Scavenger Activity in Monocyte-Derived Macrophages From Atherothrombotic Strokes

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SUMMARY Foam cells are lipid-laden macrophages derived primarily from circulating mononuclear cells and are a characteristic feature of atheromatous lesions. The exact role of these foam cells in the pathogenesis of atherosclerotic lesions remains uncertain, but one potential function is to take-up and process excess interstitial arterial lipoproteins, suggested by their extraordinary ability to engulf enormous quantities of modified low density lipoproteins by the so-called “scavenging pathway.” To test this possibility, monocytes from 15 atherothrombotic brain infarct patients and age and sex matched controls were isolated and cultured for 7–8 days in 20% normal serum. The monocyte-derived macrophages were investigated for their ability to bind, internalize and degrade both native and modified (acetylated) LDL labelled with 125I-iodine. While native LDL was metabolized similarly, stroke macrophages displayed significantly reduced ability to scavenge modified LDL. These findings suggest that insufficient processing of interstitial arterial cholesterol by monocyte-derived macrophages may contribute to the aggravation of atheromatous formation. This inadequacy is likely further compromised by reduced levels of serum high density lipoprotein since the absence of a cholesterol-acceptor will promote the slow but continued accumulation of lipids and the formation of foam cells.

ATHEROSCLEROTIC LESIONS are characterized histologically by smooth muscle cell proliferation, accumulation of cholesteryl esters, and foam cells, and each of these elements is believed to play a pathogenetic role. The last, the foam cell, representing primarily lipid-laden macrophages derived from monocytes, has an uncertain role since it is believed on the one hand to be the “messenger of destruction” by releasing its hydrolytic enzymes but on the other a “scavenger” of excess interstitial lipids in arterial wall for luminal or hepatic disposal. Supporting the latter contention is the development of receptors for modified low density lipoproteins on monocyte-derived macrophages (MDMs), termed the “scavenger pathway” by Brown and Goldstein, and the associated ability to accumulate enormous quantities of cholesterol intracellularly. Coupling this activity to clear damaged or trapped lipoproteins in arterial wall is the ability of cholesteryl ester loaded MDMs to synthesize and secrete apoprotein E. This activity has been postulated to facilitate the transport and receptor processing of HDL-transported cholesterol by liver hepatocytes. When the efficient processing of interstitial arterial cholesterol is overloaded, MDM become converted to foam cells.

Since atherothrombotic brain infarctions (ABIs) display reduced acid cholesteryl ester hydrolase activity in circulating mononuclear cells which may affect the functional activity of its derivative MDM for “scavenging activity”, we investigated the ability of ABI patients’ MDM to bind, internalize and degrade native and modified LDL labelled with 125I-Iodine, compared to age and sex-matched controls. Our results indicate that stroke patients’ MDM have defective scavenging of modified LDL, but not of native LDL, which suggests that this abnormality may play a role in provoking the atherosclerotic process.

Methods

Atherothrombotic brain infarction (ABI) patients are selected from those admitted to the neurology service of the Hermann Hospital. These patients met the standard criteria of ABI in having evidence for atherosclerotic vascular disease, particularly extracranial occlusive disease, and the absence of evidences for cardiogenic emboli, intracerebral hemorrhage, subarachnoid hemorrhage or miscellaneous vascular disorders such as arteritis. Controls are obtained from healthy elderly volunteers without cerebrovascular symptoms who attend local senior citizen groups. Sodium (123I) iodide (17 mCi/mg) and (20-14C) sodium acetate (57 mCi/mMol) is purchased from Amersham Corporation and New England Nuclear respectively.

Phosphate buffered saline and RPMI-1640 medium are purchased from Corning and New England Biologicals respectively. Phosphate buffered saline and RPMI-1640 medium are purchased from Corning and New England Biologicals respectively. RPMI medium is purchased from Flow Laboratories.

Human mononuclear cells are isolated from freshly drawn blood from control and stroke patients by Ficoll-Hypaque gradient centrifugation. The cells are washed with RPMI-1640 medium by centrifugation at 1000 rpm for 10 minutes. The washed cells are diluted with RPMI and 20% human serum to a concentration of 3-4×10^9 cells/ml. 1.5 ml aliquots are placed in 35 mm tissue culture dishes and then incubated for 2 hours at 37°C to allow monocytes to adhere to the flask. The flasks are then swirled to remove loosely adherent cells and medium is replaced with fresh medium. To minimize the possibility of any variability between cell lines due to cholesterol content of the medium, cells from control subjects and from patients are maintained in medium containing normal serum. No
significant differences have been observed in the growth and development of cells obtained from control subjects or from patients. Experiments are routinely performed on day 7 or 8 after seeding of cells.

