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Major role of HSP70 as a paracrine inducer of cytokine production in human oxidized LDL treated macrophages

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Abstract

Lipid accumulation and inflammation are key hallmarks of the atherosclerotic plaque and macrophage uptake of oxidized low-density lipoprotein (oxLDL) is believed to drive these processes. Initial experiments show that supernatants from oxLDL treated macrophages could induce IL-1 β production in naïve macrophages. To search for potential paracrine mediators that could mediate this effect a DNA microarray scan of oxLDL treated human macrophages was performed. This analysis revealed that oxLDL induced activation of heat shock protein (HSP) expression. HSPs have been implicated in the development of atherosclerosis, but the exact mechanisms for this is unclear. Extracellular heat shock protein 70 (HSP70) has been shown to elicit a pro-inflammatory cytokine response in monocytes and could therefore be a potential paracrine pro-inflammatory mediator. After 24 h of oxLDL treatment there was a significant increase of HSP70 concentrations in supernatants from oxLDL treated macrophages (oxLDLsup) compared to untreated controls ($P < 0.05$). OxLDLsup could induce both interleukin (IL)-1 β and IL-12 secretion in naïve macrophages. We also demonstrate that the effect of oxLDLsup on cytokine production and release could be blocked by inhibition of HSP70 transcription or secretion or by the use of HSP70 neutralizing antibodies. This suggests that extracellular HSP70 can mediate pro-inflammatory changes in macrophages in response to oxLDL.

Keywords

Oxidized low-density lipoprotein; Lipid; HSP70

1. Introduction

Lipid uptake by macrophages and the activation of inflammatory processes are key features in the development of atherosclerosis. Activation of endothelial cells and recruitment of monocytes and T-lymphocytes to the vascular wall are early events in the atherosclerotic process. In the intima, the monocytes differentiate to macrophages that secrete pro-inflammatory cytokines that induce inflammation in the atherosclerotic plaque. Importantly,

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the degree of inflammation correlates with plaque rupture and part of the clinical benefit from statins may involve anti-inflammatory effects [1,2].

High levels of low-density lipoprotein (LDL) cholesterol increase the risk of atherosclerosis and the cholesterol accumulated in the macrophages is derived mainly from modified forms of LDL. Oxidation of LDL is believed to be the major modification of LDL cholesterol in vivo [3]. Although the effect of oxidized LDL (oxLDL) on macrophages has been extensively studied, many aspects of foam-cell formation are still unknown.

The aim of this study was to search for paracrine proinflammatory mediators released from foam cells. We here show that supernatants from oxLDL treated macrophages stimulates the production and release of pro-inflammatory cytokines from naïve macrophages and we provide data to suggest that this effect is mediated by oxLDL induction of extracellular heat shock protein 70 (HSP70).

2. Materials and methods

2.1. Preparation and oxidation of human LDL

Preparation and oxidation of LDL was performed as previously described [4]. Briefly, human LDL was prepared by sequential ultracentrifugation (4°C) of plasma from healthy, fasted male volunteers. The LDL preparations were filtered through 0.22-µm filters, stored at 4°C, and used within 1 week of preparation. After extensive dialysis against PBS supplemented with penicillin 100 U/mL and streptomycin 100 µg/mL for 24 h, oxidation of LDL was performed by incubating 0.1 mg LDL protein/mL in PBS containing 5 µM CuSO₄ and antibiotics for 13 h at 37°C.

2.2. Preparation and cell culture of human macrophages

Monocytes were prepared and differentiated as previously described [4]. Briefly, buffy coats from blood donors were used to isolate human mononuclear blood cells using Ficoll-Paque (Amersham Pharmacia Biotech, Little Chalfont, UK). Mononuclear cells were resuspended and seeded at a density of 10⁷ cells per each 100-mm plastic dish in a serum-free medium (Macrophage-SFM; GIBCO BRL, Grand Island, NY), supplemented with penicillin 100 U/mL and streptomycin 100 µg/mL and allowed to adhere for 1 h. Non-adherent cells were removed by three washes with PBS. Adherent monocytes were cultured in Macrophage-SFM medium with antibiotics and supplemented with 70 U/mL human granulocyte macrophage colony stimulating factor (GM-CSF; R&D Systems Europe Ltd., Abingdon, UK). The medium was discarded after 3 days and the cells were washed once with PBS. The macrophages were then allowed to grow for another 3 days in the same medium, but without GM-CSF. After 6 days in culture, the cells were considered to be human monocyte-derived macrophages, denoted “macrophages”. Macrophages were incubated with oxLDL (50 µg protein/mL) or with control media without oxLDL for up to 24 h.

