skeleton [5,6]. Mechanisms by which these agents are involved in the atherosclerotic process remain unclear, mostly due to the broad spectrum of activity of OS. The effects of OS on vascular cells include toxicity to endothelium [7], decrease of endothelial barrier function [8], and inhibition of gap junctional communication between smooth-muscle cells [9]. In T-cell lineages, 25-OH, but not 7β-OH, has been shown to induce apoptosis [10], a mode of cell death known to be associated with profound membrane remodelling indicated by exposure of normally sequestered anionic phospholipids such as phosphatidylserine [11].

Anionic phospholipids, chiefly phosphatidylserine, play a key role in the coagulation cascade. They act as a template for the assembly of the characteristic vitamin K-dependent enzyme complexes by accommodating enzyme, cofactor and substrate at the membrane surface [12]. Phosphatidylserine from the inner leaflet of the plasma membrane is also required for tissue factor (TF) optimal function [13,14]. TF is thought to be the major cellular factor implicated in the spontaneous initiation of blood coagulation. It is an integral membrane glycoprotein, related to the cytokine receptor family, which contains a serine residue in its cytoplasmic tail which can be phosphorylated by a protein kinase C-dependent mechanism. TF is constitutively synthesized by a variety of extravascular cells, or by fibroblasts or adipocytes from the adventitia, or more irregularly by smooth-muscle cells from the media. Endothelial cells and monocytes express TF only after stimulation [15]. TF expression by monocytes is inducible after exposure to various agents such as lipopolysaccharide (LPS), phorbol myristate acetate (PMA), complement, or cytokines such as tumour necrosis factor-α and interleukin-1β [16]. TF, in combination with factor VII and procoagulant phospholipids, initiates the coagulation cascade [12,14,15]. However, the sole presence of TF antigen is not sufficient to bring the process to completion. Hence, the lipid environment of TF antigen on the cell surface could play a critical role in the modulation of TF activity.

Because monocytes are the sole circulating cells able to synthesize TF and these cells isolated from atherosclerotic lesions have been found to possess augmented membrane-dependent procoagulant activity [17], it was decided to study the effect of two OS molecules on procoagulant activities expressed by the monocyte-like cell line THP-1. These cells express low basal levels of TF activity and can be stimulated by agents such as LPS which elicits *de novo* TF biosynthesis [18]. Moreover, tumour cell lines also bear higher amounts of exposed phosphatidylserine than their differentiated counterparts [19,20]. These properties allow analysis of the effect of OS on both the modification of TF

Abbreviations used: FCS, fetal calf serum; FITC, fluorescein isothiocyanate; annexin V^{FITC}, FITC-conjugated annexin V; HMG-CoA, 3-hydroxy-3-methylglutaryl-CoA; LPS, lipopolysaccharide; 7β-OH, 7β-hydroxycholesterol; 25-OH, 25-hydroxycholesterol; OS, oxysterol; ox-LDL, oxidized low-density lipoprotein; PMA, phorbol myristate acetate; TF, tissue factor.

‡ To whom correspondence should be addressed.

activity due to possible up-regulation of *de novo* protein biosynthesis and rearrangement of the lipid environment associated or not with apoptosis.

