Neutralizing LDL-induced white blood cell damage: investigation of a new antioxidant route

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Abstract

Incorporation of oxidized low-density lipoprotein (ox-LDL) into macrophages adherent to the lumen of vascular tissue is a necessary step in the development of atherosclerotic plaques. Theses regions are characterized by the progressive accumulation of cells, cellular debris, lipid and extracellular matrix material and are found primarily at vascular bifurcations experiencing low levels of blood-flow mediated fluid stress. Macrophage cells found at the core of these plaques are sequestered from circulating anti-oxidants, which could otherwise neutralize damaging ox-LDL. Since regulating the interaction of LDL with macrophages is critical to inhibiting plaque development, the present studies investigated the effectiveness of mechanical and chemical preconditioning in reducing the consequences of oxidative injury mediated by LDL. The results of our studies suggest that intermediate shear stress levels, typical of bifurcated vascular regions, may contribute to LDL mediated macrophage damage. However, oxidative injury may be modulated by pre-incubation with an extracellular source of the free radical scavenger, reduced glutathione (GSH).

Introduction

Atherosclerosis is an occlusive arterial disease that causes about 75% of all adult deaths in Western society (Callow, 1989). The recruitment of monocyte/macrophage leukocytes to nascent plaque sites, their ingestion of oxidized forms of low density lipid (LDL), and their differentiation into foam cells are especially critical to the early escalation of atherosclerotic plaque formation. In fact, oxidative mediated macrophage damage may predicate the progression of other atherosclerotic events resulting in severe vascular damage (Mitchinson et al., 1995; Davies, 1986).

Prior to their entry into vascular intimal spaces, the sites of plaque development, recruited monocytes/macrophages adhere to endothelial cell layers and continue to be exposed to normal physiological blood flow and its accompanying shear forces.

Higher levels of shear forces, in turn, can regulate both macrophage phenotype and function, causing significant activation of "generic cellular stress protein based responses", which can physiologically pre-condition macrophages to resist stress mediated cell damage (Rosenson-Schloss et al., 1999).

This pattern of response to shear stress is important because atherosclerotic plaque deposition predominantly occurs at areas of vascular bifurcation characterized by disrupted blood flow (Liepsch, 1990). Since blood flow patterns around vascular bifurcations typically give rise to lower levels of shear forces, they may induce only modest levels of stress response activation.

Regions with suboptimal stresses could invariably be predisposed to the rapid onset of atherosclerosis, in part, due to deficiency in neutralizing incorporated oxLDL. Intracellular accumulation of excessive ox-LDL byproducts and reactive oxygen intermediates (ROI) promotes oxidative stress and consequent cell damage. However, intracellular oxidative damage may also be neutralized by specific biochemical anti-oxidant factors, such as reduced glutathione (GSH), a constitutive free radical scavenger. Preconditioning by incubation with extracellular GSH prior to stress exposure, may thus play important physiological roles in limiting either the oxidation of LDL, or the accumulation of intracellular reactive oxygen intermediates (Aviram, 1995) thereby reducing severe macrophage mediated oxidative damage and significantly altering their role in plaque formation dynamics, especially at vascular bifucations.

In this study we assessed the effect of differential shear on macrophage/LDL interaction. Our results indicate that cells exposed to 10 dyne/cm^2 shear, typical of bifurcated vascular regions, were prone to enhanced damaging effects following exposure to an oxidized LDL analog, AcLDL. In addition, preconditioning with the anti-oxidant tripeptide, glutathione, mitigated both the damaging effects of LDL exposure in the absence of flow, and LDL incorporation in 10 dyne/cm^2 flow exposed cells.

Materials and methods

Tissue Culture: IC21 macrophage cells (ATCC, Rockville, MD) were propagated by culture with RPMI media containing 10% Fetal Bovine Serum and Penicillin/Streptomycin, in a humidified CO_2 atmosphere as previously described (Rosenson-Schloss et al., 1999). All media components were purchased from Biowhittaker (Walkersville, MD).

Flow studies

A temperature controlled flow system was designed, as previously described (Rosenson-Schloss et al., 1999). Experiments were conducted at 25, 10 or 3 dyne/cm^2 / inverted flow chamber, in a HBSS Ca^{+2} containing buffer. The portable flow system

was attached to an inverted microscopic imaging system containing a 37°C heated stage. Static conditions (0 dyne/cm^2) were established by placing inverted slides in a 37°C, 5% CO_2 incubator. Flow experiments were conducted for 6 hours unless otherwise indicated.