2.3. DNA microarray analysis

Macrophages were washed in PBS and RNA was isolated using the RNeasy kit (Qiagen, Hilden, Germany). Gene expression in macrophages treated with oxLDL for 6 or 24 h or untreated control macrophages was analyzed on duplicate HuFL DNA microarrays (Affymetrix, Santa Clara, CA) using pooled RNA from four donors (2 µg each). Scanned output files were analyzed with MAS5 software (Affymetrix) and globally scaled to an average intensity of 500. Regulated genes were selected using the change call algorithm (Affymetrix) essentially as previously described [5]. An average fold change was calculated between the baseline DNA microarrays (control) and the experimental DNA microarrays (6 and 24 h) using

the signal ratios. The identities of the regulated genes on the DNA microarray were defined using the Netaffx database (www.affymetrix.com).

2.4. Real-time RT-PCR analysis of gene expression

Oligonucleotide primers and probes (primer and probe sequences available on request) for the HSP70-1 genes (primers detecting both HSP70-1A and -1B) were designed with Primer Express 1.5 software (Applied Biosystems, Foster City, CA). Real-time RT-PCR was performed as previously described [5]. Briefly, each sample reaction consisted of diluted cDNA (corresponding to 20 ng DNase treated total RNA), 300 nmol/L of each primer and 200 nmol/L TaqMan probe in 1× PCR reaction mix. Amplification and detection of specific products was performed with the ABI Prism 7900 sequence detection system (Applied Biosystems) using default cycle parameters. Pre-developed assay reagents for human RPLP0 (large ribosomal protein) was obtained from Applied Biosystems and used as reference to normalize the expression levels between the samples. All standards and samples were analyzed in triplicate.

2.5. Cell culture media protein measurement

HSP70 concentrations in cell culture media were analyzed by the HSP70 ELISA kit (StressGen, Vic., Canada) according to the manufacturer's instructions. The concentration of IL-1 β in the culture supernatant after 24 h treatment was measured using an IL-1 β ELISA (Pharmingen, San Diego, CA) as directed by the manufacturer. The release of IL-12 from macrophages was measured using an IL-12 ELISA kit (Pharmingen) according to the manufacturer's instructions. The total cell protein content was determined by Bradford analysis, using bovine serum albumin as a standard [6].

2.6. Intracellular cytokine expression

To measure intracellular cytokine expression human macrophages (2×10^6 cells) were washed twice in PBS and simultaneously fixed and permeabilized using 2 mL PermeaFix (OrthoDiagnostics, Raritan, NJ) for 40 min at room temperature in the dark, as previously described [7]. Cells were then treated with anti-human interleukin (IL)-1 β -PE or anti-human IL-12-FITC (1 μ L/ 10^6 cells; Becton Dickinson, Mountain View, CA) for 40 min at room temperature in the dark, and analyzed by flow cytometry. Flow cytometric analysis was performed on a FACScan with Lysis II software program (Becton Dickinson). Individual cells were gated on the basis of forward (FSC) and orthogonal scatter (SSC). The photomultiplier for FITC (FL1-height) or PE (FL2-height) was set on a logarithmic scale.

