Biochem. J. (1987) 248, 919–926 (Printed in Great Britain)

Neutrophil association and degradation of normal and acute-phase high-density lipoprotein 3

Enid G. SHEPHARD,* Frederick C. DE BEER,*‡ Maria C. DE BEER,* Mohammed S. JEENAH,† Gerhard A. COETZEE† and Deneys R. VAN DER WESTHUYZEN

*Department of Internal Medicine, University of Stellenbosch, Tygerberg 7505, and †UCT/MRC Muscle Research Unit, Department of Medical Biochemistry, University of Cape Town Medical School, Cape Town, South Africa

The interaction of normal and acute-phase high-density lipoproteins of the subclass 3 (N-HDL_3 and AP-HDL_3) with human neutrophils and the accompanying degradation of HDL_3 apolipoproteins have been studied *in vitro*. The chemical composition of normal and acute-phase HDL_3 was similar except that serum amyloid A protein (apo-SAA) was a major apolipoprotein in AP-HDL_3 (approx. 30% of total apolipoproteins). ^{125}I-labelled AP-HDL_3 was degraded 5–10 times faster than ^{125}I-labelled N-HDL_3 during incubation with neutrophils or neutrophil-conditioned medium. Apo-SAA, like apolipoprotein A-II (apo-A-II), was more susceptible than apolipoprotein A-I (apo-A-I) to the action of proteases released from the cells. The amounts of cell-associated AP-HDL_3 apolipoproteins at saturation were up to 2.8 times greater than N-HDL_3 apolipoproteins; while apo-A-I was the major cell-associated apolipoprotein when N-HDL_3 was bound, apo-SAA constituted 80% of the apolipoproteins bound in the case of AP-HDL_3. The associated intact apo-SAA was mostly surface-bound as it was accessible to the action of exogenous trypsin. α_1-Antitrypsin-resistant (α_1-AT-resistant) cellular degradation of AP-HDL_3 apolipoproteins also occurred; experiments in which pulse-chase labelling was performed or lysosomotropic agents were used indicated that insignificant intracellular degradation occurred which points to the involvement of cell-surface proteases in this degradation.

INTRODUCTION

Apo-SAA and C-reactive protein are the two proteins in man whose concentrations increase most dramatically during an acute-phase reaction. Plasma levels are elevated up to 100-fold following inflammation, infarction or infection [1]. This change is probably produced as a result of the release from macrophages of interleukin I, which alters hepatic protein synthesis [2]. In contrast with C-reactive protein, whose biological role seems to be the complement-dependent solubilization of chromatin following cell death [3], the function of apo-SAA is unknown. As some apolipoproteins have been shown to act as targeting devices for receptor-mediated cellular uptake and in others as enzymatic co-factors, further studies on the cellular interactions and metabolism of apo-SAA are essential to elucidate its function [5]. Most previous work on apo-SAA has focused on its proteolytic degradation in relation to amyloidogenesis [4,5]. Only in rare instances during chronic inflammation does the aberrant degradation of apo-SAA (M_r 12000) yield a cleavage product called amyloid A protein (M_r 8000) that polymerizes pathogenetically to amyloid fibrils [6]. The occurrence of apo-SAA in the plasma is probably of functional importance in inflammatory processes of different kinds irrespective of whether they are complicated by amyloidosis.

Neutrophil leukocytosis is a concomitant of the acute-phase response and the deposition of murine amyloid is accelerated by it [7]. Previous studies have shown that purified apo-SAA is degraded by neutrophil-derived proteolytic enzymes amongst which is an elastase that has been shown to play an important role [4]. The enzyme that cleaves apo-A-II upon incubation of normal human HDL_3 with neutrophils is also an elastase [8]. The degradation of both apo-A-II and apo-SAA is inhibited by α_1-AT and other elastase inhibitors [4,8].

Apo-SAA exists in the plasma almost exclusively as an apolipoprotein of HDL_3 [9]. It should be noted that AP-HDL_3 represents a polydisperse population of particles that differ with respect to density, size and apolipoprotein composition [10]. In this study we have investigated the association with neutrophils of N-HDL_3 and AP-HDL_3 and have examined the influence of this association on the degradation of apo-SAA and other HDL_3 apolipoproteins. Our results show that apo-SAA, like apo-A-II, is highly susceptible to degradation by neutrophil-derived proteases. In addition, significantly more AP-HDL_3 protein associates with neutrophils than N-HDL_3 protein. Apo-SAA constitutes more than 80% of the cell-associated AP-HDL_3 apolipoproteins. In the presence of α_1-AT, the significant cellular degradation of AP-HDL_3 apolipoproteins (predominantly apo-SAA) is likely to be an event that occurs at the surface.

EXPERIMENTAL

Isolation of neutrophils

Neutrophils were isolated from heparinized normal adult venous blood (5 units of preservative-free heparin/

Abbreviations used: N-HDL_3 and AP-HDL_3, normal and acute-phase high-density lipoproteins of subclass 3 respectively; apo-A-I and apo-A-II, apolipoprotein A-I and apolipoprotein A-II respectively; apo-SAA, serum amyloid A protein; α_1-AT, α_1-antitrypsin; RPMI 1640, Roswell Park Memorial Institute 1640 (culture medium).

‡ To whom correspondence should be addressed.

ml of blood) by Hypaque/Ficoll gradient centrifugation [11]; contaminating red blood cells were lysed by hypotonic shock. Cell viability was greater than 98% as determined by Trypan Blue exclusion, and Wright-Giemsa stains showed greater than 98% cell purity. Cells (suspended at 40×10^6 cells/ml of RPMI 1640) (GIBCO, Grand Island, NY, U.S.A.) were kept on ice and used within 1 h of preparation.

