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Regulation of endothelial adhesion molecules by ligands binding to the scavenger receptor

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SUMMARY

Monocyte adherence to the endothelium, their penetration to the subendothelial space and excessive lipid accumulation (foam cell formation) are the initial events in atherogenesis. Scavenger receptors have been reported to play an important role in foam cell formation, since modified low density lipoproteins can be taken up via scavenger receptors in a non-down-regulated fashion. In this study we demonstrate that stimulation of scavenger receptors in endothelial cells induces the expression of endothelial adhesion molecules. Polyinosinic acid (poly I), a known scavenger receptor ligand, significantly induced the expression of intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1) and E-selectin on human umbilical vein endothelial cells when compared with polycytidylic acid (poly C), a structurally related compound to poly I, which does not bind to the scavenger receptor. The effect of scavenger receptor ligands on the endothelial cell line EA hy. 926 was also tested. Poly I up-regulated ICAM-1 expression also on EA hy. 926 cells, while it had no effect on IL-1β or tumour necrosis factor-alpha (TNF-α) production on the same cell line. Poly I-induced ICAM-1 expression on EA hy. 926 cells could be inhibited by H7, a protein kinase C inhibitor, while HA 1004, a preferential protein kinase A inhibitor, had no effect on ICAM-1 expression. The role of protein kinase C in scavenger receptor-mediated adhesion molecule up-regulation was confirmed by the ability of poly I to directly activate protein kinase C, when measured with ^{3}H-phorbol dibutyrate binding to EA hy. 926 cells, while poly C again was ineffective.

Keywords atherosclerosis endothelial cell scavenger receptor
intercellular adhesion molecule-1 vascular cell adhesion molecule-1 E-selectin

INTRODUCTION

The earliest sign of atherosclerosis in the vessel wall is the formation of a fatty streak, which consists mainly of lipid-loaded monocytes, the foam cells [1,2]. Adherence of circulating monocytes to the arterial endothelium is first seen in high cholesterol diet-induced experimental atherosclerosis in non-human primates [3]. Subsequently, monocytes penetrate through the endothelium and migrate subendothelially, where they accumulate excessive amounts of lipids, and turn into foam cells.

The endothelium is capable of controlling the binding of leucocytes by differential expression of adhesion molecules, which are expressed either constitutively, or in response to various inflammatory stimulators. Circulating leucocytes respectively display receptors for these adhesion molecules (reviewed in [4]). The recruitment of monocytes to early atheromatous lesions suggests an up-regulation of monocyte-specific adhesion molecules on the endothelial cells in atheroma formation. Recently the expression of vascular cell adhesion molecule-1 (VCAM-1), one of the adhesion molecules mediating the binding of monocytes to the endothelium [5], was reported on the aortic endothelium overlying early foam cell lesions in dietary hypercholesterolaemic and Watanabe heritable hyperlipidaemic rabbits [6]. VCAM-1 is not expressed on unstimulated endothelial cells, but can be up-regulated by the inflammatory cytokines IL-1 and tumour necrosis factor-alpha (TNF-α) [7]. The molecular mechanisms of VCAM-1 up-regulation in the pathogenesis of atherosclerosis are unknown.

Elevated levels of plasma low density lipoproteins (LDLs) are associated with an increased incidence of atherosclerosis [3]. Cells express specific LDL receptors, which are strictly regulated by the cholesterol needs of the cell [8]. By various modifications (oxidation, acetylation, endothelial cell modification) LDL is converted to a form recognized by another cell surface receptor, the scavenger receptor [9–11]. Modified LDL is internalized in a non-down-regulated fashion via the scavenger receptor, result-

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ing in foam cell formation. Oxidative modification of LDL has been reported to occur *in vivo*, thus generating a physiological ligand to the scavenger receptor [12]. Though scavenger receptors are mainly expressed on cells from the monocyte-macrophage lineage [9], endothelial cells [13,14], smooth muscle cells and fibroblasts [15] have also been reported to express scavenger receptors. The cloning of two forms of scavenger receptors from bovine, murine and human macrophages with high (>70%) amino acid homology has recently been reported [16–19]. The receptors are integral membrane proteins consisting of three polypeptide chains. Whether the existence of these two receptors and their possible heterotrimers accounts for all the scavenger receptor activity is not yet clear.

Activation of cells by ligands binding to the scavenger receptor *in vitro* has been reported previously. Maleyl-BSA and fucoidan trigger protease secretion, cytolytic activity and the expression of early proteins in primed murine macrophages [20,21]. We have recently reported that stimulation of the scavenger receptor activates human monocytes to produce IL-1 [22]. Since endothelial cells also express scavenger receptors [13,14], we wanted to study the effect of ligand binding to the scavenger receptor in this cell type. Our results indicate that intercellular adhesion molecule-1 (ICAM-1), VCAM-1 and E-selectin, which are all endothelial adhesion molecules involved in monocyte binding to the endothelium [5,23,24], are up-regulated via scavenger receptor stimulation in a non-cytokine-mediated fashion.