2.7. HSP70 antibody blockade

A neutralizing anti-HSP70 monoclonal antibody was synthesized and tested for specificity and ability to neutralize recombinant human HSP70 by Multiple Peptide Systems (San Diego, CA). Briefly, recombinant human HSP70 was conjugated to a carrier protein through a cysteine residue at the N-terminal and immunized into five mice. An HSP70 ELISA was developed to test the serum at various periods and spleen cells were fused with murine myeloma cells. The hybridoma cultures were selected and cloned. Successful clones (Ab48) were expanded and monoclonal antibodies were recovered using affinity purification. Supernatants from oxLDL treated macrophages or control supernatants from non-oxLDL treated macrophages were pre-treated with either anti-HSP70 neutralizing antibody (Ab48, 10 μ g/mL) or control antibody (10 μ g/mL IgG) for 1 h at 37°C. The pre-treated supernatants were added to human macrophages for 24 h at 37°C.

2.8. Mutated HSP1 expression plasmid

Human macrophages were transiently transfected with a plasmid expressing dominant negative heat shock factor-1 (HSF1) that inhibits the formation of active HSF1 homotrimers (HSF1mut) [8] essentially as previously described [9]. In brief, empty plasmids (25 µg) or HSF1mut plasmids (25 µg) were mixed with 50 µg *N*-[1-(2,3-dioleoyloxypropyl)]-*N,N,N*,-trimethylammonium methylsulfate (DOTAP) in 500 mL OptiMEM (Roche, Mannheim, Germany) and incubated at room temperature for 20 min before addition to the cultured macrophages for 2.5 h. After 24 h the macrophages were then treated with 50 µg/mL oxLDL or control medium without oxLDL for 24 h at 37°C. Freshly recovered human macrophages (10⁶) were then treated with supernatant recovered from the mock or HSF1mut transfected oxLDL treated macrophages or untreated macrophages.

2.9. Methyl-β-cyclodextrin treatment

Macrophages were incubated with 5.0 mmol/L methyl-β-cyclodextrin (MβD) in PBS or PBS for 6 h at 37°C and where then rinsed in PBS three times. The macrophages were then treated with oxLDL or culture media alone for a further 24 h at 37°C. Freshly recovered human macrophages were then treated with supernatant recovered from the MβD treated or MβD untreated oxLDL treated macrophages or untreated macrophages.

2.10. Statistical analysis

The data were analyzed using a two-tailed Student's *t*-test or by ANOVA follow by a two-tailed Student's *t*-test. Differences were considered significant when $P < 0.05$.

3. Results

To identify potential paracrine factors that could mediate the pro-inflammatory signals from the oxLDL treated macrophages we performed a DNA microarray scan of oxLDL treated macrophages. The results from the DNA microarray scan show that 5 of the 17 up regulated genes after 6 h of oxLDL treatment belonged to the heat shock protein (HSP) family (Fig. 1A). Three of these genes belonged to the HSP70 family (HSP70-1A, -1B and HSP70 6). The two remaining HSP genes were DNAJB1 (member of the HSP40 family) and heme oxygenase 1 (HMOX1, also referred to as HSP32). The increased levels of HSP70-1 (HSP70-1A and -1B) mRNA in response to oxLDL was verified by real-time-PCR ($P < 0.05$; Fig. 1B). Extracellular HSP70 has recently been shown to elicit a pro-inflammatory cytokine response in monocytes [7,10] and could therefore be a potential paracrine pro-inflammatory mediator.

To determine if the increased HSP70-1 gene expression also resulted in increased HSP70 release from the macrophages, HSP70 concentrations in cell culture media from oxLDL treated macrophages was determined by ELISA. After 24 h there was a significant increase in HSP70 in the culture media of oxLDL treated macrophages compared to untreated controls ($P < 0.05$, Fig. 2A and B).

To evaluate if extracellular HSP70 in the oxLDLsup could be a paracrine factor mediating the observed pro-inflammatory response, experiments where extracellular HSP70 was blocked were performed. In the first experiment, we used a neutralizing HSP70 antibody. Supernatant recovered from macrophages, treated with or without oxLDL, was admixed with naïve macrophages and intracellular IL-1β expression was determined by flow cytometry. In naïve macrophages, oxLDLsup increased IL-1β concentrations ($P < 0.05$; Fig. 3), and pre-treatment of the oxLDLsup with blocking antibodies against HSP70 inhibited this increase whereas addition of a control antibodies was without effect. In the next experiment, supernatant recovered from macrophages, treated with or without oxLDL, was admixed with naïve macrophages and IL-1β concentrations in the cell culture media were determined by ELISA.