MATERIALS AND METHODS

Proteins and reagents

Human blood coagulation factors VII and X, and prothrombin, were purified from vitamin K-dependent protein concentrates kindly provided by Dr. H. P. Schwarz (Immuno AG, Vienna, Austria) [21]. Human α-thrombin [3000 National Institutes of Health (NIH) units/mg of protein] was prepared from purified prothrombin according to the method of Freyssinet et al. [22]. Factor Xa was obtained from purified factor X as also described in [22]. Factor V was purchased from Diagnostica Stago (Asnières, France). Human placenta annexin V (placental anticoagulant protein-I) was purified according to the method of Funakoshi et al. [23], characterized as published in [24] and conjugated with fluorescein isothiocyanate (FITC) (annexin V^{FITC}) following the procedure described by Dachary-Prigent et al. [25]. FITC was a product from Aldrich Chemical Co. (Milwaukee, WI, U.S.A.). Anti-(human TF) monoclonal antibody IgG1, HTF1-7B8, was a kind gift from Professor S.D. Carson (University of Nebraska at Omaha, NE, U.S.A.) [26]. Mouse IgG1 control immunoglobulin, human serum albumin, DMSO and LPS (*E. coli*, serotype 055:B5) were products from Sigma Chemical Co. (St. Louis, MO, U.S.A.). FITC-conjugated $F(ab')_2$ fragment of goat anti-(mouse immunoglobulins) was from DAKO A/S (Glostrup, Denmark). *N*-α-Benzyloxycarbonyl-D-arginyl-L-glycyl-L-arginine *p*-nitroanilide dihydrochloride (S-2765) and H-D-phenylalanyl-L-pipecolyl-L-arginine *p*-nitroanilide dihydrochloride (S-2238) were purchased from Chromogenix AB (Mölndal, Sweden). Cell culture reagents and media, i.e. Trypan Blue dye, RPMI 1640 medium with Phenol Red and with glutamax-I, RPMI 1640 medium without Phenol Red, non-essential amino acid solution, sodium pyruvate, penicillin, streptomycin and fetal calf serum (FCS) were from Life Technologies (European Division, Paisley, U.K.).

Cells

THP-1 cell line

THP-1 cell line was obtained from the American Type Culture Collection (reference A.T.C.C. TIB 202). Cells were cultured in RPMI 1640 medium with glutamax-I and Phenol Red, supplemented with 1% (v/v) non-essential amino acids, sodium pyruvate (1 mM), penicillin G (1 unit/ml) and 10% (v/v) heat-inactivated FCS, at 37 °C in a humidified 5% CO_2 atmosphere. Cell counts were determined using a haemocytometer.

Monocytes

Monocytes from healthy consenting donors (Strasbourg University Hospital, with approval of the Local Ethics Committee) were collected by leukapheresis and then purified by counterflow elutriation (up to 10^9 cells, with purity $> 90\%$) as described in [27]. Cells were maintained in RPMI 1640 medium with glutamax-I and Phenol Red, supplemented with 1% (v/v) non-essential amino acids, sodium pyruvate (1 mM), streptomycin (17.2 μM), penicillin G (1 unit/ml) and 10% (v/v) heat-inactivated FCS, at 37 °C in a humidified 5% CO_2 atmosphere, in Teflon culture bags (TechGen, Les Ulis, France) in order to minimize adherence.

Cell stimulation

Before each test, cells were seeded at 5×10^5 cells per ml (THP-1) or 2×10^6 cells per ml (monocytes) in the above culture medium or in RPMI without Phenol Red supplemented with 1% amino acids and sodium pyruvate but without serum when supernatant analysis was performed. Then OS previously solubilized in DMSO was added to cell suspensions at different final concentrations as indicated in the Results section. Incubation conditions were the same as those followed for culture. For each experiment a control was performed in which the DMSO concentration was the same as that contributed by OS addition.

TF and prothrombinase functional assays

After stimulation, the cell suspension was centrifuged at 600 ***g*** for 10 min at room temperature. The supernatant was collected when indicated. The cell pellets were washed once more and, after counting, cells were resuspended in RPMI medium without Phenol Red at a final concentration of 5×10^5 cells per ml. Cells were then allowed to incubate in this medium without FCS for 30 min at 37 °C under 5% CO_2 in order to avoid excessive background activity due to serum.

In each assay, concentrations of blood-clotting factors and cell numbers have been determined to ensure that TF or phosphatidylserine concentrations are the rate-limiting parameters of linear reactions of activation of factor X or prothrombin to factor Xa and thrombin respectively. In each case, less than 20% of total protein substrate was converted into its activated form. Measurements were performed in 96-well microtitration plates (Maxisorp, Nunc, Roskilde, Denmark) precoated with albumin at 76 μM (in 50 mM Tris buffer containing 120 mM NaCl, 2.7 mM KCl and 1 mM $CaCl_2$, adjusted to pH 7.5 with HCl). The final incubation volume was 150 μl.