MATERIALS AND METHODS

Reagents

Polyinosinic acid (poly I), polycytidylic acid (poly C) and phorbol 12-myristate-13-acetate (PMA) were purchased from Sigma Chemical Co. (St Louis, MO). Lipopolysaccharide (LPS) (*Escherichia coli* 026:B6) was purchased from Difco Laboratories (Detroit, MI). Protein kinase C inhibitor H7 (1-[5-isoquinolone-sulphonyl]-2-methylpiperazine dihydrochloride) and protein kinase A inhibitor HA 1004 (N-(2-guanidinoethyl)-5-isoquinolinesulphonamide hydrochloride) were purchased from Seikagaku Kogyo Co. (Tokyo, Japan). Recombinant TNF-α ($2{\cdot}0 \times 10^7$ U/mg) was purchased from Boehringer Mannheim (Mannheim, Germany). Acetylated LDL labelled with 1,1′-dioctadecyl-1-3,3,3′,3′-tetramethyl-indocarbocyanine perchlorate was purchased from Biomedical Technologies Inc. (Stoughton, MA).

Endothelial cells

Human endothelial cells (HUVEC) were isolated from fresh umbilical cords, cultured as described [25] and used at the second to fourth passage.

Human endothelial cell line EA hy. 926 was obtained from C.-J. S. Edgell (University of North Carolina, NC). This cell line is an intraspecies hybrid derived from fusing HUVEC with the permanent cell line A549, which originally derived from a human lung carcinoma [26]. EA hy. 926 has been shown to express several characteristics common to endothelial cells, it expresses von Willebrand factor, and has the capacity to produce prostacyclin [27]. The expression of scavenger receptors on this cell line was confirmed by its ability to take up acetylated LDL labelled with 1,1′-dioctadecyl-1-3,3,3′,3′-tetramethyl-indocarbocyanine perchlorate (results not shown). EA hy. 926 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS), gentamycin and HAT (100 μM hypoxantine, 0·4 μM aminopterin, 16 μM thymidine) and were subcultured twice a week at a ratio of 1:4.

Antibodies

Anti-ICAM-1 (CD54, clone 84H10) MoAb was from Immunotech (Marseille, France). The MoAb recognizing VCAM-1, clone 4B9 [28] was a gift from Dr John Harlan (University of Washington, Seattle, WA). The anti-E-selectin MoAb BB 11 was a gift from Dr Roy Lobb (Biogen Inc., Cambridge, MA).

Flow cytometry

Normal indirect flow cytometry was used to measure the amount of various molecules expressed on the cell surface. For flow cytometry analysis, endothelial cells were cultured with the indicated stimulators for 20 h and detached from the culture flasks with versene (1:5000; Gibco, Grand Island, NY). Cells (5×10^5) were exposed to primary MoAbs at +4°C for 30 min; followed by washings and addition of fluorescein-conjugated rabbit anti-mouse immunoglobulin (Dakopatts, Glostrup, Denmark). After a second washing, the cells were analysed with flow cytometry for antigen expression.

Protein kinase C assay

Activation of protein kinase C (PKC) was determined by estimation of phorbol ester binding capacity of whole cells as described [29]. Briefly, endothelial cells on microtitre plates (2×10^4 cells/well) were left untreated or treated as triplicates at 37°C with the indicated stimulators for various time periods. Cells were then washed with PBS and divided in two groups, and incubated for 10 min at 37°C in PBS containing 4 mg/ml bovine serum albumin (BSA) and 40 nM ^{3}H-phorbol-12,13-dibutyrate (^{3}H-PBt$_2$; Radiochemical Centre, Amersham, UK) with or without 3 mM of unlabelled competitor PMA. Cell-non-associated ^{3}H-PBt$_2$ was washed away with PBS/BSA. Each sample was tested in triplicate. Specific binding was estimated by subtracting the mean non-specific binding in the presence of 250-fold excess of unlabelled PMA. The data are shown as fold of increase of stimulated cells when compared to the specific binding in unstimulated cells.

Cytokine induction and assays

EA hy. 926 cells ($0{\cdot}5 \times 10^6$) were cultured on a Petri dish (10 cm^2) and stimulated with 100 μg/ml poly I or poly C. Cells were then collected with the supernatants to get total IL-1β and TNF-α produced. Cells were disrupted with three cycles of freezing and thawing, and all the samples were stored at −20°C before being assayed for their cytokine contents in IL-1β- and TNF-α-specific ELISAs (Endogen, Boston, MA). The assays were performed according to the manufacturer's instructions.