In naïve macrophages, oxLDLsup increased IL-1 β concentrations ($P < 0.05$; Fig. 4), and pre-treatment of the oxLDLsup with blocking antibodies against HSP70 inhibited this increase. The increased IL-1 β concentrations was also reduced when using oxLDLsup from macrophages transfected with a plasmid expressing mutated HSF1 (pHSF1mut) that inhibits HSP production [8] (Fig. 4). These results strongly suggest that extracellular HSP70 is a paracrine mediator of proinflammatory responses in oxLDL treated macrophages.

To investigate if oxLDLsup stimulates the expression of other cytokines we repeated the HSP70 neutralizing antibody experiments and analyzed the expression of intracellular IL-12 by flow cytometry. OxLDLsup increased intracellular IL-12 production in naïve macrophages ($P < 0.05$; Fig. 5), and pre-treatment of the oxLDLsup with neutralizing antibodies against HSP70 inhibited this increase.

HSP70 release from epithelial cells is mediated by sphingolipid-cholesterol-rich structures named lipid rafts [11]. To study the mechanism of oxLDL-induced HSP70 release in human macrophages, the cells were depleted of cholesterol using methyl- β -cyclodextrin before oxLDL treatment. When supernatant from macrophages pre-treated with M β D before exposure to oxLDL were admixed with naïve macrophages the increase of IL-12 concentrations was reduced ($P < 0.05$; Fig. 6).

4. Discussion

In this study, we show that HSP70 is released from macrophages in response to oxLDL treatment and that HSP70 may be a major paracrine inducer of cytokine expression and release in human macrophages. This provides further support for a role of extracellular HSP70 in addition to the well established cytoprotective effects of intracellular HSPs [12].

HSPs have been implicated in several diseases, such as arthritis, diabetes mellitus, schizophrenia and atherosclerosis [13]. In the field of atherosclerosis, heat shock protein 60 (HSP60) and HSP70 are the most widely studied stress proteins. Accumulating evidence suggest that HSP70 mediates myocardial protection, particularly in experimental models of ischemia and reperfusion injury [14-16]. Several studies have investigated the link between cardiovascular disease (CVD) and serum/plasma levels of HSP or HSP auto-antibodies. A clear link between HSP60 serum levels and CVD has been shown [17]. However, the connection between HSP70 or anti-HSP70 antibody levels and CVD or CVD risk phenotypes is unclear. Low serum levels of HSP70 has been associated with atherosclerosis in subjects with established hypertension [18] and coronary artery disease [19]. However, patients with intermittent claudication, critical lower limb ischaemia, aneurysms [20], hypertension [21] and cerebral ischemia [22] have elevated anti-HSP70 antibody levels. It is unclear how these epidemiological studies relate to local events in the vascular intima. Clearly, a cytoprotective intracellular effect of HSP and a pro-inflammatory extracellular effect makes serum levels of HSPs difficult to interpret.

HSP70 has been detected in both fibrotic and necrotic atherosclerotic plaques [23] and macrophages are an important source of HSP70 production in atherosclerotic plaques [24, 25]. These immunohistochemical analysis clearly demonstrate that local expression of HSP70 within the atherosclerotic plaque occurs, however, it is unknown if the HSP70 is intracellular or extracellular.

Several studies have shown that HSP70 can be actively released from cultured cells via a mechanism suggested to involve lipid-rafts [11,26,27]. A recent study has demonstrated that this also occurs in mononuclear cells [28]. In addition, HSP70 and other intracellular proteins are released when cells undergo necrosis. The formation of a necrotic core in advanced atherosclerotic plaques clearly involves macrophage cell death [29,30], indicating that both

cell death-mediated and active release of HSP70 may occur within the atherosclerotic plaque. OxLDL have been shown to have cytotoxic properties, and therefore cell death-mediated release of HSP70 to the cell culture media cannot be ruled out. However, our experiments where cholesterol depletion of macrophages before oxLDL treatment almost completely abolished the release of IL-12 in naïve macrophages indicate that active release is a major component of HSP70 in the cell culture media.