TF was measured through its ability to promote the activation of factor X (150 nM) by factor VII(a) (5 nM) in the presence of 1 mM $CaCl_2$ and 5×10^5 cells/ml. The reaction was allowed to proceed for 15 min at 37 °C and was stopped by the addition of an excess of EDTA (5 mM final concentration). Chromogenic substrate for factor Xa, S-2765, was then added at a final concentration of 0.1 mM and the changes in absorbance at 405 nm versus time were immediately recorded using a microtitre plate reader equipped with kinetics software (Ceres 900 HDI; Bio-Tek, Winooski, VT, U.S.A.). The linear absorbance changes were converted into concentrations of factor Xa generated in the assay by reference to a standard curve constructed with known amounts of factor Xa. In controls, either factor VII or factor X, or both were omitted.

In order to assess specificity of the assay further, inhibition of TF was achieved by a specific monoclonal antibody added at a final concentration of 42.5 nM and allowed to incubate for 30 min at 4 °C before factor X and Ca^{2+} addition.

Phosphatidylserine exposure was detected through its ability to promote the activation of prothrombin (2 μM) by factor Xa (5 pM) in the presence of factor V(a) (50 pM) and $CaCl_2$ (1 mM), and with 5×10^5 cells/ml. The incubation procedure was exactly the same as that followed for TF. Chromogenic substrate for thrombin, S-2238, was used at 0.1 mM final concentration. Linear absorbance changes were converted into concentrations of generated thrombin by reference to a standard curve constructed with known amounts of thrombin. In controls either factor Xa or prothrombin, or both, were omitted. In order to avoid any effect from factor V possibly contributed by the cells [28], the concentration of exogenous factor V was chosen to be 10-fold that of factor Xa.

Inhibition assays of TF or prothrombinase activities by annexin V were carried out by adding this phospholipid an-

tagonist, at a final concentration indicated in the Results section, to the respective media just before coagulation factors.

Flow cytometry analysis

Dilution or suspension buffer for flow cytometry experiments was Hanks' balanced salt solution. Primary antibodies were added to suspensions at a final ratio of 42.5 pmol per 5×10^5 cells for anti-TF monoclonal antibody and control IgG1. Incubations were allowed to proceed for 30 min at 4 °C. Antigen labelling was achieved following another 15 min incubation at 4 °C with conjugated $F(ab')_2$ fragments of goat anti-(mouse immunoglobulin) at a final proportion of 95 pmol per 5×10^5 cells.

Phosphatidylserine probing was achieved using annexin V^{FITC} added at a final concentration of 140 nM. Incubation at room temperature was allowed to proceed for 10 min before data acquisition.

Samples were analysed using a Becton Dickinson (San Jose, CA, U.S.A.) FACScan flow cytometer. The sheath fluid was Isoton II balanced electrolyte solution (Coulter, Krefeld, Germany). Suspensions to be analysed contained 1×10^6 cells/ml. Data acquisition and analysis were carried out with Lysis II software. The light scatter were set at linear gain and fluorescence channels at logarithmic gain. The forward light scatter setting was E00, with a threshold of 16. A total of 10000 events were analysed for each sample.

Extranuclear DNA analysis

After counting, a total of 5×10^6 control and stimulated cells were removed. Cells were centrifuged at 200 ***g*** for 10 min and washed once with RPMI medium. The cells were lysed with 0.2 ml of cold hypotonic lysis buffer (10 mM Tris/HCl, 10 mM EDTA and 0.2% Triton X-100). The lysate was centrifuged at 13000 ***g*** for 10 min. The supernatant was separated from the pellet of cell nuclei and then extracted with an equal volume of phenol/chloroform (1:1, v/v) and once more with an equal volume of chloroform alone. The DNA in the aqueous phase was precipitated overnight at −20 °C with ammonium acetate and isopropanol. The DNA pellet was dissolved in TE buffer (10 mM Tris, pH 7.5, 1 mM EDTA) containing RNase A type I (50 μg/ml). The samples were analysed by electrophoresis at 10 V/cm in a 1.8% (w/v) agarose gel containing ethidium bromide (0.1 mg/ml).