HDL_3 preparation and iodination

Plasma was collected, as previously described [10], from normal adults for the preparation of N-HDL_3 and from patients with myocardial infarction, bacterial pneumonia or after abdominal surgery, for preparation of AP-HDL_3. An HDL_3 fraction with a density of 1.13–1.18 g/ml was obtained by discontinuous gradient ultracentrifugation as previously described [10,12]. Protein was determined by the Lowry method [13], using bovine serum albumin (Fraction V; Miles Laboratories) as a standard. HDL_3 composition and the relative content of apolipoproteins was determined as previously described [10,12]. The apolipoprotein (apo-A-I, apo-A-II, apo-SAA) and lipid composition of three normal and four acute-phase HDL_3 batches used in this study are shown in Table 1. Except for the apolipoprotein profiles, no significant differences between AP- and N-HDL_3 were observed. Iodination of lipoproteins was done by a modified iodine monochloride method using [^{125}I]NaI (Amersham International) [14].

Neutrophil association and degradation of ^{125}I-labelled N-HDL_3 and ^{125}I-labelled AP-HDL_3

Neutrophils (10×10^6) were incubated in borosilicate glass tubes (Kimble, Owens, IL, U.S.A.) [37 °C, under CO_2/air (19:1)] with ^{125}I-labelled N-HDL_3 or ^{125}I-labelled AP-HDL_3 in a final volume of 2 ml of RPMI 1640, with or without α_1-AT (Sigma, cat. no. 9024; 1.5 mg/ml). Prior titration confirmed that this α_1-AT concentration (1.5 mg/ml) was unequivocally on a plateau of inhibition with respect to AP-HDL_3 degradation for all conditions described. The medium was supplemented with bovine serum albumin to give a final (α_1-AT plus bovine serum albumin) protein concentration of 2 mg/ml. The incubation with HDL_3 was done according to the regimes described in the Results section. Reactions were stopped by centrifugation at 2000 rev./min for 4 min at 4 °C. Cell-free supernatants (1 ml portions) were immediately precipitated with trichloroacetic acid at a final concentration of 12% (w/v). Inorganic iodide from trichloroacetic acid-soluble material was extracted with $CHCl_3$ following oxidation with H_2O_2 [15]. This procedure eliminated any inorganic iodide that existed in the ^{125}I-labelled HDL_3 preparation (< 1% of the total added radioactivity) and any iodide that was released during the incubation.

The cells were washed at 4 °C by centrifugation, once with 10 mM-phosphate-buffered saline (pH 7.4) containing 0.2% (w/v) bovine serum albumin, and twice with the same buffer but without bovine serum albumin. With the final wash, cells were transferred to clean tubes. Total radioactivity associated with the cells was determined by gamma counting (LKB gamma counter, LKB Instruments, Turku, Finland). The results are averages of duplicate incubations (Fig. 2 and Fig. 3). Duplicates did not differ by more than 12% from each other. In some experiments, the nature of the associated apolipoproteins was determined by adding an equal volume of 4% SDS/24% glycerol in 12 mM-Tris, pH 6.8, either before or after cell trypsinization (see below), followed by SDS/polyacrylamide-gel electrophoresis and autoradiography (Kodak XAR film) [16].

Table 1. Composition of N- and AP-HDL_3

Composition (w/w) values represent average values ±S.D. of three N- and four AP-HDL_3 preparations. Apolipoprotein content values are in % colour yields after SDS/polyacrylamide-gel-electrophoresis separation and Coomassie Blue staining (not including minor apolipoproteins). Results represent average values ±S.D. of three N- and four AP-HDL_3 preparations. *P* values are normal versus acute phase, student *t*-test.

	Composition (% w/w)		
	N-HDL_3	AP-HDL_3	*P*
Proteins	53.2±4.1	59.5±7.1	> 0.05
Phospholipids	21.2±4.7	24.5±3.1	> 0.05
Cholesterol esters	15.7±4.8	11.1±7.4	> 0.05
Free cholesterol	2.2±0.3	2.0±1.1	> 0.05
Triacylglycerols	7.8±5.6	2.8±0.8	> 0.05
	Colour yield (%)		
Apolipoproteins			
Apo-A-I	86.9±2.8	62.3±5.9	< 0.01
Apo-A-II	13.1±2.8	5.3±1.9	< 0.01
Apo-SAA	–	32.4±4.4	–

Cell-conditioned medium was also obtained from neutrophils to investigate the degradation of ^{125}I-labelled N-HDL_3 and ^{125}I-labelled AP-HDL_3 by secreted proteases. Cell-conditioned medium was prepared by incubating 10×10^6 neutrophils in 2 ml of standard incubation medium containing α_1-AT and bovine serum albumin but without HDL_3 present. The cell-free cell-conditioned medium was mixed with ^{125}I-labelled N-HDL_3 or ^{125}I-labelled AP-HDL_3 within 5 min of collection and the reaction conditions were as described in the Results section. Incubation mixtures were analysed for the generation of trichloroacetic acid/soluble degradation products or by SDS/polyacrylamide-gel electrophoresis and autoradiography as described above.

In other experiments, after incubation of cells with ^{125}I-labelled AP-HDL_3 (25 μg/ml, 60 min, 37 °C), the fate of the associated ^{125}I-labelled AP-HDL_3 was investigated. The cells (10×10^6) were first washed in phosphate-buffered saline (4 °C) by two brief (< 30 s) centrifugation steps in a Microfuge (Beckman Instruments, La Jolla, CA) and then incubated at 37 °C in HDL_3-free medium (2 ml) containing α_1-AT and bovine serum albumin (as indicated above) for various time periods (pulse-chase experiments). Cell-associated radioactivity and radioactivity released into the medium were determined at the end of the chase times.

Surface-bound radioactivity was determined by treating the cells at the end of incubation periods or at the end of chase periods with 1 ml of 0.05% trypsin (1:250; Difco Laboratories) containing 0.02% EDTA, for 10 min at 37 °C. This treatment did not affect cell viability as judged by Trypan Blue exclusion. Reactions were stopped by washing the cells as above. Radioactivity

Table 2. Effect of α_1-AT on the association and degradation of HDL_3 by neutrophils

Incubations were carried out with 10×10^6 cells and ^{125}I-labelled HDL_3 (25 μg of protein/ml) for 60 min at 37 °C in the absence (−) or presence (+) of α_1-AT (1.5 mg/ml). The results of three experiments using three different batches each of ^{125}I-labelled N-HDL_3 and ^{125}I-labelled AP-HDL_3 and neutrophils from three different subjects are given.