LDL-induced damage

Five thousand cells were incubated in 96 well microtiter plates for 2-24 hours with 25 μg/ml of a fluorescently labeled, oxidized LDL analog AcLDL (Molecular Probes, Eugene, OR). Plates were introduced onto a heated humidified 37°C optical stage of a computer-interfaced inverted Nikon Diaphot fluorescence microscope. Images of selected fields were captured via Optronics DEI-75- camera using Image Pro image analysis software (Media Cybernetics), and morphologically intact cells were enumerated.

Cell preconditioning

Five thousand cells were plated in 96 well microtiter plates in the presence of reduced glutathione (GSH), acetylsalicylic acid (ASA) or salicylic acid (SA) (Sigma, St. Louis, MO) for 15-24 hours. Preconditioning media was aspirated and replaced with AcLDL. Normal tissue culture media was used as a control for both GSH and AcLDL conditions. Morphologically intact cells were enumerated following image capture as described above.

LDL incorporation

IC21 cells were preconditioned with either 5mM GSH or control media conditions as described above, and exposed to 10 dyne/cm^2 flow or static control conditions for 2 hours. Cells were subsequently recultured with fluorescently labeled 25 μg/ml AcLDL and images were captured, as described above, at 10-minute intervals.

Results and discussion

Conversion of macrophages to LDL containing foam cells and consequent LDL mediated injury and cell death, initiate the development of atherosclerotic plaque development and severe vascular occlusion. The progression of these events occurs predominantly along regions of vascular bifurcation, characterized by altered blood flow (Liepsch, 1990). Macrophages adherent to these surfaces are thus exposed to shear stress conditions intermediate to those at non-branched arterial (25 dyne/cm^2) or venous (3 dyne/cm^2) vascular channels. In order to determine the contribution of differential shear conditioning to the progression of atherosclerotic plaque formation the effect of venous, arterial, and intermediate shear levels on adherent macrophages was assessed in a macrophage cell line, IC21. This cell line exhibits normal macrophage specific morphology and function.

Effect of LDL on shear stressed cells

The interaction of macrophages with oxidized LDL results in oxidative stress, oxidative byproduct induced cell injury, and ultimately augmented plaque deposition at these vascular bifurcations. Experiments were designed to examine the effects of primary shear stress exposure on resistance to secondary oxidized LDL exposure. Intermediate and arterial shear were evaluated as preconditioning regimens against subsequent exposure to the oxidized LDL analog, AcLDL. As indicated in Figure 1, although cells exposed to either control or 25 dyne/cm^2 conditions and subsequently LDL, developed LDL containing vacuoles typical of foam cells, surviving cells were still morphologically intact. In fact 25 dyne/cm^2 exposed cells still exhibited flowinduced cell elongated cell morphology. However, cells exposed to intermediate shear (10 dyne/cm^2) demonstrated a total loss of cell integrity, with only cell fragments remaining after a 24 hour LDL exposure. Therefore, intermediate shear exposure did not sufficiently protect cells against subsequent oxidative injury imposed by LDL. In contrast arterial level shear exposed cells maintained cellular integrity as well as control cells.

Evaluation of chemical preconditioners

Our experiments suggested that inadequate shear preconditioning was at least partially responsible for LDL mediated cell death and could explain the progression of atherosclerosis at bifurcated vascular regions characterized by intermediate shear levels. Therefore, we hypothesized that supplemental anti-oxidant chemical preconditioning could mediate the cellular consequences of LDL exposure. A series of known anti-oxidants or stress response preconditioners was therefore evaluated for reduction of LDL mediated cell injury at concentrations previously reported to be optimal (Amici et al., 1995; Peter et al., 1997; Polasek, 1997; Cotgreave and Gerdes, 1998; Steer et al., 1997). These results are summarized in figure 2. Salicylic acid (300 □g/ml) preconditioned cells were just as susceptible to LDL mediated morphological changes as control cells. In contrast preconditioning with equal concentrations of acetylsalicylic acid, did preserve cell integrity. However, 5 mM GSH was most effective at minimizing LDL induced cell destruction.
Since GSH was determined to be the most effective chemical preconditioner against LDL mediated macrophage cell damage, the dose dependency of this response was examined. As indicated in Figure 3, the GSH protective effect was dose dependent with optimal concentrations determined to be between 5-8 mM. Therefore, GSH, a physiologic intracellular free radical scavenger, which normally functions to neutralize oxidative byproducts, could also effectively precondition cells against AcLDL mediated oxidative injury.