RNA isolation and analysis

At the times indicated after stimulation, endothelial cells were harvested and Poly (A) mRNA was extracted according to standard protocols [30]. The mRNA isolated was quantified spectrophotometrically and 4-μg samples were size-fractionated on 0·8% agarose-formaldehyde gels, transferred to a nylon filter, dried and baked at 80°C and hybridized with cDNA probes. A 2·4-kb ICAM-1 cDNA probe was obtained from

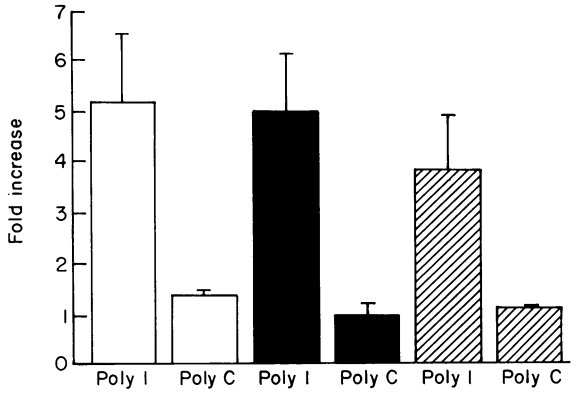

Fig. 1. Human umbilical vein endothelial cells (HUVEC) were either left unstimulated or cultured with 100 μg/ml polyinosinic acid (poly I) or polycytidylic acid (poly C). After 20 h, cells were collected and analysed for the expression of intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1) and E-selectin with flow cytometry. The data are shown as mean ($n=4$) fold of increase ± s.e.m. of mean fluorescence intensities (MFIs) in poly I- and poly C-stimulated cells when compared with unstimulated cells. With all three adhesion molecules poly I *versus* poly C stimulation: $P<0.05$. □, ICAM-1; ■, VCAM-1; ▨, E-selectin.

Professor T. A. Springer [31] (Centre for Blood Research, Boston, MA), and glyceraldehyde phosphate dehydrogenase (GAPDH), used as a control probe for the amount of RNA loaded, was obtained from Dr Kari Alitalo (Department of Pathology, University of Helsinki, Finland). The cDNA probes were labelled with ^{32}P by nick-translation. After washings the filters were exposed to x-ray film.

Statistical analysis

When comparing the fold increases between poly I and poly C stimulations and H7 and HA 1004 inhibitions, statistical analysis was performed using one-tailed paired Student's *t*-test.

RESULTS

Ligands binding to the scavenger receptor induce increased expression of ICAM-1, VCAM-1 and E-selectin in HUVEC cells

HUVEC cells were either left unstimulated or stimulated with 100 μg/ml poly I, a known ligand for the scavenger receptor [16,32] or poly C, a structurally related compound to poly I, which does not bind to the scavenger receptor. After 20 h, cells were collected and the expression of ICAM-1, VCAM-1 and E-selectin was analysed with flow cytometry. The data are shown as the mean fold of increase ± s.e.m. of the mean fluorescence intensity (MFI) of stimulated cells when compared with unstimulated cells from four independent experiments (Fig. 1). Poly I clearly up-regulated the expression of ICAM-1, VCAM-1 and E-selectin on HUVEC when compared with poly C. Though the MFI levels between individual experiments varied, the MFI for VCAM-1 and E-selectin was several fold lower than for ICAM-1 in each experiment (MFI ranges for poly I stimulation: ICAM-1 39–213, VCAM-1 9–37 and E-selectin 4–40). This has been reported previously with VCAM-1 after phorbol ester stimulation in the endothelial cell line EA hy. 926 [33].