Experiment number	α_1-AT	Cell-association (ng of protein/10×10^6 cells)		Degradation (ng of protein/10×10^6 cells)	
		^{125}I-labelled N-HDL_3	^{125}I-labelled AP-HDL_3	^{125}I-labelled N-HDL_3	^{125}I-labelled AP-HDL_3
1	+	42	135	22	182
	−	24	43	57	595
2	+	33	95	33	253
	−	15	25	80	712
3	+	138	420	42	322
	−	42	79	117	699

released into the medium and radioactivity remaining with the cells were then determined as before.

Influence of AP-HDL_3 and N-HDL_3 on release of neutrophil proteolytic enzymes

The influence of N-HDL_3 or AP-HDL_3 on the release of proteolytic enzymes was studied essentially as described by Polacek *et al.* [21]. This involved evoking the release of proteolytic enzymes by the respective HDL_3 preparations, followed by ultracentrifugal flotation of the HDL_3 and recovery of proteolytic activity in the infranatants. Specifically, 80×10^6 neutrophils were incubated in 5 ml of RPMI 1640/25 mM-Hepes buffer (pH 7.4) containing either 1 mg of AP-HDL_3/ml, 1 mg of N-HDL_3/ml or no HDL_3 (control). Incubations were in sterile Greiner polycarbonate tubes for 60 min at 37 °C. At the end of the incubation, cells were spun at 2000 rev./min; the density of 4 ml of each of the respective supernatants was increased to 1.25 g/ml with KBr, and they were layered under 2 ml of KBr (1.21 g/ml) and spun for 38 h at 45000 rev./min in a Beckman T65 rotor at 10 °C. The upper 2 ml containing the HDL_3 was removed and the infranatants containing the released proteases were dialysed against 0.15 M-NaCl/0.02% azide, pH 7.0. The three infranatants constituting 'N-HDL_3-conditioned medium', 'AP-HDL_3-conditioned medium' and 'control-conditioned medium' were obtained, and medium from 2×10^6 cells, were each incubated in triplicate with ^{125}I-labelled AP-HDL_3 or ^{125}I-labelled N-HDL_3, and apolipoprotein degradations were determined as described above.

RESULTS

The influence of α_1-AT on cell-association and degradation of ^{125}I-labelled HDL_3

Physiological interactions between HDL_3 and neutrophils occur in the presence of α_1-AT. Given the known susceptibility of apo-A-II [8] and purified apo-SAA [4] to proteolytic cleavage by elastases, the influence of α_1-AT (1.5 mg/ml) on the association and degradation of ^{125}I-labelled N-HDL_3 and ^{125}I-labelled AP-HDL_3 was investigated. This concentration of α_1-AT amounted to an excess and the use of higher concentrations yielded similar results. Our results (Table 2) show greater total cell-association of both ^{125}I-labelled N-HDL_3 and ^{125}I-labelled AP-HDL_3 in the presence than in the absence of α_1-AT. Although some degradation of HDL_3 protein did occur in the presence of α_1-AT, this was greatly increased in the absence of the elastase inhibitor. In the experiments with α_1-AT present, about 3 times more ^{125}I-labelled AP-HDL_3 than ^{125}I-labelled N-HDL_3 was associated with neutrophils. In the absence of α_1-AT, this difference was smaller. Possibly, this reflected proteolytic damage to the apolipoproteins of ^{125}I-labelled N-HDL_3 and especially ^{125}I-labelled AP-HDL_3 particles prior to or during association.

^{125}I-labelled HDL_3 degradation by neutrophil cell-conditioned medium was analysed next by SDS-electro-

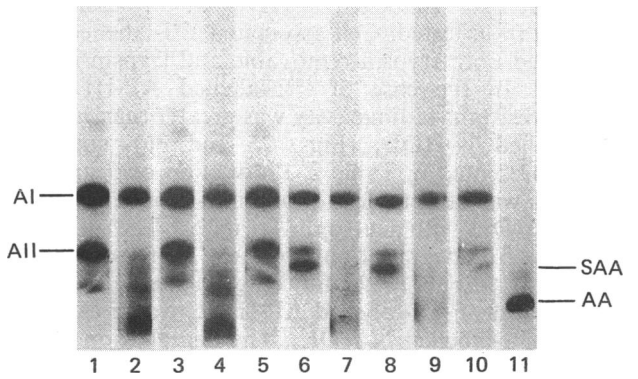

Fig. 1. SDS-electrophoresis of HDL_3 degradation products formed during incubation with cell-conditioned medium

^{125}I-labelled N-HDL_3 and ^{125}I-labelled AP-HDL_3 (25 μg of protein/ml) were incubated with cell-conditioned medium prepared from 10×10^6 cells for 60 min at 37 °C in the presence and absence of α_1-AT (1.5 mg/ml) respectively. Tracks 1, 6 and 11, ^{125}I-labelled N-HDL_3, ^{125}I-labelled AP-HDL_3 and ^{125}I-labelled amyloid A protein (AA) respectively. Tracks 2 and 3, ^{125}I-labelled N-HDL_3 degraded for 2.5 h without and with α_1-AT respectively. Tracks 4 and 5, ^{125}I-labelled N-HDL_3 degraded for 18 h without and with α_1-AT respectively. Tracks 7, 8, 9 and 10; ^{125}I-labelled AP-HDL_3 degraded as for ^{125}I-labelled N-HDL_3 in tracks 2, 3, 4 and 5 respectively.