Chemical preconditioning of flow exposed cells

Although GSH could precondition IC21 macrophages against subsequent AcLDL exposure, its effectiveness in modulating oxidative damage in cells exposed to suboptimal shear conditions was unknown. Therefore, experiments were designed to evaluate the effect of GSH preconditioning on cells exposed subsequently to both intermediate shear and AcLDL. As indicated in figure 4, flow exposed cells, preconditioned with 5mM GSH, did not contain detectable AcLDL levels. In contrast, incorporation was dramatic as early as 2 hours post exposure in the absence of GSH preconditioning (data not shown). Therefore, GSH mediation against LDL effects is apparent within two hours of LDL exposure. It is currently unclear whether diminished intracellular LDL in GSH preconditioned and flow exposed cells is due to either neutralization of incorporated LDL or diminished LDL uptake kinetics. In either case our experiments suggest that GSH can be used to protect cells against oxidative injury mediated by intermediate shear and AcLDL exposure.

In summary, we have identified preconditioning regimens to protect cells against LDL mediated cell damage. Arterial level shear exposed cells are more resistant to LDL mediated oxidative injury than cells exposed to intermediate shears typical of bifurcated regions. Differential shear exposure may thus explain the predominant atherosclerotic plaque formation at these vascular regions. In addition we have identified a chemical preconditioner, reduced glutathione, that may mediate LDL induced damage, even at susceptible areas of vascular bifurcations. Further study is necessary to determine the mechanism of both chemical and mechanical macrophage preconditioning in effecting survival from LDL mediated oxidative injury. However, regardless of the mechanism, the results of our studies may be used to develop therapeutic strategies to prevent or reduce atherosclerotic vascular occlusion precipitated by the interaction of LDL with adherent macrophages.

Figures

Fig. 1: Macrophages were exposed to 0 (A,D), 10 (B,E) or 25 (C,F) dyne/cm^2 shear stress, subsequently incubated with fluoresceinated acetylated LDL, and their morphology and survival were assessed using fluorescence (A,B,C) and phase contrast microscopy (D,E,F). LDL exposed macrophages were resistant to damage when pre-exposed to high, but not intermediate levels of shear.

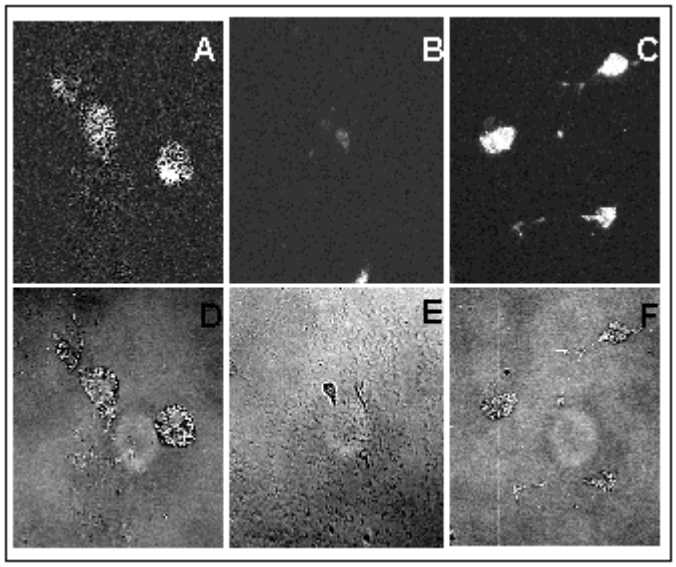


Fig. 2: In our experiments, we compared the ability of various stress regulators to neutralize LDL-induced oxidative damage to IC21 macrophages in culture. The antioxidant efficacy of glutathione (GSH), was compared with that of acetyl salicylic acid (ASA) and salicylic acid (SA). The transmitted light micrographs of the adjoining figure panel show that LDL incubated IC21 cells exposed to GSH best withstood damage and exhibited normal cellular morphology, with highest levels of membrane integrity. This suggests that GSH protection is more effective than that of other radical quenchers, ASA and SA, against LDL induced damage. Glutathione is both a scavenger of hydroxyl radicals and a biochemical pathway regulator, and is implicated to protect against lipid related oxidative injury.