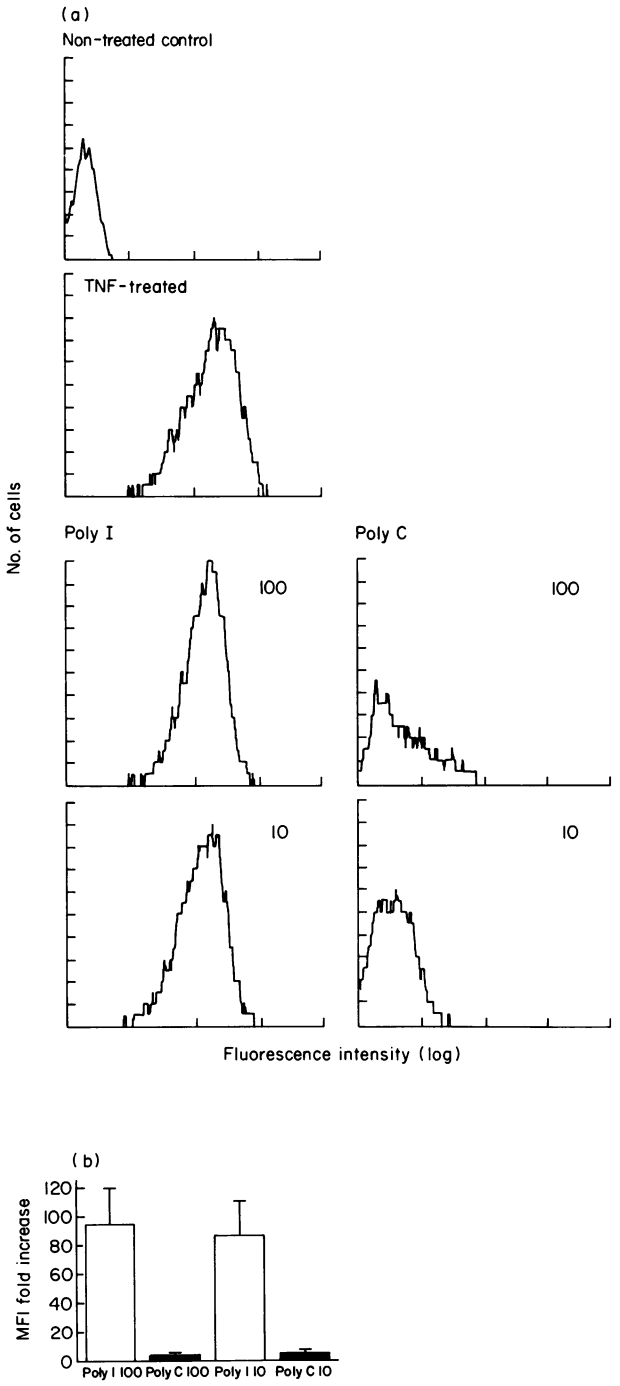

Fig. 2. Endothelial cell line EA hy. 926 was cultured with 10 or 100 μg/ml polyinosinic acid (poly I) or polycytidylic acid (poly C), or 10 ng/ml tumour necrosis factor-alpha (TNF-α). After 20 h, endothelial cells were collected and the expression of intercellular adhesion molecule-1 (ICAM-1) was analysed with flow cytometry. (a) Flow cytometry data from one representative experiment. (b) The mean ($n=4$) fold of increase ± s.e.m. of mean fluorescence intensities (MFIs) in poly I- and poly C-stimulated cells when compared with unstimulated cells. In 10 μg/ml and 100 μg/ml poly I *versus* poly C stimulations: $P<0.05$.