phoresis of reaction products followed by autoradiography (Fig. 1). In the absence of α_1-AT, apo-A-II of N-HDL_3 was degraded almost completely to a band of approx. M_r 7000 after a 2.5 h incubation with cell-conditioned medium (Fig. 1, track 2). Further incubation for up to 18 h did not substantially alter the pattern of protein bands except for some degradation of apo-A-I (Fig. 1, track 4). In the case of AP-HDL_3, both apo-A-II and apo-SAA were completely degraded by 2.5 h, with apo-A-I remaining apparently resistant in the absence of α_1-AT in the medium (Fig. 1, track 7). However, definite degradation of apo-A-I was visible by 18 h under these conditions (Fig. 1, track 9). No low-M_r protein fragments or amyloid A-like protein band was detected even during shorter incubation times (results not shown). With α_1-AT present in the medium, no degradation of apo-A-I or apo-A-II on the N-HDL_3 particles was apparent even after incubation for 18 h (Fig. 1, tracks 3 and 5). Apo-A-II and apo-SAA on AP-HDL_3 were not visibly degraded after a 2.5 h incubation with α_1-AT (Fig. 1, track 8), but some degradation of these apolipoproteins was clearly detected by 18 h (Fig. 1, track 10). Degradation of apo-A-I on the AP-HDL_3 particles was not visible even after incubation for up to 18 h (Fig. 1, track 10). This pattern of degradation was observed in the case of three different batches of N-HDL_3 and AP-HDL_3. All further experiments were carried out in the presence of α_1-AT (1.5 mg/ml).

Association of ^{125}I-labelled N-HDL_3 and ^{125}I-labelled AP-HDL_3 with neutrophils as a function of time and concentration

The cell-association of both ^{125}I-labelled N-HDL_3 and ^{125}I-labelled AP-HDL_3 approached a steady-state at 60 min (Fig. 2*a*), and was a saturable process, as shown by concentration-dependence curves (Fig. 3*a*). The level of associated ^{125}I-labelled AP-HDL_3 material was greater than that of ^{125}I-labelled N-HDL_3 at any comparable concentration tested. Cell-associated ^{125}I-labelled HDL_3 comprised both trypsin-removable and trypsin-resistant proteins. In the case of ^{125}I-labelled AP-HDL_3, the trypsin-resistant radioactivity was greater than in that of ^{125}I-labelled N-HDL_3 (Figs. 2*b* and 3*b*), confirming results presented in Table 2. Production of degradation products was detectable within 2 min of incubating ^{125}I-labelled HDL_3 with neutrophils (Fig. 2*c*). The production of iodide-free degradation products from both ligands was linear for up to 90 min (Fig. 2*c*) and the rate of degradation of ^{125}I-labelled AP-HDL_3 was greater than that of ^{125}I-labelled N-HDL_3. The degradation rates of ^{125}I-labelled HDL_3 approached saturation at high concentrations of ^{125}I-labelled HDL_3 (Fig. 3*c*).

The contribution of proteases released from cells to the degradation of ^{125}I-HDL_3 was investigated to ascertain whether a cell-mediated proteolytic component exists in addition to proteases released into the medium as demonstrated by the action of α_1-AT (see above). Far fewer iodide-free degradation products were produced (Fig. 2*d* and 3*d*) by the cell-free system (cell-conditioned medium) than were released in the presence of cells (Fig. 2*c* and 3*c*).

The differences between AP-HDL_3 and N-HDL_3 with respect to the above-mentioned parameters were consistent features when neutrophils from six different subjects, and HDL_3 preparations from three normal and four acute-phase patients, were used in similar experiments. For example, the cell-association of AP-HDL_3 was 2.84 (±0.14)-fold ($n = 15$) higher than that of N-HDL_3, and the corresponding degradation rate was 8.11 (±0.71)-fold ($n = 15$) higher when HDL_3 (25 μg of protein/ml) was incubated with neutrophils for 60 min.

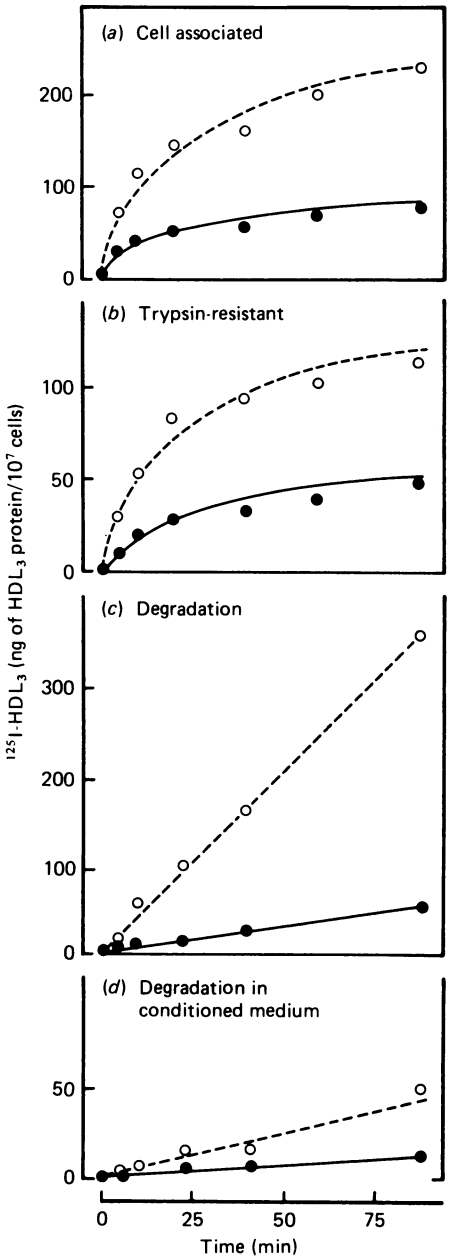

Fig. 2. Metabolism of HDL_3 by neutrophils as a function of time

^{125}I-labelled N-HDL_3 (●) and ^{125}I-labelled AP-HDL_3 (○) were incubated at 28 μg of protein/ml with neutrophils (10×10^6 cells) at 37 °C. (*a*) Total cell-associated ^{125}I-labelled HDL_3, (*b*) trypsin-resistant ^{125}I-labelled HDL_3, (*c*) iodide-free degradation products, (*d*) iodide-free degradation products generated by incubating cell-conditioned medium (prepared from 10×10^6 cells, various time intervals) with ^{125}I-labelled HDL_3 for time periods equivalent to that of the production of this medium.