RESULTS

Effect of OS molecules on the exposure of procoagulant phospholipids

When prothrombinase activity was measured in THP-1 cells treated for 24 h with OS at various final concentrations, a significant increase was observed (Figure 1a). This was more pronounced with 7β-OH than 25-OH. Significant increase of prothrombinase activity was observed with 7β-OH and reached a maximum at about 30 μM. When 25-OH was used, prothrombinase activity enhancement was also dose-dependent but the maximal amplitude was much lower than that obtained with 7β-OH. In contrast, addition of 20 μM cholesterol for 24 h to THP-1 cells was without effect on prothrombinase activity (results not shown). In order to better understand the mechanism by which OS molecules act on THP-1, the time course of prothrombinase activity was also analysed (Figure 1b). It increased significantly between 12 and 24 h after addition of 20 μM 7β-OH. With 25-OH, the increase in prothrombinase activity was slower and did not reach a plateau at 36 h. Flow

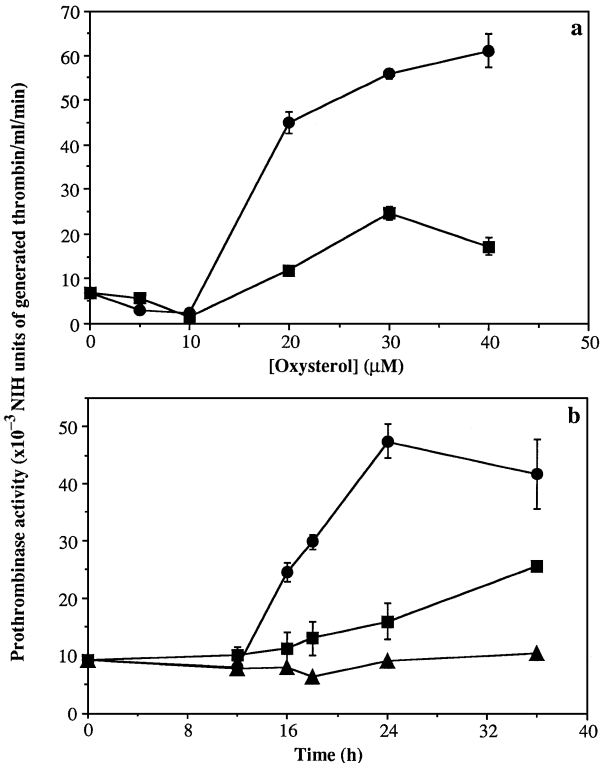

Figure 1 Prothrombinase activity in THP-1 cells treated with 7β-OH (●) or 25-OH (■)

Cells were treated (**a**) for 24 h with different OS concentrations and identical final DMSO concentrations adjusted to 0.4% (v/v), or (**b**) with either 20 μM OS or control [0.4% (v/v) DMSO; ▲] for variable time periods. Results represent means ± S.D. of five determinations performed with 5×10^5 cells/ml.

cytometry analysis was also performed. Cells were incubated as described in the Materials and methods section with annexin V^{FITC}, a protein which binds aminophospholipids with a high affinity [23]. A significant increase in the binding of annexin V was observed after treatment with OS (Figure 2). Table 1 shows the variation of the mean fluorescence intensity recorded during the above experiments and confirms that OS-treated cells exposed more anionic phospholipids than control cells and that the binding of annexin V^{FITC} increased with time and OS concentration.

Effect of annexin V on prothrombinase activity

In order to confirm the exposure of phosphatidylserine detected by the prothrombinase assay, annexin V was used as an inhibitor of its catalytic potential, as described in the Materials and methods section. Figure 3 shows the effect of annexin V on 7β-OH-induced prothrombinase activity in THP-1 cells. Prothrombinase activity progressively decreased as the annexin V concentration was increased. Basal THP-1 prothrombinase activity was totally inhibited at 10 nM annexin V whereas approx. 100 nM of annexin V was required to elicit 100% inhibition of OS-induced prothrombinase activity.

Effect of OS molecules on TF activity in THP-1 cells

THP-1 cells exposed to various OS concentrations presented a significant increase in TF activity at 20 μM of 7β-OH (Figure

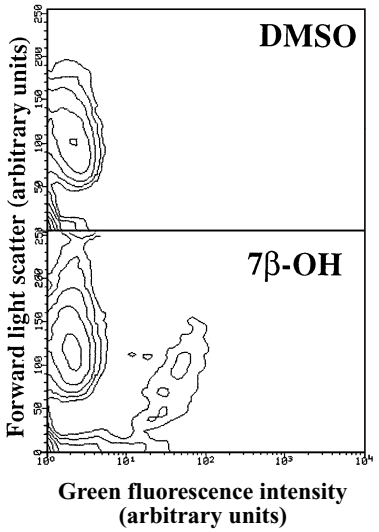

Figure 2 Flow cytometry analysis of THP-1 cells treated with 0.4% DMSO or 20 μM 7β-OH for 24 h and labelled with annexin V^{FITC} as described in the Materials and methods section

Cell size is related to forward light scatter.