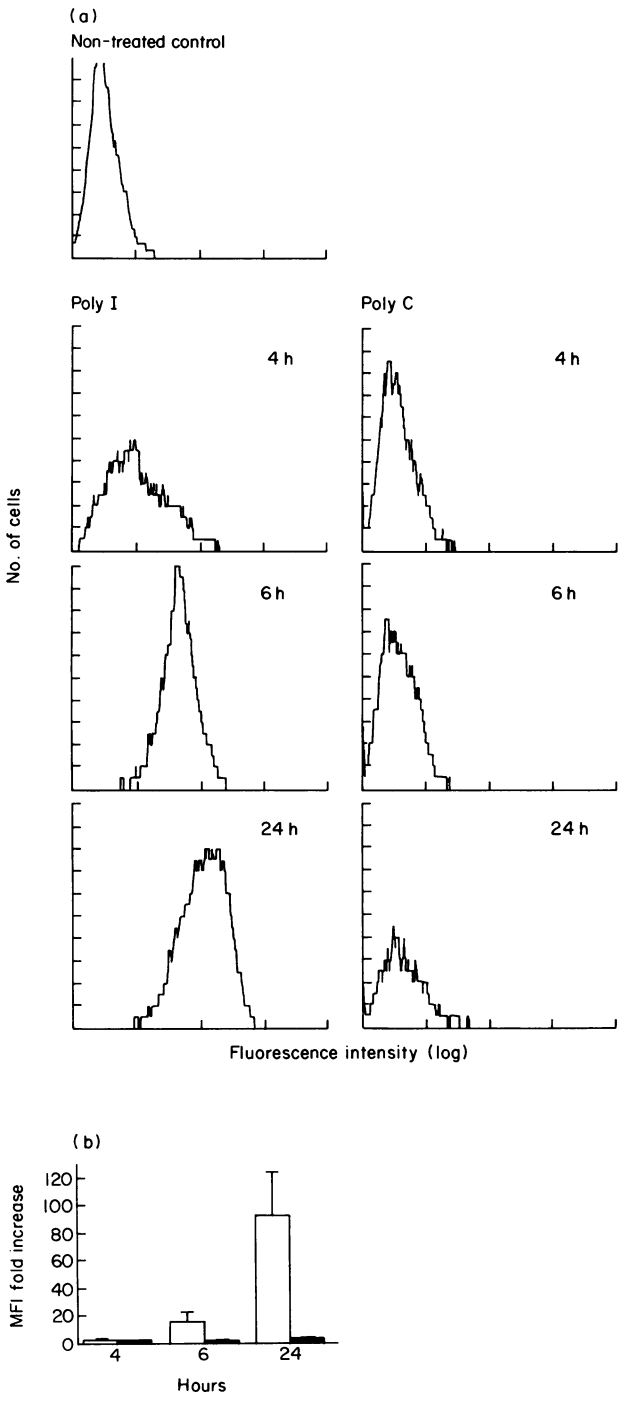

Fig. 3. EA hy. 926 cell line was left untreated or stimulated with 10 μg/ml polyinosinic acid (poly I) or polycytidylic acid (poly C) and cells were harvested at time points indicated and analysed for intercellular adhesion molecule-1 (ICAM-1) expression by flow cytometry. (a) Flow cytometry data from one representative experiment. (b) Mean ($n=3$) fold of increase $\pm$ s.e.m. of mean fluorescence intensities (MFIs) in poly I and poly C stimulations when compared with unstimulated cells. The difference in poly I *versus* poly C stimulation at 4 h was not statistically significant. At 6 h and at 24 h $P<0{\cdot}05$. □, Poly I; ■, poly C.

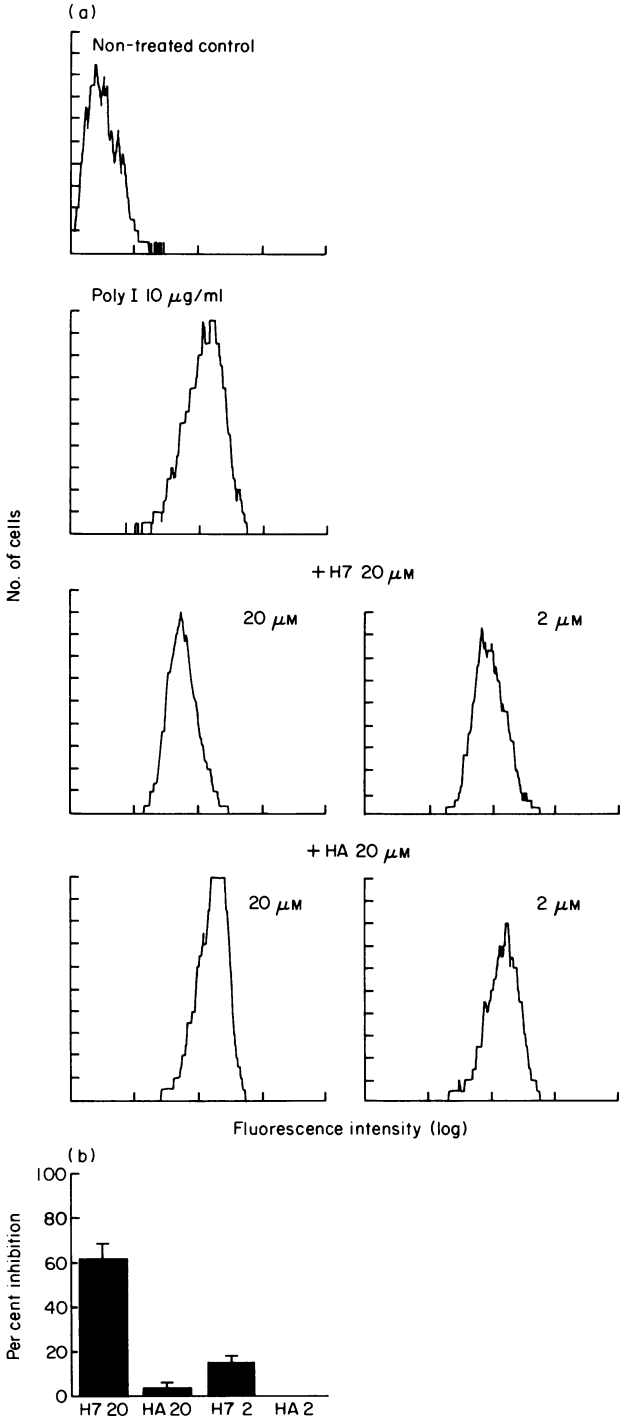

Fig. 4. EA hy. 926 cell line was left untreated or pre-incubated for 30 min with the indicated concentrations of H7 or HA 1004 and subsequently stimulated with 10 μg/ml polyinosinic acid (poly I) for 24 h. Cells were then collected and intercellular adhesion molecule-1 (ICAM-1) expression was determined with flow cytometry. (a) Flow cytometry data from one experiment. (b) The mean ($n=3$) per cent of inhibition $\pm$ s.e.m. of H7 and HA 1004 pre-incubated cells when compared with untreated cells in poly I stimulation. In the 20 μM H7 *versus* HA 1004 inhibition per cent, $P<0{\cdot}01$.