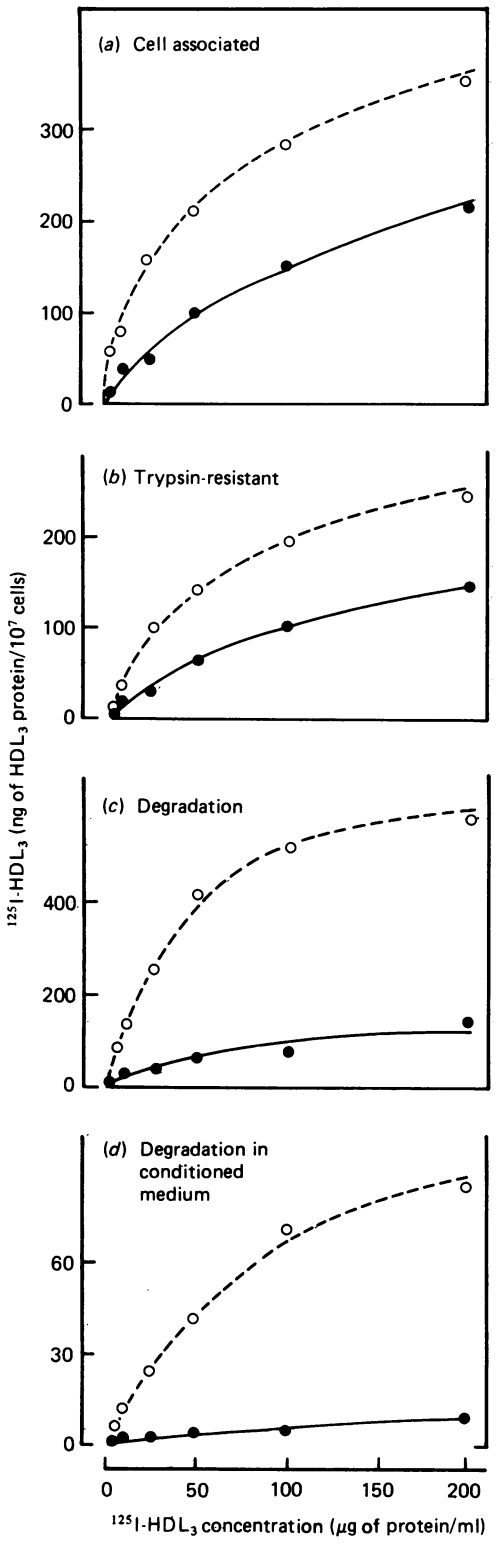

Fig. 3. Metabolism of HDL_3 by neutrophils as a function of HDL_3 concentration

^{125}I-labelled N-HDL_3 (●) and ^{125}I-labelled AP-HDL_3 (○) were incubated with neutrophils (10×10^6 cells) at 37 °C for 60 min. (*a*) Total cell-associated ^{125}I-labelled HDL_3, (*b*) trypsin-resistant ^{125}I-labelled HDL_3, (*c*) iodide-free degradation products, (*d*) iodide-free degradation products generated by cell-conditioned medium (prepared from 10×10^6 cells for 60 min) after incubation with ^{125}I-labelled HDL_3 for 60 min.

Influence of AP-HDL_3 and N-HDL_3 on release of proteolytic enzymes from neutrophils during cellular degradation of HDL_3

Linear concentration-dependent release by N-HDL_3 of an elastase with proteolytic activity directed against apo-A-II from neutrophils has been described [8,21]. In order to clarify the observed cell-associated degradation of HDL_3 (Figs. 2*c*, 2*d*, 3*c* and 3*d*), we measured the release from neutrophils of proteolytic enzymes capable of degrading AP-HDL_3 apolipoproteins (especially apo-SAA). Although only 25 μg of HDL_3/ml was available to elicit release during cell-associated degradation experiments, the results in Table 3 show the influence of 1 mg each of AP-HDL_3 or N-HDL_3/ml on the release of proteolytic enzymes. Even at these high concentrations of HDL_3, the release of enzymes by either AP-HDL_3 or N-HDL_3 could not fully account for the observed rates of total cellular degradation (Figs. 2*c*, 2*d*, 3*c* and 3*d*). Enzymes released by AP-HDL_3 or by N-HDL_3 resulted in about 30% and 90% increases in the degradation of AP-HDL_3, in the absence and presence of α_1-AT respectively. Total cellular degradation rates of ^{125}I-labelled AP-HDL_3 (when 1 mg of HDL_3/ml was available to evoke release of proteolytic enzymes) were about three times higher than those brought about by conditioned medium lacking α_1-AT and about 15 times higher when α_1-AT was present (Table 3). No proteolytic activity was lost during the different steps employed to prepare the conditioned medium, since similar degradation rates of ^{125}I-labelled AP-HDL_3 were obtained when freshly-prepared conditioned medium (in the absence of HDL_3) was compared with conditioned medium subjected to all the preparation steps referred to above ('control conditioned medium'). These findings therefore suggested than an α_1-AT-resistant cellular proteolytic component was present in the overall degradation of HDL_3 apolipoproteins by neutrophils.

The possible contribution of lysosomes to the degradation of ^{125}I-AP-HDL_3 during incubation with neutrophils was investigated using the lysosomotropic inhibitors chloroquine (100 μM) and NH_4Cl (25 mM). No significant inhibitory effect on the production of iodide-free degradation products was found using either of these inhibitors of lysosomal function (results not shown).

Nature of cell-associated apolipoproteins

Apo-A-I was the major cell-associated apolipoprotein when ^{125}I-labelled N-HDL_3 was incubated with neutrophils (Fig. 4*a*). When ^{125}I-labelled AP-HDL_3 was used (Fig. 4*b*), however, apo-SAA was the predominant apolipoprotein associated with the cells. This apparently selective association of apo-SAA was reproducibly demonstrated by the binding of three different batches of ^{125}I-labelled AP-HDL_3 to three different neutrophil preparations for 60 min (Fig. 4*d*). The enrichment of apo-SAA in cell-associated fractions was not due to apolipoproteins non-specifically associated to the reaction tubes (results not shown). The proportion of ^{125}I-apo-SAA in the starting HDL_3 was $36 \pm 5\%$ ($n = 3$), whilst $82 \pm 7\%$ ($n = 3$) of the cell-associated apolipoprotein was ^{125}I-apo-SAA. Fig. 4(*c*) shows that following trypsinization, the cell-associated apo-SAA was mostly degraded when compared with untreated samples. This indicates that trypsin gained access to the intact cell-associated ^{125}I-labelled apo-SAA, and confirms the cell-surface location of the associated ^{125}I-apo-SAA.