Table 1 Variation of the binding of annexin V^{FITC} to THP-1 cells after OS treatment

Control cells presented a mean fluorescence intensity of 20 arbitrary units of fluorescence. Results are from a single experiment representative of three performed under the same conditions where 1×10^4 events were analysed, S.D. values did not exceed 10%.

		Mean fluorescence (% of control)	
OS concentration (μM)	Incubation time (h)	7β-OH	25-OH
0	24	100	100
5	24	199	75
10	24	183	132
20	24	296	147
30	24	576	121
40	24	566	163
20	0	100	100
20	12	117	128
20	16	170	128
20	20	214	121
20	24	238	140
20	36	366	241

4a). After 24 h exposure to 20 μM 7β-OH, TF activity was enhanced by up to approx. 3-fold. With 25-OH, the increase of TF activity was less marked. As observed for prothrombinase, TF activity of OS-treated cells also increased with time (Figure 4b). Again, cholesterol had no effect. In order to confirm that the activity observed was indeed due to TF, a monoclonal antibody raised against TF was used. When cells were preincubated with 42.5 nM of antibody for 30 min at 4 °C before assay, no TF activity could be detected (results not shown). It has also been verified that factor VII and factor X are both required to reveal TF activity, and that 7β-OH and 25-OH do not interfere in the TF assay using crude TF preparation (thromboplastin) as a positive control (results not shown).

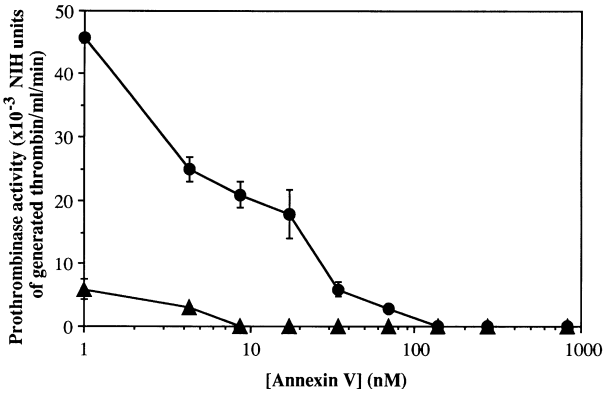

Figure 3 Effect of variable concentrations of annexin V on prothrombinase activity expressed by intact THP-1 cells

Cells were treated with 0.4% (v/v) DMSO (▲) or 20 μM 7β-OH (●) for 24 h. Results represent means ± S.D. of five determinations performed with 5×10^5 cells/ml.

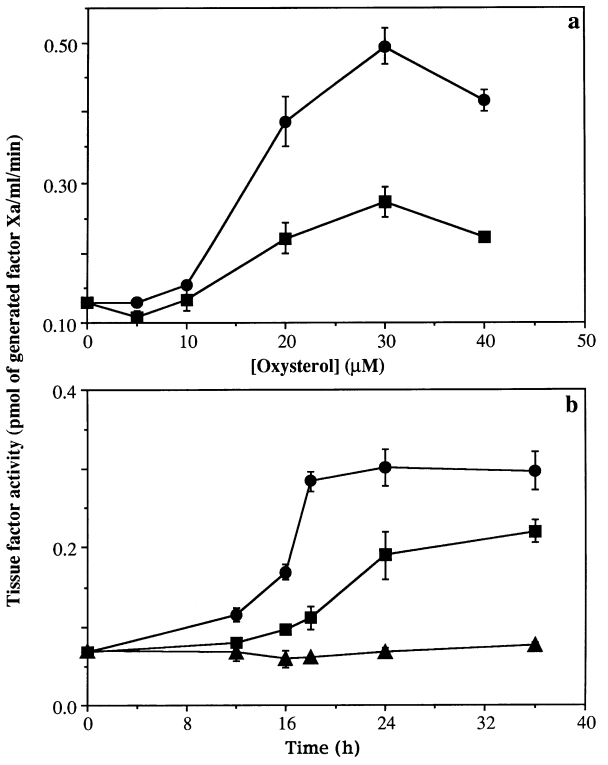

Figure 4 TF activity in THP-1 cells treated with 7β-OH (●) or 25-OH (■)