There is an ongoing debate whether recombinant HSP70 has the ability to activate cytokine release or if these findings are due to lipopolysaccharide (LPS)-contamination of recombinant HSP70 (rHSP70) preparations [31]. This is a serious problem for researchers interested in examining the mechanism of HSP70-induced effects in vitro. This concern was recently addressed by MacAry et al. using physical and functional assays to ensure the “cleanliness” of the rHSP70 protein preparations. These included physical assays like adding an additional microdialysis step and passing all HSP70 protein preparations through polymyxin B column three times. They also used functional assays like boiling HSP70 protein preparations or pre-treatment of HSP70 proteins with proteinase K. This destroys HSP70 proteins, but not LPS and inhibited HSP70-induced, but not LPS induced-cytokine release from dendritic cell (DC) [32]. When similar measures were used to control for LPS contamination of rHSP70 preparations, Millar et al. demonstrated that HSP70 augments DC effector functions and when admixed with specific antigens, triggers autoimmune diseases in vivo [33]. Although the most up to date methods to eliminate endotoxin contamination may be used, it is virtually impossible to completely eliminate this possibility. Still, taken together these results strongly suggest that when special care is taken to control for LPS contamination, clear effects of rHSP70 can be demonstrated. These experiments clearly show that rHSP70 has cytokine activation (chaperokine) properties. In this study, we extend these findings by showing that also macrophage-derived HSP70 (non-recombinant) can induce cytokine production in naïve macrophages. The experiments showing that cytokine release is almost completely abolished by the HSP70 neutralizing antibody argue strongly against that the effects could be explained by endotoxin contamination in the oxLDL preparation.

In conclusion, these studies show that oxLDL induces the release of HSP70 by human macrophages, and that extracellular HSP70 may be major paracrine inducer of cytokine expression and secretion in human macrophages. Our study suggests that extracellular HSP70 may be an additional link between oxLDL-induced macrophage lipid accumulation and the initiation of inflammatory processes in the atherosclerotic plaque.

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References

1. van der Wal AC, Becker AE, van der Loos CM, Das PK. Site of intimal rupture or erosion of thrombosed coronary atherosclerotic plaques is characterized by an inflammatory process irrespective of the dominant plaque morphology. *Circulation* 1994;89:36–44. [PubMed: 8281670]
2. Ross R. Atherosclerosis—an inflammatory disease. *N Engl J Med* 1999;340:115–26. [PubMed: 9887164]
3. Steinberg D. Low density lipoprotein oxidation and its pathobiological significance. *J Biol Chem* 1997;272:20963–6. [PubMed: 9261091]
4. Ohlsson BG, Englund MC, Karlsson AL, et al. Oxidized low density lipoprotein inhibits lipopolysaccharide-induced binding of nuclear factor-kappaB to DNA and the subsequent expression