Normal serum lipoproteins are prepared as previously described;¹³ low density lipoproteins (LDL) are isolated in the density range 1.019–1.063, high density lipoproteins (HDL) in the range 1.063–1.21. Each lipoprotein fraction is repurified once by ultracentrifugation and dialysed extensively against 5 mM phosphates, 1 mM EDTA, 0.15M NaCl (pH 7.4). Purified lipoproteins are filtered through a 0.45 mM filter into a sterile vial and aliquots are taken for protein and lipid extraction.¹⁴ Acetylated LDL is prepared as described by Basu et al¹⁵ and the lipoproteins are iodinated using a modification¹⁶ of the method of McFarlane.¹⁷ The range of specific activities are 250–350 cpm/ng. The amount of radioactivity associated with lipids was less than 2% of the total trichloracetic acid precipitable radioactivity.

Cholesterol synthesis by macrophages is estimated by measuring by incorporation of (2-¹⁴C) acetate into cholesterol as described earlier.¹⁸ The cells are washed with RPMI, then medium containing 1500 µM (Z-¹⁴C) acetate, 5 µCi/dish is added and incubated at 37°C in a humidified incubator for 5 hours. The cells are then washed three times with phosphate buffered saline and harvested by scraping the dishes with a teflon policeman, (1, 2-³H) cholesterol is added as an internal standard to correct for procedural losses. Lipids are extracted with chloroform/methanol (1/1, v/v) and protein residues sedimented.

Lipid-free cell pellets was dissolved in 0.1M NaOH and the protein contents are determined by the method of Lowry et al.¹⁹ The lipid extracts are partitioned with water, the chloroform extract taken to dryness and redissolved in chloroform. An aliquot is saponified by 1 M KOH in ethanol for 1 hour at 37°C. Sterols are extracted with 3 × 4 ml of hexane and non-saponifiable lipids further separated by thin layer chromatography on silica gel-G in the solvent system ethyl acetate/hexane/diethyl ether (10/40/10, v/v/v). The plate is then removed, allowed to dry briefly and re-run in the second solvent system heptane/diethyl ether (95/20, v/v). The cholesterol containing band is counted. Very little radioactive cholesterol is recovered from the incubation media under all assay conditions. Total and HDL cholesterol of the plasma of 5 stroke patients and in the incorporation of (2-¹⁴C) acetate into cholesterol is determined (table 1). Preliminary experiments show that acetate incorporation is relatively low for the first two days and become maximum between the 6th and 9th days of culture (data not shown). The amount of acetate incorporated into cholesterol is significantly higher in stroke patients’ macrophages compared to controls (p < .001). The amount of radioactivity secreted in the media as ¹⁴C-cholesterol during the 5 hour incubation period is negligible.

Table 2 shows the degradation of native LDL and acetyl LDL in MDM from controls and from stroke patients. For the determination of lipoprotein degradation, cells are incubated in lipoprotein deficient medium for 4 hours, since longer periods of incubation in LPDS cause a fall in the activity of certain enzymes.²⁰ Although the standard culture medium already contains approximately 150 µg LDL protein/ml, which fully saturates the LDL receptors, addition of LDL (200 µg/ml) provokes a reduction in LDL degradation and in the incorporation of (2-¹⁴C) acetate into cholesterol (data not shown). Four hour preincubation in LPDS causes a significant increase in these activities.
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without any morphological changes. Comparison of LDL and acetyl LDL degradation by MDM from 15 control and 15 stroke patients show that macrophages from stroke patients degrade less acetyl LDL (table 2, p < .01) than controls. In contrast, LDL receptor activity is maintained under the same conditions. Table 4 shows the cell association and degradation of acetyl-LDL by MDM from 4 controls and 4 stroke patients. In all concentrations used cell association rate is lower in stroke than control cells and the trichloracetic acid soluble radioactivity appearing in the medium is also lower.

Total plasma cholesterol and HDL cholesterol concentrations as well as the HDL/total cholesterol ratio in 5 controls and 4 stroke patients are shown in table 3. HDL cholesterol and HDL/total cholesterol ratio are lower in subjects with atherothrombotic strokes than in age matched control subjects.

Discussion

Monocyte-derived macrophages (MDM), converted to lipid-laden foam cells, are a recognized constituent of atheromas, along with an increase in the proliferation of smooth muscle cells, in the content of cholesterol esters, and in the deposition of connective tissue substances.\(^1\)\(^2\) But the mechanisms of foam cell formation, regulation and precise role remain uncertain, particularly on whether foam cells play a crucial role in either inducing or aggravating the atherosclerotic process. Recent investigations indicate, however, that MDMs may function instead to be protective by their ability to reduce the lipid content in the arterial interstitium.