Table 3. Influence of AP-HDL_3 and N-HDL_3 on the release of proteolytic enzymes from neutrophils

Degradation is that of 5 μg of ^{125}I-labelled AP-HDL_3 or ^{125}I-labelled N-HDL_3 (200 μl of RPMI 1640; 60 min; 37 °C; with or without α_1-AT; 1.5 mg/ml); by either 2×10^6 neutrophils (total), or conditioned media representing release from 2×10^6 neutrophils (ultracentrifugal separation of HDL_3 from medium after release). AP-HDL_3, N-HDL_3 and controls were used as described in the Experimental section to evoke proteolytic enzyme release.

	Total release (ng)	N-HDL_3 released (conditioned) (ng)	AP-HDL_3 released (conditioned) (ng)	Control (conditioned) (ng)
Degradation of AP-HDL_3				
$-\alpha_1$-AT	424±27	141±19	145±11	109±4
$+\alpha_1$-AT	207±26	14±1	14±2	8±2
Degradation of N-HDL_3				
$-\alpha_1$-AT	32±6	37±2	47±5	43±5
$+\alpha_1$-AT	13±2	0.2±0.06	0.6±0.01	0.8±0.05

Fate of cell-associated ^{125}I-labelled AP-HDL_3

The fate of cell-associated ^{125}I-labelled AP-HDL_3 was studied in pulse-chase experiments. Cells were pulse-labelled with ^{125}I-labelled AP-HDL_3 and then incubated in an HDL_3-free medium at 37 °C for various time periods. The results of a typical experiment are summarized in Fig. 5. A rapid initial rate of release of ^{125}I-labelled material was observed. Analysis of the integrity of the HDL_3 protein that was released into the medium, measured by iodide-free degradation products in the chase medium, revealed that 85% of the ^{125}I-labelled AP-HDL_3 that was released was trichloroacetic acid-precipitable (Fig. 5) and detectable as intact apo-SAA on SDS-gels (results not shown). A small but significant fraction of the released material was nevertheless recovered as trichloroacetic acid-soluble degradation products. Although ±50% of the cell-associated material was not released from the cells even after prolonged post-incubation periods (1 h; Fig. 5), this material represented relatively degraded apo-SAA (Fig. 5 inset). Less than 10% of the original cell-associated apo-SAA at zero time (Fig. 5 inset, track 2) could be resolved as intact apo-SAA during any time of the chase period (Fig. 5 inset, tracks 3–5). Less than 15% of cell-associated material dissociated from the cells at 4 °C in 5 min, indicating that only minimal losses of bound material occurred during the wash process (4 °C, less than 3 min) employed between the pulse- and chase-incubation periods.

DISCUSSION

Apo-SAA, which is produced by the liver during the acute-phase response, exists in the plasma mainly as an apolipoprotein of HDL_3 [9,10]. It generally constitutes 15–50% of the total HDL_3 apolipoproteins but in extreme circumstances the percentage of apo-SAA can rise to 80% of the total HDL_3-apolipoprotein content [17,18]. Newly synthesized apo-SAA seems to associate with existing HDL_3 in the circulation with resultant remodelling of the surface of such particles yielding larger HDL_3 particles which are denser and depleted of apo-A-I [10,18]. The protein–lipid ratios and lipid composition of AP-HDL_3 and N-HDL_3 are similar both in man [10] and monkey [19].

As neutrophil leukocytosis occurs together with increases in plasma apo-SAA levels during the acute-phase period [7], our study has focused on a comparison of the association of N-HDL_3 and AP-HDL_3 with neutrophils and the accompanying degradation of the HDL_3 during association. Our results have clearly shown that more ^{125}I-labelled AP-HDL_3 associates with neutrophils than ^{125}I-labelled N-HDL_3. The presence of physiological concentrations of α_1-AT in the incubation medium increases the association of ^{125}I-labelled HDL_3 with neutrophils. The proteolytic alteration of the apolipoproteins in the absence of α_1-AT could account for the decreased association of ^{125}I-labelled HDL_3.

The association of N-HDL_3 with cells, including neutrophils, is believed to be apo-A-I mediated but the nature of this has not been completely resolved [20,21]. Our results do show that when ^{125}I-labelled N-HDL_3 is associated with neutrophils, apo-A-I is the major apolipoprotein that can be visualized after SDS electrophoresis (Fig. 4*a*). However, in the case of the association of ^{125}I-labelled AP-HDL_3 with neutrophils, apo-SAA is the predominant associating apolipoprotein. This association is apparently saturated at about 150 μg of total AP-HDL_3 protein/ml. Scatchard analysis of the results is not feasible because of ligand degradation. It remains unresolved whether apo-SAA dissociates from the AP-HDL_3 particle to bind to neutrophils or whether a subset of apo-SAA-rich particles preferentially bind to the cells. AP-HDL_3 has been shown to be polydisperse with respect to apolipoprotein distribution; particles do exist where apo-SAA is the major apolipoprotein [10,26]. In any event, our results may explain the observed greater metabolic turnover rate of apo-SAA compared with that of any other HDL_3 apolipoprotein [23–25].

In analysing the intact apo-SAA associated with neutrophils, it is obvious that although a fraction exists that is resistant to trypsin release, this is not likely to be intracellular as analysis after SDS-electrophoresis revealed degradation of the apo-SAA after exposure to trypsin, reflecting accessibility of surface-bound intact apo-SAA to this exogenous protease.

