Cytotoxic and chemotactic potencies of several aldehydic components of oxidised low density lipoprotein for human monocyte-macrophages

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Abstract We have investigated the cytotoxic and chemotactic potencies of malondialdehyde (MDA), hexanal, 4-hydroxyhexenal (HHE), 4-hydroxynonenal (HNE) and 4-hydroxyoctenal (HOE), which are aldehydes found in oxidised low density lipoprotein (LDL), for human monocyte-macrophages. They were toxic in the following order: hexanal < HHE = HOE < HNE. HNE was toxic at 20 μM and chemotactic at 2.5 μM. The other aldehydes tested had no chemoattractant activity. Our results suggest that HNE arising from LDL oxidation could attract monocytes into the human atherosclerotic lesion. A direct cytotoxic role of aldehydes in foam cell death in the lesion is less likely.

Key words: Aldehydes; Cytotoxicity; Chemotaxis; Monocyte-macrophage (human); Oxidised LDL; Atherosclerosis

1. Introduction

Among the events of atherosclerosis that still remain unresolved are the reasons for monocyte accumulation in the artery wall leading to the formation of the fatty streak and the cause of macrophage death that contributes to the lipid core of the advanced lesion. The death of macrophage foam cells may be crucial for progression to the advanced lesion, which is responsible for clinical complications of myocardial and cerebral infarction [1].

Oxidation of LDL, the physiologically most likely modification of LDL in vivo, is implicated in the progression of atherosclerotic lesions [2]. Quinn and co-workers have shown that oxidised LDL induces chemotaxis in human monocytes, with lysophosphatidyl choline being one of its active components [3,4]. Further, oxidised LDL has been shown to be toxic for a variety of cell types including smooth muscle cells, fibroblasts [5] and more recently mouse peritoneal macrophages [6,7], the macrophage-like cell line P388D$_1$ [8] and human monocyte-macrophages [9]. However, oxidised LDL is a complex mixture of various components including lipid hydroperoxides, oxysterols and aldehydes [10]. Among the active products of LDL oxidation the aldehydes have recently attracted special interest, for example as inducers of cytokine release [11] and markers of atherosclerosis [12]. Curzio et al. have extensively published on the effect of aldehydes on the motility of rat neutrophils [13,14], but the possibility of their involvement in affecting monocyte migration, or macrophage death has not hitherto been investigated.

The aim of this study was to investigate the chemotactic activity and cytotoxic potencies of some of the aldehydes found in oxidised LDL for human monocyte-macrophages.

2. Materials and methods

All chemicals were purchased from Sigma Chemical Co. Ltd (Poole, Dorset, UK), unless otherwise indicated and were of the highest grade of purity available.

The 4-hydroxyalkenals, 4-hydroxyhexenal (HHE), 4-hydroxyoctenal (HOE) and 4-hydroxynonenal (HNE), were prepared as diethylacetals using an established method [15]. Prior to use, the acetals were converted to the free aldehydes by acid hydrolysis with 1 mM HCl for 1 h at room temperature. The exact concentrations of the aldehyde stock solutions (approximately 20 mM) were determined by measuring the UV spectrum (ε_{223nm} = 13750, 13800 and 13780 l mol^{-1} cm^{-1} for HNE, HHE and HOE, respectively). The malondialdehyde (MDA) stock solution was prepared by incubating 1 M 1,1,3,3-tetramethoxypropane in 1 mM HCl for 1 h at room temperature. During acid hydrolysis the diethylacetal moiety is converted to two molecules of ethanol, resulting in a concentration of 40 mM (0.184%) in the 4-hydroxyalkenal stock solutions. In the MDA stock solution, methanol is formed during hydrolysis resulting in a concentration of 40 mM (0.128%) in 10 mM MDA. These concentrations of ethanol and methanol, even if undiluted, had no detectable effect on chemotaxis or cytotoxicity (the authors, unpublished observation). The MDA, HHE, HNE and HOE stock solutions were kept at 4°C and used within 7 days of preparation. Hexanal (Aldrich, Gillingham, Dorset, UK) was kept at room temperature. For the chemotaxis and cytotoxicity experiments the aldehyde stock solutions were diluted in the respective assay medium to give the desired concentrations.