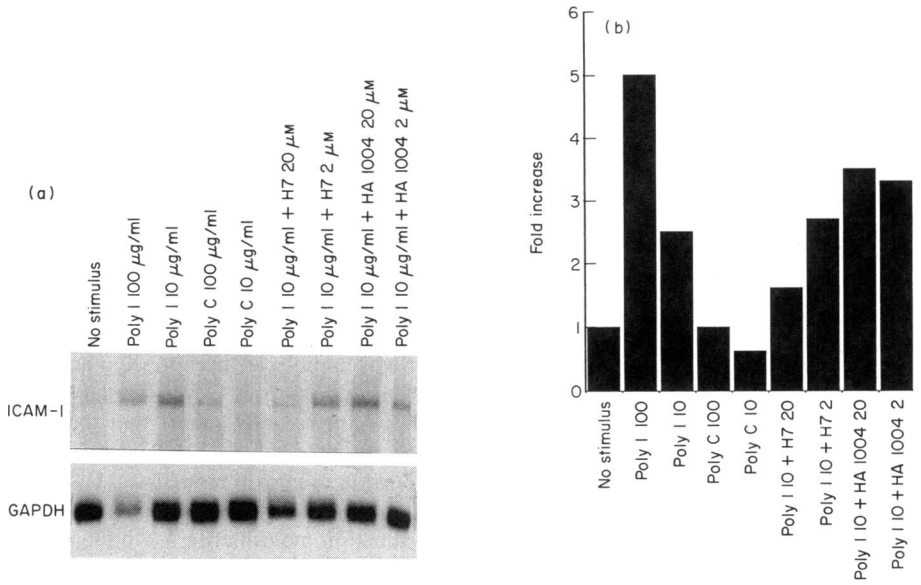

Fig. 5. EA hy. 926 cell line was left untreated or pre-incubated for 30 min with indicated concentrations of the protein kinase C inhibitor H7, or the protein kinase A inhibitor HA 1004 and subsequently stimulated with indicated concentrations of polyinosinic acid (poly I) or polycytidylic acid (poly C) for 6 h. mRNA was then isolated and analysed for intercellular adhesion molecule-1 (ICAM-1) expression as described in Materials and Methods. (a) The relative expression of ICAM-1 in the groups indicated in (a) were determined by laser densitometric scanning after standardization of the mRNA amounts according to the expression of a constant gene GAPDH (b).

Scavenger receptor-induced ICAM-1 expression is not mediated via induction of inflammatory cytokines

We have recently reported that ligands binding to the scavenger receptor induce IL-1 production in human monocytes [22]. Since IL-1 is a potent inducer of adhesion molecules [4,34], an endothelial cell line EA hy. 926 was used in the remaining experiments to avoid leucocyte contamination and subsequent cytokine production. Also, the use of this cell line facilitated the further characterization of scavenger receptor-mediated signal transduction mechanisms, which requires large numbers of cells.

The ability of scavenger receptor ligands to up-regulate adhesion molecule expression in EA hy. 926 cells was first determined. EA hy. 926 cells were either left untreated or stimulated with various concentrations of poly I, poly C or TNF-α as a positive control. Cells were harvested after 20 h and the expression of ICAM-1 was analysed with flow cytometry. Both 100 μg/ml and 10 μg/ml of poly I clearly up-regulated the cell surface expression of ICAM-1 (Fig. 2) and VCAM-1 (results not shown) on EA hy. 926 cell line when compared with the same concentrations of poly C. The results are shown both as flow cytometry data of poly I, poly C or TNF-α stimulation of one representative experiment (Fig. 2a), and as the mean fold of increase ± s.e.m. of the mean of MFI in poly I- and poly C-stimulated cells when compared with unstimulated cells of four independent experiments (Fig. 2b) (100 μg/ml poly I stimulation: MFI range 33–151).