Pulse-chase experiments have shown a rapid loss from the cell of intact apo-SAA; a significant pool of relatively degraded apo-SAA remained associated with the neutrophils. The nature and function of this pool is unknown,

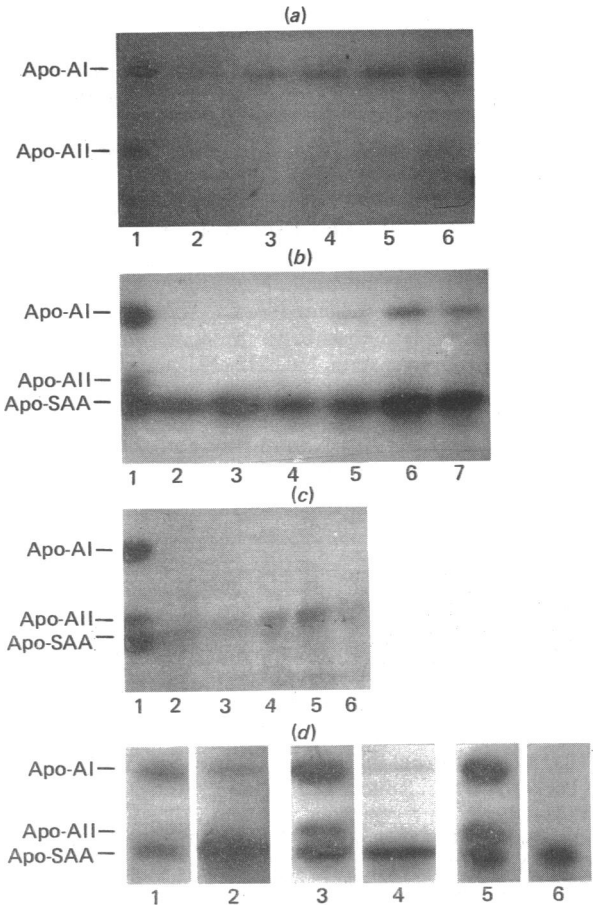

Fig. 4. Nature of cell-associated apolipoproteins

Neutrophils (10×10^6) were incubated with various concentrations of HDL_3 (60 min, 37 °C) and the nature of the cell-associated apolipoproteins was analysed by 5–20% SDS/polyacrylamide-gel electrophoresis. In (*a*), (*b*), and (*c*) the starting HDL_3 apolipoproteins are shown in each case in track 1. (*a*) Association of ^{125}I-labelled N-HDL_3 apolipoproteins. Tracks 2 to 6 represent the apolipoproteins associated with 250 μg of cellular protein after incubation with 10, 25, 50, 100 and 150 μg of ^{125}I-labelled N-HDL_3/ml respectively. (*b*) Association of ^{125}I-labelled AP-HDL_3 apolipoprotein. Tracks 2 to 7 represent the apolipoprotein associated with 250 μg of cellular protein after incubation with 10, 25, 50, 100, 150 and 200 μg of ^{125}I-labelled AP-HDL_3/ml respectively. (*c*) ^{125}I-labelled AP-HDL_3 apolipoprotein remaining cell-associated after trypsinization of the cells following incubation with 10, 25, 50, 100 and 150 μg of ^{125}I-labelled AP-HDL_3/ml (tracks 2 to 6 respectively). These represent the apolipoprotein remaining associated with 250 μg of cellular protein. In each case the radioactivity associated with intact apo-SAA represents less than 10% of the corresponding total cell-associated ^{125}I-labelled apo-SAA. (*d*) Preferential apo-SAA cell-association (25 μg/ml, 60 min, 37 °C). Nature of ^{125}I-labelled AP-HDL_3 (three different batches) is shown in tracks 1, 3, and 5. Corresponding cell-associated ^{125}I-labelled AP-HDL_3 is shown in tracks 2, 4, and 6 (three different neutrophil preparations).

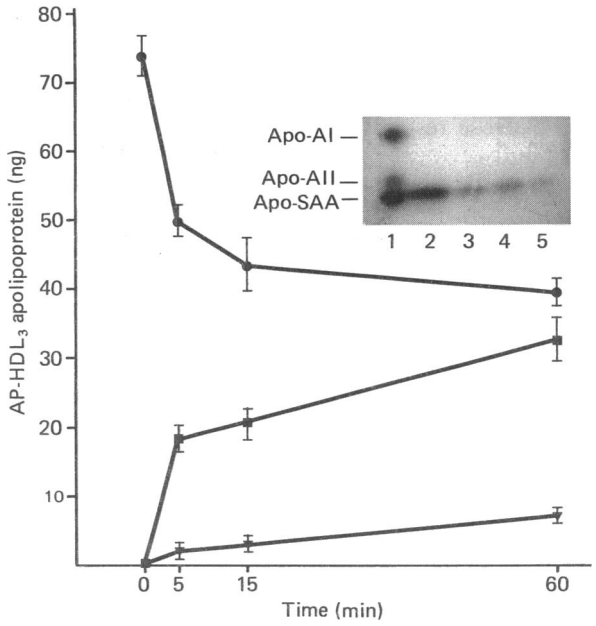

Fig. 5. Fate of cell-associated ^{125}I-labelled AP-HDL_3

Neutrophils (10×10^6 cells) were incubated with ^{125}I-labelled AP-HDL_3 (25 μg/ml) for 60 min at 37 °C, washed and then incubated (37 °C) as described in the Experimental section in an HDL_3-free medium (37 °C). ●, Total cell association; ■, released in supernatant; ▼, degraded apolipoproteins. Inset: Track 1 offered ^{125}I-labelled AP-HDL_3. Tracks 2 to 5, apolipoproteins remaining cell-associated after 0, 5, 15 and 60 min respectively. For each of tracks 2 to 5, 250 μg of cellular protein was loaded.

but it is tempting to speculate that in certain individuals, after prolonged exposure to AP-HDL_3 in areas of inflammation, this pool of intact SAA can be the source of amyloid A protein for polymerization into amyloid fibrils.