Human monocyte-macrophages (HMM) were isolated from peripheral blood from healthy volunteers as described previously [9]. Blood was diluted with phosphate-buffered saline (PBS) and layered onto Lymphoprep (Nycomed Pharma AS, Oslo, Norway). Following centrifugation at 300×*g*, mixed mononuclear cells were removed from the interface. Following washing steps to remove platelets (PBS/centrifugation at 150×*g*), cells were plated in 48-well plastic culture plates (Becton-Dickinson, New Jersey, USA) at a density of 3×10^6 cells/ml in serum-free medium (Macrophage-SFM, Gibco, Paisley, Scotland, UK). After 1 h, non-adherent cells were removed, leaving adherent HMM. This technique gave HMM cultures of >90% purity (based on Giemsa staining).

Monocytes destined for use in the assessment of chemotaxis were obtained by centrifugation on a Percoll gradient [16]. Following the washing steps to remove the platelets (see above), the mixed mononuclear cells were resuspended in 4 ml of PBS and mixed with 8 ml Percoll:Hank's balanced salt solution (6:1, pH 7.0) and spun for 30 min at 400×*g* at room temperature. HMM were collected from the top of the gradient, washed once with PBS and used immediately.

Chemotaxis was estimated in a 48-well Microchemotaxis Chamber (Neuro Probe Inc., Cabin John, MD, USA) using 5 μm polyvinylpyrrolidone-free polycarbonate filters [17]. Dilutions of potential chemoattractant and cell suspensions (10^6 cells/ml) were prepared in RPMI 1640 supplemented with 0.25% fatty acid-free BSA. 30 μl/well of medium control or potential chemoattractant was applied to the bottom plate and after insertion of the filter, 50 μl/well cell suspension was put in the upper plate. After incubation at 37°C for

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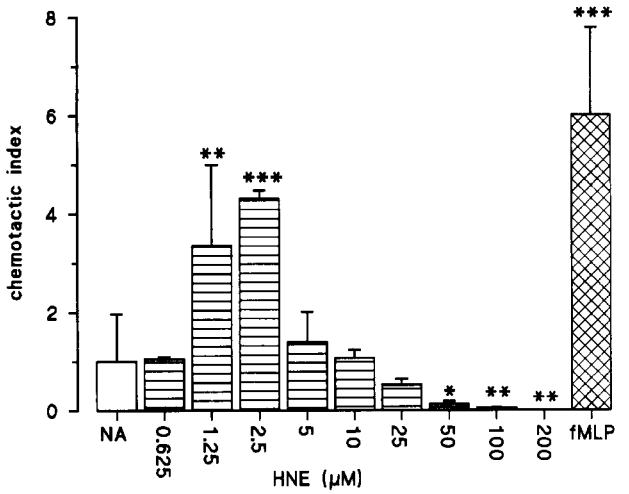

Fig. 1. Chemotactic activity of HNE. Human monocyte-macrophages were assessed for chemotaxis in response to 0.625–200 μM HNE. fMLP (10 nM) was employed as a positive control. Cells incubated in the absence of aldehydes are denoted by no additions (NA). The results shown are the mean chemotactic indices ± S.D. of one experiment carried out in triplicate which was representative of three. Statistically significant differences from the no additions control are denoted by: $*P \leq 0.1$, $**P \leq 0.01$ and $***P \leq 0.001$.

90 min, the filter was removed and stained with Diff-Quik (Browne Ltd, Reading, Berkshire, UK). Then, non-migrated cells were wiped off the top side, and the filter was mounted on a glass slide using oil. All samples were tested in triplicate. To assess chemotaxis, migrated cells in 4 high power fields (HPFs, magnification ×400) were counted for each well. Results are given as the chemotactic index (CI), = the ratio of cells in 4 HPFs with chemoattractant to cells in 4 HPFs in medium without chemoattractant. Inhibition of random migration was assessed as the decrease of migrated cells with sample solution as compared to the medium control (50% inhibition = chemotactic index of 0.5).

For the measurement of cytotoxicity HMM were preloaded with tritiated adenine (Amersham Radiochemicals, Amersham, Bucks, UK, 24 Ci/mmol) for 1 h (0.5 μCi/well) using an established technique [9]. After washing with PBS, cells were incubated in Macrophage-SFM in the presence or absence of the various aldehydes. After the appropriate incubation, samples of medium (200 μl) were counted using a liquid scintillation counter and OptiPhase 'HiSafe' scintillant (Fisons Scientific Equipment, Loughborough, Leicestershire, UK). Intracellular radioactivity was determined by lysing the cells with 1% (v/v) Triton X-100, followed by liquid scintillation counting. The percentage of total radioactivity leakage into the medium was calculated.