The possibility of cytokines mediating the scavenger receptor ligand-induced adhesion molecule expression on EA hy. 926 cells was also investigated. EA hy. 926 cells ($0{\cdot}5 \times 10^6$) were cultured with 100 μg/ml poly I. After 20 h, cells and supernatants were collected and analysed for IL-1β and TNF-α production. No detectable (> 16 pg/ml) IL-1β or TNF-α production was found with specific ELISAs (results not shown). Since endothelial cells have not been reported to produce interferon-gamma (IFN-γ), the effect of these cytokines, known to up-regulate adhesion molecule expression on endothelial cells, was excluded.

Kinetics of scavenger receptor-mediated ICAM-1 expression

The kinetics of scavenger receptor-mediated ICAM-1 expression was then determined. EA hy. 926 cell line was stimulated with 10 μg/ml poly I or poly C and cells were harvested at time points indicated and analysed for ICAM-1 expression by flow cytometry. ICAM-1 cell surface expression was up-regulated with poly I stimulation already at 6 h and the levels increased up to 24 h, while Poly C had no effect on ICAM-1 expression at the same time points. The results are shown both as flow cytometry data from one experiment (Fig. 3a) and the mean ($n = 3$) fold of increase ± s.e.m. of MFI in stimulated cells when compared with unstimulated cells at the indicated time points (Fig. 3b).

Scavenger receptor-mediated endothelial adhesion molecule up-regulation is inhibited by a PKC inhibitor

To further characterize the signal transduction mechanisms involved in scavenger receptor-mediated ICAM-1 up-regulation, we tested the effect of H7, a preferential PKC inhibitor, and HA 1004, a preferential protein kinase A inhibitor, on 10 μg/ml poly I-induced ICAM-1 expression. H7 (20 μM) clearly inhibited poly I-induced expression of ICAM-1 when determined by flow cytometry, while the same concentration of HA 1004 had no effect on ICAM-1 expression. The results are shown both as

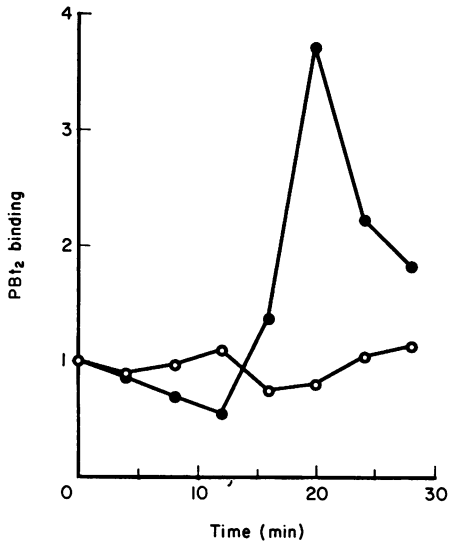

Fig. 6. Activation of protein kinase C (PKC) was determined by phorbol ester binding capacity of whole cells. EA hy. 926 cells were either left unstimulated or stimulated at time points indicated with 100 μg/ml polyinosinic acid (poly I) or polycytidylic acid (poly C) and analysed for their phorbol binding capacity as described in Materials and Methods. Each time point was determined in triplicate and s.e.m. was always less than 10%. The data are shown as the mean fold of increase of specific binding in stimulated cells when compared with the specific binding in unstimulated cells. The assay was repeated three times with analogous results. ●, Poly I; ○, poly C. PBt_2, phorbol-12,13-dibutyrate.

flow cytometry data (Fig. 4a) and as the mean ($n=3$) per cent of inhibition $\pm$ s.e.m. of ICAM-1 expression in H7- and HA 1004-treated cells, when compared with poly I stimulation alone (Fig. 4b).

The scavenger receptor-mediated ICAM-1 up-regulation is at the transcriptional level, since poly I up-regulated ICAM-1 mRNA expression on EA hy. 926 cells, while poly C had no effect on ICAM-1 expression (Fig. 5a). Poly I-induced ICAM-1 mRNA expression could also be inhibited with PKC inhibitors, since 20 μM H7 inhibited ICAM-1 mRNA expression on EA hy. 926 cells, while HA 1004 did not inhibit, but rather up-regulated, adhesion molecule expression in the same experimental conditions. The data are also shown as the relative expression of ICAM-1, when determined by laser densitometric scanning after standardization of the mRNA amounts according to the expression of the constant gene GAPDH (Fig. 5b). The mean ($n=3$) per cent of inhibition $\pm$ s.e.m. of 20 μM H7 on 10 μg/ml poly-I-induced ICAM-1 mRNA expression is 49 ± 15.