Cells were treated (**a**) for 24 h with different OS concentrations at identical final DMSO concentrations adjusted to 0.4% (v/v), or (**b**) with either 20 μM OS or under control conditions [0.4% (v/v) DMSO; ▲] for variable time periods. Results represent means ± S.D. of three determinations performed with 5×10^5 cells/ml.

Comparison of the effect of OS molecules versus LPS on TF activity

The incubation period required for maximum TF activity is different for OS or LPS treatment. Stimulation by LPS was maximal after 6 to 8 h, in agreement with other reports [29], whereas 24 h was needed with OS (Table 2). The extent of

Table 2 Comparison of TF activity of LPS- and OS-treated THP-1 cells

LPS was added at a final concentration of 10 μg/ml and 7β-OH at 20 μM. Control cells presented a TF activity of 0.13 pmol of generated factor Xa/ml per min. Results are mean ± S.D. of three determinations.

Cell treatment	TF activity (% of control)	
	6 h	24 h
DMSO	100 ± 20	100 ± 2
DMSO + LPS	289 ± 8	200 ± 24
7β-OH	102 ± 2	497 ± 19
7β-OH + LPS	256 ± 20	681 ± 6
25-OH	84 ± 15	377 ± 24
25-OH + LPS	219 ± 37	555 ± 34

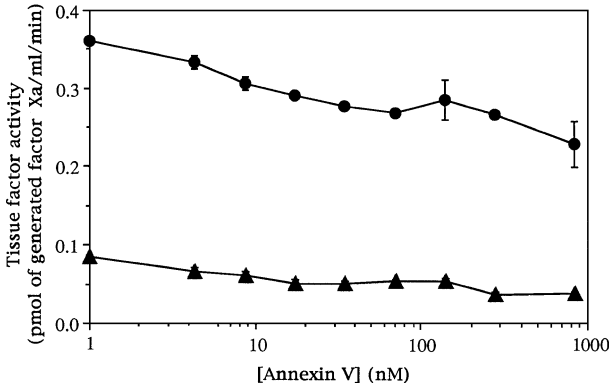

Figure 5 Effect of annexin V at various concentrations on TF activity expressed by intact THP-1 cells treated for 24 h with 0.4% (v/v) DMSO (▲) or 20 μM 7β-OH (●)

Results represent means ± S.D. of three determinations performed with 5×10^5 cells/ml.

enhancement of TF activity in intact cells was 2.5 times the basal level after 6 h of culture with 10 μg/ml LPS. Co-incubation of LPS in the presence of either 7β-OH or 25-OH for 6 h did not change anything. After 24 h, an additive effect between LPS and OS was observed.

Effect of annexin V on TF activity in the presence of OS

In order to assess the importance of phospholipids on TF activity, annexin V was used at various concentrations in the assay as described in the Materials and methods section. The results are reported in Figure 5. In contrast to what was observed for prothrombinase activity (Figure 3), TF activity was only inhibited by about 30% at 1 μM annexin V.

Comparison of TF activity between normal and disrupted cells and lack of TF expression by monocytes following OS treatment

Since THP-1 cells seem to express TF constitutively at a basal level it appeared important to determine whether OS would be able to promote exposure of TF from an intracellular pool. When TF activity of lysed control cells was compared with that of lysed OS-treated cells it remained mostly unchanged whatever the incubation conditions with OS (results not shown). When a lack of TF activity with respect to total activity after lysis of OS-treated cells was noticed, the difference could be recovered in the cell supernatant. No difference of TF antigen level could be detected by flow cytometry between untreated and 7β-OH-treated THP-1 cells. Finally no TF activity appeared in human monocytes after OS treatment. Such cells did not express significant basal TF activity.