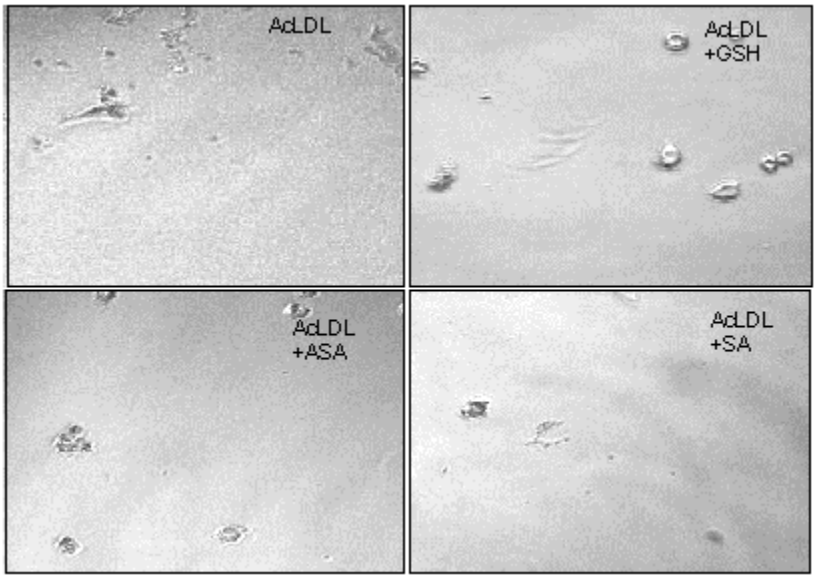


Fig. 3: Dose response of GSH neutralizing capacity against LDL-induced oxidative stress. Cells were incubated overnight with glutathione at varying concentrations. The cells were subsequently exposed to LDL and macrophage damage was determined by morphological changes.

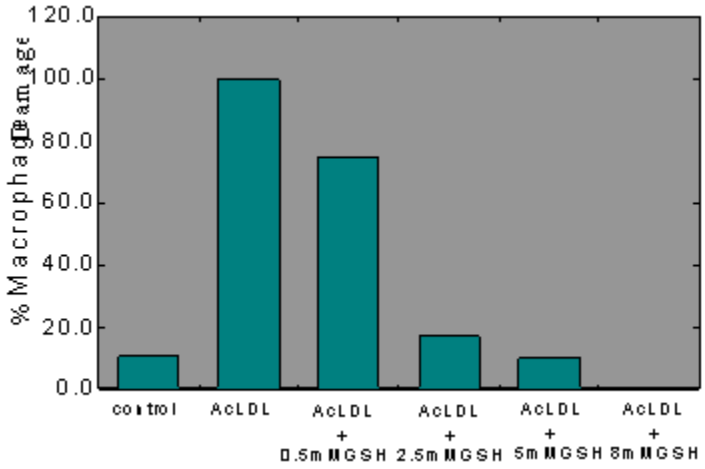
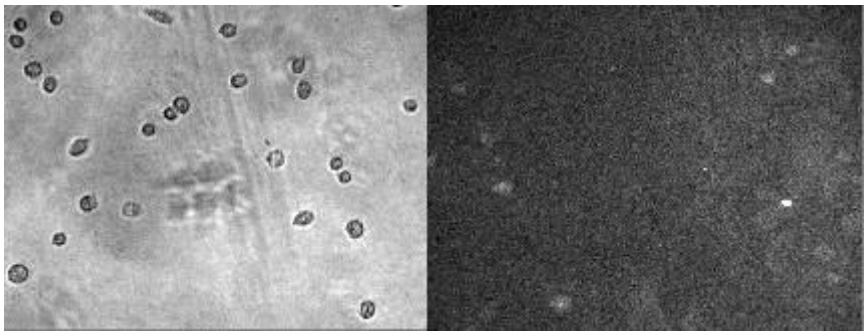


Figure 4: Cells were incubated overnight with 5mM GSH and subsequently exposed to 10 dyne/cm^2 flow conditions for 6 hours. Fluorescent AcLDL (25 mg/ml) was added to the flow exposed cells over a two hour incubation period, and the cells were microscopically imaged under either epifluorescence or phase contrast microscopy.



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