The statistical significance of the data was determined, where appropriate, using unpaired Students' *t*-tests either alone, or in combination with Analysis of Variance (ANOVA) using Microsoft Excel software for Microsoft Windows 3.1.

3. Results

The ability of MDA, hexanal, HHE, HNE and HOE to attract HMM was compared with *N*-formyl-leucine-methionine-phenylalanine (fMLP) at 10 nM, as a positive control. Since many chemoattractants induce chemotaxis only around an optimal concentration, the aldehydes were tested over a wide concentration range, initially. MDA, hexanal and HHE (10^{-3} M to 10^{-16} M) and HOE (10^{-5} M to 10^{-18} M) did not induce chemotaxis in HMM at any of the concentrations tested (data not shown). In contrast, HNE elicited chemotaxis (Fig. 1) and a narrower concentration range (0.625 μM to 200 μM) was examined to assess the optimal concentration. Significant chemotactic activity with HNE was observed at concentrations of 1.25 and 2.5 μM with a maximum at 2.5 μM (4.7 ± 2.9, mean chemotactic index ± S.D. of three separate experiments). On average the maximal chemotactic activity of HNE was slightly lower than that of 10 nM fMLP (chemotactic index of 6.0 ± 1.8). Occasional preparations of monocytes, however, showed no response to HNE.

Concentrations in the range of 0.1 μM up to 100 mM, for MDA, hexanal, HHE, HNE and HOE, were examined for a possible inhibitory effect on random cell migration, i.e. cells migrating spontaneously in the absence of chemoattractant. Below, the aldehydes are arranged in ascending order of inhibitory potency. Figures in parentheses are the concentrations required to inhibit random migration by 50%. Hexanal (22 mM) < MDA (3 mM) < HHE (90 μM) < HOE (80 μM) < HNE (18 μM).

Hexanal was not toxic at concentrations up to and including 800 μM, as determined by the tritiated adenine method. Statistically significant leakage of radiolabel was obtained at 8 and 80 mM, but this effect is of dubious biological relevance (Fig. 2A). Menadione, a lipophilic quinone which possesses both redox cycling and arylating activity, induces oxidative stress and is cytotoxic and was included as a positive control at 200 mM. It induced significant toxicity after 2 h, and by 6 h leakage of radioactivity was near-total (data not shown). The extent of leakage of radioactivity from no addition controls also increased with time over the 24 h period investigated (Fig. 2A). Exposure to MDA induced leakage at 1 mM. Concentrations below 1 mM were not toxic at any time. Exposure of cells to 8 and 16 μM MDA produced an extent of leakage which was not significantly different from no addition controls up to and including 24 h (Fig. 2B). The leakage of radioactivity from cells resulting from exposure to concentrations of HNE of 20 μM was significantly different from no addition controls and was near-total at 24 h (Fig. 2C). HHE also exhibited significant toxicity (Fig. 2D) at concentrations of 50 μM and above. HOE produced concentration-dependent toxicity at 24 h. Marked toxicity was observed at 6 h with 200 μM HOE. Significantly increased leakage compared with no addition controls was obtained with 100 μM HOE (Fig. 2E). It can be seen from the data in Fig. 1 that the aldehydes showed an ascending order of toxicity: hexanal < HHE = HOE < HNE. The use of two other cytotoxicity assays (MTT dye reduction, a measure of mitochondrial integrity, and neutral red staining, a measure of cell adherence) confirmed the results obtained with tritiated adenine (data not shown).

4. Discussion

In the present study five aldehydes which can be found in oxidised LDL were examined for their chemotactic and cytotoxic potencies for human monocyte-macrophages. The issue we were attempting to address was whether these aldehydes could contribute to the recruitment or the death of macrophages, in vitro, at concentrations actually present in oxidised LDL.