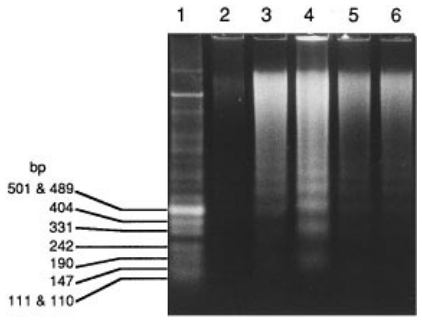

Figure 6 Agarose gel analysis of extranuclear DNA from THP-1 cells

Lane 1, molecular-mass markers; lane 2, control THP-1 cells treated with 0.4% (v/v) DMSO; lane 3, THP-1 cells treated with 20 μM 7β-OH; lane 4, THP-1 cells treated with 30 μM 7β-OH; lane 5, THP-1 cells treated with 20 μM 25-OH; lane 6, THP-1 cells treated with 30 μM 25-OH. Each treatment was for 24 h.

DNA analysis

As 7β-OH-treated cells exhibited an important morphological change with blebbing, and because it has been described that cells dying by apoptosis express high levels of phosphatidylserine it was decided to examine apoptosis by analysis of the extranuclear DNA. As shown in Figure 6, DNA extracted from OS-treated cells exhibited the usual ladder pattern of apoptotic DNA, reflecting the regular fragmentation in multimers of approx. 200 bp. Apoptosis was also more pronounced, as revealed by the DNA ladder intensities, in 7β-OH-treated THP-1 cells than in cells treated with 25-OH at equivalent concentrations.

DISCUSSION

From the above results it is clear that OS molecules are not 'neutral' with regard to membrane-dependent procoagulant reactions. Phosphatidylserine exposure has been described as a key step in the blood-coagulation cascade, and particularly required for optimal TF activity [12–14] by allowing the assembly of the characteristic enzyme complexes [12]. 7β-OH, and to a lesser extent 25-OH, were observed to enhance anionic phospholipid exposure by THP-1 cells in a dose- and time-dependent manner. Phosphatidylserine exposure was assessed by two different methods: prothrombinase activity, which reflects functional activity, and annexin V, which binds this phospholipid in the presence of Ca^{2+}. Hence, the addition of one hydroxy group on a cholesterol molecule seems sufficient for the promotion of the enhancement of phospholipid-dependent procoagulant activities since cholesterol was without effect in our system.

When the TF assay was performed in parallel with prothrombinase, TF activity was found to behave in a similar manner. TF activity also increased with time and concentration in the presence of OS. Again, 7β-OH was more efficient than 25-OH. The variability of the amplitude of the increase of both activities observed in independent experiments could be due to phase differences in the cell cycle. Such a variability has already

been reported after OS treatment [8]. TF activity in THP-1 is unaffected by cholesterol whereas this agent has been previously reported to increase TF expression in monocytes [30]. However, in the latter study, TF activity was measured in disrupted cells which were monocytes and not THP-1 cells. The increase of TF activity due to OS treatment is significant since 7β-OH can apparently be 2 to 3 times more effective than LPS in the enhancement of TF activity expressed by THP-1 cells (Table 2). However, in contrast to LPS, OS was unable to induce TF expression by monocytes and did not induce *de novo* protein biosynthesis as suggested by flow cytometric analysis and similar TF activities recovered in normal and disrupted OS-treated THP-1 cells. The latter feature was confirmed by full recovery of TF activity distributed between OS-stimulated THP-1 cells and corresponding supernatants with respect to disrupted non-stimulated cells. TF activity in supernatants could be explained by membrane shedding of vesicles bearing TF as already observed with tumour cell lines [31,32] or LPS-stimulated monocytes [33]. Hence, it seems that OS-induced TF activity only results from OS-induced phosphatidylserine exposure. Annexin V could abolish only 30 % of OS-induced TF activity in intact cells at 1 μM, whereas prothrombinase activity was fully inhibited at less than 100 nM. Other investigators reported similar observations where 270 nM annexin V was without effect on TF activity induced in an umbilical vein segment [34]. In another study [35], it was observed that 16 μM annexin V could only inhibit 80 % of TF activity in PMA-stimulated endothelial cells. On the other hand, both groups found that much lower concentrations of annexin V were able to completely inhibit the activity of TF reconstituted into liposomes containing phosphatidylserine. This suggests different conformations or accessibilities between reconstituted TF and cellular TF, or restricted accessibility of annexin V to the close TF phospholipid environment in the cell membrane.