Degradation of ^{125}I-labelled N-HDL_3 and ^{125}I-labelled AP-HDL_3 by cell-conditioned medium revealed degradation of apo-A-II on ^{125}I-labelled N-HDL_3, as previously reported [8], and apo-A-II and apo-SAA on ^{125}I-labelled AP-HDL_3. It thus seems likely that the greater degradation of ^{125}I-labelled AP-HDL_3 observed during association with neutrophils is due to the degradation of both apo-A-II and especially apo-SAA.

The degradation rates of ^{125}I-labelled HDL_3 during association with cells and by cell-conditioned medium increase hyperbolically at increasing concentrations both of ^{125}I-labelled N-HDL_3 and ^{125}I-labelled AP-HDL_3. Since degradation products are detectable within 2 min after the start of the ^{125}I-labelled HDL_3 incubations, it is unlikely to be the result of internal cellular catabolism. In addition, results from pulse-chase experiments and the inability of lysosomotropic inhibitors to affect degradation indicate that intracellular lysosomal processing, as suggested by Silverman *et al.* [4], is not likely to contribute to the observed degradation, even though it has been shown that lysosomal neutrophil elastase can degrade lipid-free SAA [4].

Some of our observed cell-mediated degradation could have been due to a concentration-dependent HDL_3-mediated egress of elastase from neutrophils, which in the case of N-HDL_3, has been reported to result in the degradation of apo-A-II [8]. We have found, however, that cellular degradation, in addition to being relatively α_1-AT-resistant, is of such magnitude that 'conditioning' of the medium cannot fully account for it. Our findings rather point to degradation activity being at least partly

a cell-surface event, making it conceivable that membrane-associated enzymes play an important role in the degradation of apo-SAA and other apolipoproteins of HDL_3. Lipid-free SAA has been shown to be degraded by an elastase residing on the surface of monocytes [5] and recently evidence of the existence of a neutral serine protease in the membranes of neutrophils has been found [22]. The exact mechanism of apo-SAA breakdown needs to be elucidated in view of the possible importance of such events in various pathological processes.

K. Lyner, A. E. M. Strümpher, S. Gopal and S. Beer are thanked for their technical assistance and W. Gevers for critically reading the manuscript. The financial support of the South African Medical Research Council is acknowledged.

REFERENCES

1. Morley, J. J. & Kushner, I. (1982) Ann. N.Y. Acad. Sci. **389**, 406–418
2. Ramadori, G., Sipe, J. D., Dinarello, C. A., Mizel, S. B. & Colten, H. R. (1985) J. Exp. Med. **162**, 930–942
3. Shephard, E. G., Van Helden, P. D., Strauss, M., Böhm, L. & De Beer, F. C. (1986) Immunology **58**, 489–494
4. Silverman, S. I., Cathcart, E. S., Skinner, M. & Cohen, A. S. (1982) Immunology **46**, 737–744
5. Lavie, G., Zucker-Franklin, D. & Franklin, E. C. (1980) J. Immunol. **125**, 175–180
6. Husebekk, A., Skogen, B., Husby, G. & Marhaug, G. (1985) Scand. J. Immunol. **21**, 283–287
7. Kisilevski, R., Bondreau, L. & Forster, D. (1983) Lab. Invest. **48**, 60–67
8. Byrne, R. E., Polacek, D., Gordon, J. I. & Scanu, A. M. (1984) J. Biol. Chem. **259**, 14537–14543
9. Benditt, E. P. & Eriksen, N. (1977) Proc. Natl. Acad. Sci. U.S.A. **74**, 4025–4028
10. Coetzee, G. A., Strachan, A. F., Van der Westhuyzen, D. R., Hoppe, H. C., Jeenah, M. S. & De Beer, F. C. (1986) J. Biol. Chem. **261**, 9644–9651
11. Böyum, A. (1968) Scand. J. Clin. Lab. Invest. **21** (Suppl. **97**), 77–89
12. Godenir, N. L., Jeenah, M. S., Coetzee, G. A., Van der Westhuyzen, D. R., Strachan, A. F. & De Beer, F. C. (1985) J. Immunol. Methods **83**, 217–225
13. Lowry, O. H., Rosenbrough, N. J., Farr, A. L. & Randall, R. J. (1951) J. Biol. Chem. **193**, 265–275
14. Bilheimer, D. W., Eisenberg, S. & Levy, R. I. (1972) Biochim. Biophys. Acta **260**, 212–221
15. Bierman, E. L., Stein, O. & Stein, Y. (1974) Circ. Res. **35**, 136–150
16. Laemmli, U. K. (1970) Nature (London) **227**, 680–685
17. Eriksen, N. & Benditt, E. P. (1984) Clin. Chim. Acta **140**, 139–149
18. Clifton, P. M., Mackinnon, A. M. & Barter, P. J. (1985) J. Lipid Res. **26**, 1389–1398
19. Parks, J. S. & Rudel, L. L. (1985) J. Lipid Res. **26**, 82–91
20. Eisenberg, S. (1984) J. Lipid Res. **25**, 1017–1058
21. Polacek, D., Byrne, R. E., Burrous, M. & Scanu, A. M. (1984) J. Biol. Chem. **259**, 14531–14536
22. Pontremoli, S., Melloni, E., Michetti, M., Sacco, O., Sparatore, B., Salamino, F., Damiani, G. & Horecker, B. L. (1986) Proc. Natl. Acad. Sci. U.S.A. **83**, 1685–1689
23. Parks, J. S. & Rudel, L. L. (1983) Am. J. Pathol. **112**, 243–249
24. Hoffmann, J. S. & Benditt, E. P. (1982) J. Biol. Chem. **257**, 10510–10517
25. Bausserman, L. L., Herbert, P. N., Rodger, R. & Nicolosi, R. J. (1984) Biochim. Biophys. Acta **792**, 186–191
26. Strachan, A. F., De Beer, F. C., Coetzee, G. A., Hoppe, H. C., Jeenah, M. S. & Van der Westhuyzen, D. R. (1986) Protides Biol. Fluids **34**, 359–362

Received 20 March 1987/8 July 1987; accepted 27 August 1987