The composition of oxidised LDL varies strongly with oxidation conditions and also somewhat with individual batches of the native LDL from which it is prepared. Published data of aldehyde concentrations in strongly oxidised LDL (55 μg protein/ml with 1.66 μM Cu^{2+} for 24 h, leading to complete consumption of polyunsaturated fatty acids) give the follow-

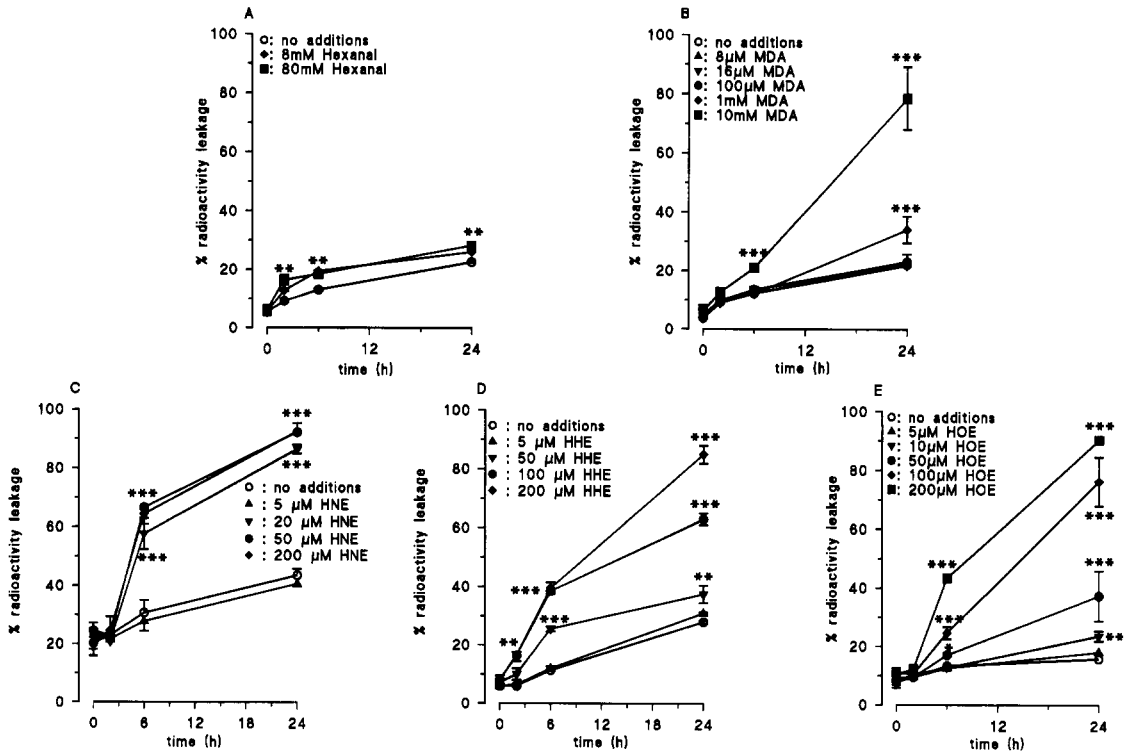

Fig. 2. Cytotoxicity of aldehydes. Human monocyte-macrophages were preloaded with tritiated adenine and then exposed to hexanal (A), MDA (B), HNE (C), HHE (D) and HOE (E), for up to 24 h. Results shown are the mean percentage of total radioactivity leakage into the medium ± S.D. for triplicate wells from one experiment typical of three. Statistically significant differences from the no addition controls (NA) are indicated by: $*P \leq 0.1$, $**P \leq 0.01$, $***P \leq 0.001$.

ing levels for hexanal, MDA, HNE, and HHE: 229, 114, 114 and 49 nmol/mg LDL protein [10,18].

In previous studies with human monocyte-macrophages, we obtained reproducible cell-killing with copper-oxidised LDL at a concentration of 200 μg/ml in culture medium [9]. Analysis of this less extensively oxidised LDL (1 mg protein/ml using 5 μM Cu^{2+} for 24 h, resulting in consumption of approximately 70% of the polyunsaturated fatty acids) using the same analytical method as Esterbauer and colleagues indicated concentrations of hexanal, HNE and HHE as 71, 22 and 4 nmol/mg LDL protein (unpublished data). Extrapolating from these data, we suggest that the final concentrations of aldehydes in our previous cultures exposed to oxidised LDL were: hexanal 14 μM, MDA ~17 μM, HNE 4.4 μM, HHE 0.8 μM and HOE ~1 μM. Taken together with the results of this study, it appears unlikely that any of these aldehydes alone could account for the observed cytotoxicity of oxidised LDL for HMM. However, the combined effect of the various aldehydes might be greater and result in increased toxicity, and toxicity of oxidised LDL will also depend on oxidation conditions. Furthermore, hexanal, HNE and HHE are lipophilic compounds and remain largely associated with the LDL particle [19]. 'Local' concentrations of these aldehydes in the lipid phase of oxidised LDL would therefore be much higher and have been estimated to be ~300 mM for hexanal and ~150 mM for HNE in strongly oxidised LDL [10]. Also, oxidised LDL contains lipid hydroperoxides which are potentially able to decompose to generate aldehydes in situ. The modification of membrane proteins for example by these aldehydes might then result in damage to the cell. HNE was shown to bind to Na^+-K^+-ATPase in vitro, leading to a loss in enzyme activity [20]. Furthermore, HNE binds to apoB-100 to give a form of LDL avidly phagocytosed by mouse peritoneal macrophages resulting in intracellular lipid accumulation [21,22]. In addition to aldehydes, oxidised LDL contains other cytotoxic components, e.g. lipid hydroperoxides and oxysterols [23,24]. Thus toxicity of oxidised LDL could arise from the combined effect of these and other as yet unidentified cytotoxins.