Poly I directly activates PKC on endothelial cells

The ability of poly I to directly activate PKC on endothelial cells was then characterized. The effect of poly I on ^{3}H-labelled ^{3}H-PBt_2 binding capacity of whole cells was measured as described in Materials and Methods [29]. This assay determines PKC activation by binding of ^{3}H-PBt_2 to activated PKC [35]. Poly I up-regulated ^{3}H-PBt_2 binding capacity at 20 min in EA hy. 926 cells, while poly C had no effect on phorbol binding during the 30-min assay time (Fig. 6). Each time point was tested in triplicate and the assay was repeated three times with similar results.

DISCUSSION

In the data presented we show that ligand binding to the scavenger receptor up-regulates endothelial adhesion molecules. Since poly I, the scavenger receptor ligand used in this study, may not exert all its effects specifically via the scavenger receptor, we used poly C, a structurally analogous compound to poly I, which has been reported not to bind to the scavenger receptor [16,32], as a negative control. Poly I induced ICAM-1, VCAM-1 and E-selectin expression on HUVEC, while the same concentration of poly C was virtually ineffective.

We have recently shown that ligands binding to the scavenger receptor induce IL-1 [22] and TNF-α (unpublished data) production in human monocytes. IL-1 and TNF-α are inflammatory cytokines which up-regulate the expression of endothelial adhesion molecules in 4–6 h [36]. To exclude the effect of leucocyte contamination and cytokine production on scavenger receptor-mediated endothelial adhesion molecule expression, the ability of poly I to up-regulate ICAM-1 expression was also determined on the endothelial cell line EA hy. 926. Poly I rapidly induced ICAM-1 expression on EA hy. 926 cell line, while poly C had no effect on adhesion molecule expression in 24 h. IL-1β and TNF-α protein production was also determined on EA hy. 926 cells with specific ELISAs and could not be detected after 24 h stimulation with optimal concentrations of poly I. The inability of EA hy. 926 to produce detectable levels of IL-1β or TNF-α protein, and the rapid kinetics of scavenger receptor-mediated ICAM-1 up-regulation, exclude the role of these cytokines in scavenger receptor-mediated adhesion molecule up-regulation.

Little is known about the signal transduction mechanisms of adhesion molecule expression on endothelial cells. PKC has been shown to mediate IFN-γ-induced ICAM-1 expression [37], while TNF-α-mediated ICAM-1 expression is PKC-independent [38]. IL-1 utilizes the cyclic AMP pathway when mediating increased lymphocyte penetration through the endothelium [39]. We investigated the signal transduction pathways of scavenger receptor-mediated ICAM-1 up-regulation. H7, a preferential PKC inhibitor, inhibited the cell surface expression of ICAM-1, while HA 1004, a preferential protein kinase A inhibitor, had no effect on adhesion molecule expression. The inhibition was at the transcriptional level, since H7 also inhibited poly I-induced ICAM-1 mRNA levels. The ability of poly I to directly activate PKC was confirmed with labelled phorbol dibutyrate binding assay on whole cells. Poly I up-regulated phorbol dibutyrate binding in 20 min, while poly C had no effect during the 30-min assay time. Our data thus indicate that the regulation of ICAM-1 expression via scavenger receptors is mediated through activation of PKC.

Inflammatory mechanisms have been implicated in the pathogenesis of atherosclerosis [40]. Atherosclerotic plaques have many hallmarks of inflammation, such as the expression of inflammatory cytokines (e.g. IL-1, transforming growth factor-beta 1 (TGF-β1) and platelet-derived growth factor-B) [40] and adhesion molecules (e.g. VCAM-1) [6]. Since hypercholesterolaemia is a well known risk factor for atherosclerosis, the possibility of modified lipoproteins having an activating effect on cells involved in atheroma formation is of considerable

clinical interest. However, conflicting data on the ability of modified LDL to activate cells *in vitro* exist. Both stimulatory [41–43], and inhibitory [44,45] effects of modified lipoproteins *in vitro* have been reported in monocyte/macrophages and smooth muscle cells. Our own experience in the use of lipoproteins in cell culture has also been controversial. When testing the effect of modified lipoproteins on monocyte IL-1 production, conflicting data within one lot as well as with different lots were obtained [22]. This could reflect difficulties in maintaining the same degree of LDL modification during cell culture, as has been reported with endothelial cells [10]. In contrast to the difficulties in using modified lipoproteins, the ligands used in this work provide a reproducible model of studying scavenger receptor-mediated functions *in vitro*.

Antibodies against human scavenger receptors have revealed the existence of immunoreactive scavenger receptor protein in human atherosclerotic lesions [19]. However, direct evidence of scavenger receptors functioning as signal-transducing molecules mediating activating signals during atherogenesis *in vivo* is still lacking. Further studies will reveal whether monocyte-specific adhesion to atheromatous areas *in vivo* is mediated via scavenger receptor-induced up-regulation of monocyte binding adhesion molecules as demonstrated *in vitro* in this study.

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