The exact mechanisms by which OS molecules induce phospholipid expression remain unclear. They are known to interfere in cholesterol uptake and block cholesterol biosynthesis by inhibiting HMG-CoA reductase. Such an inhibition involves an OS-binding protein and depends on the nature of OS, which determines the affinity for this OS-binding protein [36]. Variations of cellular cholesterol could affect membrane stability and phospholipid distribution. Nevertheless, it does not seem that inhibition of cholesterol biosynthesis could be involved since 25-OH is more effective than 7β-OH in inhibiting HMG-CoA reductase [1], whereas it is less potent than 7β-OH in the enhancement of anionic phospholipid exposure, at least in THP-1 cells. Furthermore, OS-induced procoagulant activities are similar in the presence or absence of serum which provides cholesterol in large excess (results not shown). Because of their cholesterol-like structure, OS molecules can insert into membranes and cause, by themselves, pertubation in lipid distribution and fluidity [37]. In addition, a recent study reports that OS interacts preferentially with inner phospholipids and inhibits their methylation, whereas cholesterol preferentially interacts with the outer phospholipids [38]. OS also interferes in ion transport. 25-OH has been reported to favour calcium influx [39,40]. After rapid elevation of cytosolic calcium, TF activity enhancement was almost completely due to exposure of phosphatidylserine in the outer leaflet of the plasma membrane in fibroblasts [13]. Calcium interferes in the transporter function of aminophospholipid translocase, a Mg^{2+}-ATPase involved in the sequestration of aminophospholipids in the inner leaflet of the plasma membrane of most resting cells [41].

Apoptosis is known to be associated with enhanced phosphatidylserine exposure [11] which constitutes one of the major signals for recognition, by the cells of the reticulo-endothelial system [42]. Phosphatidylserine-mediated clearance of apoptotic and damaged cells is thought to involve the ox-LDL receptor [43,44]. Moreover the loss of membrane asymmetry has been shown to occur at an early phase of apoptosis concomitantly with chromatin condensation and before membrane damage [45]. The analysis of extranuclear DNA from OS-treated cells revealed that both compounds induced this type of cell death in the THP-1 cell line. The efficiency of 7β-OH to induce apoptosis, revealed by the relative intensity of the DNA ladder (Figure 6), is slightly higher than that of 25-OH. This is in agreement with corresponding procoagulant activity measurements. As a consequence, OS-induced phospholipid exposure may result from apoptosis induction. This hypothesis deserves further investigation.

A concentration of 20 μM 7β-OH represents a 100-fold excess with respect to that measured in normal serum [46]. Such concentrations had to be used in order to observe a biological effect after 1 or 2 days, but it seems reasonable to assume that sustained subtoxic concentrations of OS could produce effects comparable with short exposure to higher concentrations. Under pathological situations such as atherosclerosis, the development of lesions spans several years and lipid accumulation in crystal form is responsible for high local concentrations of cholesterol and derivatives [4]. Moreover, it was recently shown that one of the major modifications of ox-LDL caused by mouse peritoneal macrophages or copper-catalysed reaction results in the transformation of cholesterol into 7β-OH. These ox-LDLs have been implicated in LPS-induced TF enhancement [47] and are able by themselves to induce apoptosis of monocytic cells [48]. In addition, the rate of degradation of these ox-LDLs by macrophages increases in parallel with their 7β-OH content [49]. Hence, OS present in ox-LDL [49] and atheromatous plaque [5] can induce anionic phospholipid exposure. This is not sufficient to trigger coagulation but OS could act synergistically with cholesterol [30] or inflammation mediators such as tumour necrosis factor-α and interleukin-1β [50] which have been shown to induce TF synthesis by monocytes and endothelium. These results establish a possible link between an atherogenic process associated with OS accumulation and the hypercoagulable state resulting from procoagulant phospholipid exposure and TF activity potentiation.

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