The “scavenging pathway”, described by Goldstein et al,\(^2\) provide cells without adequate membrane LDL-receptors, as in familial hypercholesterolemia, a mechanism to satisfy membrane cholesterol requirements not met by endogenous synthesis, and by LDL receptors.\(^3\) The lipoprotein pinocytosed is for “modified” or negatively charged LDL, as by acetylation, commonly used experimentally.\(^2\)\(^4\) Although the “scavenging pathway” is well characterized, the in vivo existence of mLDL has eluded detection. However, modification of LDL by platelets,\(^3\) endothelial cell,\(^4\) and a lipid transfer factor\(^2\)\(^4\) suggests that biological alterations of LDL are likely. In the present study we compared the relative ability of MDM from control subjects and stroke patients to take up and degrade acetylated LDL. Both uptake and degradation data (Table II and IV) indicate that in stroke MDM the processing of modified lipoproteins is significantly reduced. It has been shown that the expressions of lipoprotein receptor activity in MDM is a function of the time in culture as well as the sterol content in the medium.\(^25\) The differences in our results for the acetyl LDL uptake and degradation cannot be attributed to these variables since all cells are used at the same stage of maturation and cultured in 20% normal serum.

Recently, Traber et al\(^26\) utilized indirect immunoper-
oxidase techniques and electronmicroscopy to distinguish the binding characteristics of MDM surface membranes for LDL and acetylated LDL. Unlike LDL receptors, acetylated LDL receptors are uniformly distributed on the membrane surface and not at the "coated pits". These findings indicate that the "scavenging pathway" is not a modification of the LDL receptor but a wholly different and novel mechanism for efficient uptake.

Whether MDM plays a role in either provoking or preventing atherosclerosis remains controversial, as noted above and recently reviewed by Steinberg. However, increasing attention has been focused on the protective role of MDM in scavenging arterial wall to clear damaged lipoproteins. Human MDM normally possess an efficient efllux mechanism for accumulated cholesteryl esters, shown recently to be associated with apo E secretion. Whether defective influx of mLDL in ABI MDMs, as well as the efficient extrusion of cholesteryl esters, relates to an impairment of factors regulating apo E secretion is under current investigation in our laboratory.

It is of interest that the ABI patients investigated bind and internalize native LDL at a comparable level of controls, inferred from similar levels of LDL degradation (table 2), quantitated by the TCA soluble label and representing the amino acid product of lipoprotein hydrolysis. On the other hand, scavenging of mLDL, which can normally lead to an enormous increase in cholesteryl accumulation, is significantly reduced in stroke MDM (table 2). Since scavenging MDMs have an enhanced need for membrane synthesis to accomodate its increased capacity for scavenging, the requirement for cholesterol has a parallel increase. Enhanced synthesis of endogenous cholesterol in ABI MDMs (table 1) is interpreted to reflect a cellular compensatory mechanism to provide the MDM membrane constituent.

The precise pathogenesis and role of foam cells in atherosclerosis remains uncertain. Nonetheless, the dynamic nature of the foam cell and of macrophages is demonstrated autoradiographically by their ability to take up and degrade actively LDL in cerebral and carotid arteries of subhuman primates. In addition, the lymphatics and lymph nodes draining the carotid arteries of subhuman primates is infiltrated with lipid-laden macrophages. Aside from the transport of these macrophages from the arterial interstitium, macrophages require an acceptor for their intracellular cholesterol, such as HDL, to both prevent excess intracellular accumulation of cholesterol and to facilitate "reverse cholesterol transport" by unloading its cholesterol at hepatocytes.

Stroke patients may be vulnerable to accelerated atherogenesis in part because the arterial interstitial scavenging activity is reduced, as suggested by our data, but also by reduced serum HDL (table 3), a consistent finding in stroke patients. The data in table 3 show a trend toward statistical significance but larger numbers of patients are needed for meaningful interpretation.

Reduced concentrations of HDL may compound the efficient processing of cholesterol for its role in "reverse transport" for liver disposal. Recent evidence from our laboratory demonstrates "HDL retroendotheliosis" in stroke patients (data not shown) which may play a role in cholesterol removal from MDM. Thus, while "scavenging system" deficiency should prevent foam cell formation by restricting cholesterol uptake, the added effect for cholesterol efflux may result in lipid laden foam cells.

The importance of the "scavenging" activity in vivo is uncertain, but the more dynamic and functional molecular indices of atherosclerosis risk should be of clinical relevance, as opposed to the static measures of lipid concentration such as LDL, HDL or apoproteins B and AI. These dynamic molecular indices may offer more sensitive means of identifying individuals prone to accelerated atherogenesis. Interventional therapeutic maneuvers to minimize the defect include reduction of the cholesterol "load", despite its apparently "normal" serum concentration, with the use of low cholesterol diets or resins such as cholestyramine. In addition, measures to enhance the "scavenging activity" by MDM may prove feasible, particularly with calcium channel blockers. On the basis of our findings, we conclude that ABI's MDM is defective in scavenging mLDL; this impairment may provoke the atherosclerotic process and may identify individuals at potential risk for accelerated atherosclerosis prone to atherothrombotic brain infarction (ABI).

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