Previously, strongly oxidised LDL was shown to induce chemotaxis in human monocytes at a concentration of 100 μg/ml, with lysophosphatidyl choline being one of its active components. A synthetic lysophosphatidyl choline, 1-palmitoyl-lysophosphatidyl choline, had comparable chemotactic activity with an optimum concentration at 25 μM [3,4]. Our experiments demonstrate that HNE is a strong chemoattractant for human monocytes at concentrations which can be found in oxidised LDL. With an optimum at 2.5 μM, HNE was about ten-fold more effective on a molar basis than 1-palmitoyl-lysophosphatidyl choline. HNE could, therefore, contribute significantly to the chemotactic activity of oxidised LDL observed in vitro. Curzio and colleagues showed that HNE elicits chemotaxis in rat pleural neutrophils at similar concentrations (3.7–8.4 μM), but the number of neutrophils responding to HNE was only about 50% of that for fMLP and occasional preparations of neutrophils showed no response at all [25,26]. It was suggested that either only a certain subpopulation of neutrophils react to HNE, or that the neutrophils, which were derived from pleural exudate, had been desensitized by previous exposure to HNE in vivo. Indeed, HNE was demonstrated to act as a chemoattractant for rat neutrophils in vivo and to desensitize the cells to subsequent

HNE exposure [27,28]. In our experiments the number of monocytes responding to HNE was only slightly lower than that for fMLP (78%). Therefore, monocytes appear to be more homogenous in their response to HNE. However, occasional preparations of monocytes also did not show any response to HNE. In contrast to rat pleural neutrophils [25,29], human monocytes showed no significant chemotaxis towards HOE. Hexanal is ineffective for both cell types [30]. Several hydroxyalkenals, including HNE and HOE, inhibit random migration of rat neutrophils at concentrations above 10^{-5} M, an effect which was not due to toxicity [14,25]. In our experiments, HNE, HOE and HHE displayed inhibitory activity for monocyte random migration at similar concentrations. The mechanism by which hydroxyalkenals inhibit cell migration is unknown.

One characteristic of the atherosclerotic lesion is the absence of neutrophils and the mechanism by which monocytes are selectively attracted into the intima is still a matter of intense study. Minimally-modified LDL was found to selectively promote the adhesion of monocytes to rat and human endothelial cells [31] by inducing the expression of a glycoprotein on the surface of endothelial cells [32]. This monocyte-specific adhesion molecule has not been fully characterized so far, but it is different from E-selectin, VCAM-1 and ICAM-1. Furthermore, minimally-modified LDL stimulates endothelial cells and smooth muscle cells to express a monocyte-specific chemoattractant, MCP-1 [33]. Oxidised LDL stimulates the expression of P-selectin by rat endothelial cells [34], facilitating leukocyte-endothelium interactions, and lysophosphatidyl choline, which was reported to selectively attract monocytes [4], stimulates the expression of leukocyte-adhesion molecules ICAM-1 and VCAM-1 on rat and human endothelial cells [35].

Our data suggest that the potent chemoattractant activity of HNE may contribute to the attraction of monocytes into atherosclerotic lesions by oxidised LDL. As HNE also attracts neutrophils, selectivity for monocytes in vivo may be achieved by other mechanisms, for example by expression of monocyte-specific adhesion molecules on endothelial cells. However, the role of aldehydes in foam cell death appears less certain, and other components of oxidised LDL are more likely to be responsible for cell-killing.

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