- of tumor necrosis factor-alpha and interleukin-1beta in macrophages. *J Clin Invest* 1996;98:78–89. [PubMed: 8690807]
5. Svensson PA, Englund MC, Markstrom E, et al. Copper induces the expression of cholesterologenic genes in human macrophages. *Atherosclerosis* 2003;169:71–6. [PubMed: 12860252]
  6. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 1976;72:248–54. [PubMed: 942051]
  7. Asea A, Rehli M, Kabingu E, et al. Novel signal transduction pathway utilized by extracellular HSP70: role of toll-like receptor (TLR) 2 and TLR4. *J Biol Chem* 2002;277:15028–34. [PubMed: 11836257]
  8. Chen C, Xie Y, Stevenson MA, Auron PE, Calderwood SK. Heat shock factor 1 represses Ras-induced transcriptional activation of the c-fos gene. *J Biol Chem* 1997;272:26803–6. [PubMed: 9341107]
  9. Koido S, Kashiwaba M, Chen D, et al. Induction of antitumor immunity by vaccination of dendritic cells transfected with MUC1 RNA. *J Immunol* 2000;165:5713–9. [PubMed: 11067929]
  10. Asea A, Kraeft SK, Kurt-Jones EA, et al. HSP70 stimulates cytokine production through a CD14-dependant pathway, demonstrating its dual role as a chaperone and cytokine. *Nat Med* 2000;6:435–42. [PubMed: 10742151]
  11. Broquet AH, Thomas G, Masliah J, Trugnan G, Bachelet M. Expression of the molecular chaperone Hsp70 in detergent-resistant microdomains correlates with its membrane delivery and release. *J Biol Chem* 2003;278:21601–6. [PubMed: 12682040]
  12. Jaattela M. Heat shock proteins as cellular lifeguards. *Ann Med* 1999;31:261–71. [PubMed: 10480757]
  13. Xu Q. Role of heat shock proteins in atherosclerosis. *Arterioscler Thromb Vasc Biol* 2002;22:1547–59. [PubMed: 12377729]
  14. Marber MS, Latchman DS, Walker JM, Yellon DM. Cardiac stress protein elevation 24 h after brief ischemia or heat stress is associated with resistance to myocardial infarction. *Circulation* 1993;88:1264–72. [PubMed: 8353888]
  15. Mestrlil R, Chi SH, Sayen MR, O'Reilly K, Dillmann WH. Expression of inducible stress protein 70 in rat heart myogenic cells confers protection against simulated ischemia-induced injury. *J Clin Invest* 1994;93:759–67. [PubMed: 8113409]
  16. Radford NB, Fina M, Benjamin IJ, et al. Cardioprotective effects of 70-kDa heat shock protein in transgenic mice. *Proc Natl Acad Sci USA* 1996;93:2339–42. [PubMed: 8637874]
  17. Mehta TA, Greenman J, Ettelaie C, et al. Heat shock proteins in vascular disease-a review. *Eur J Vasc Endovasc Surg* 2005;29:395–402. [PubMed: 15749041]
  18. Pockley AG, Georgiades A, Thulin T, de Faire U, Frostegard J. Serum heat shock protein 70 levels predict the development of atherosclerosis in subjects with established hypertension. *Hypertension* 2003;42:235–8. [PubMed: 12900429]
  19. Zhu J, Quyyumi AA, Wu H, et al. Increased serum levels of heat shock protein 70 are associated with low risk of coronary artery disease. *Arterioscler Thromb Vasc Biol* 2003;23:1055–9. [PubMed: 12730089]
  20. Chan YC, Shukla N, Abdus-Samee M, et al. Anti-heat-shock protein 70 kDa antibodies in vascular patients. *Eur J Vasc Endovasc Surg* 1999;18:381–5. [PubMed: 10610825]
  21. Pockley AG, De Faire U, Kiessling R, et al. Circulating heat shock protein and heat shock protein antibody levels in established hyper-tension. *J Hypertens* 2002;20:1815–20. [PubMed: 12195124]
  22. Gromadzka G, Zielinska J, Ryglewicz D, Fiszer U, Czlonkowska A. Elevated levels of anti-heat shock protein antibodies in patients with cerebral ischemia. *Cerebrovasc Dis* 2001;12:235–9. [PubMed: 11641589]
  23. Johnson AD, Berberian PA, Tytell M, Bond MG. Differential distribution of 70-kD heat shock protein in atherosclerosis. Its potential role in arterial SMC survival. *Arterioscler Thromb Vasc Biol* 1995;15:27–36. [PubMed: 7749813]
  24. Kanwar RK, Kanwar JR, Wang D, Ormrod DJ, Krissansen GW. Temporal expression of heat shock proteins 60 and 70 at lesion-prone sites during atherogenesis in ApoE-deficient mice. *Arterioscler Thromb Vasc Biol* 2001;21:1991–7. [PubMed: 11742875]
  25. Berberian PA, Myers W, Tytell M, Challa V, Bond MG. Immunohistochemical localization of heat shock protein-70 in normal-appearing and atherosclerotic specimens of human arteries. *Am J Pathol* 1990;136:71–80. [PubMed: 2297051]

26. Hightower LE, Guidon PT Jr. Selective release from cultured mammalian cells of heat-shock (stress) proteins that resemble glia-axon transfer proteins. *J Cell Physiol* 1989;138:257–66. [PubMed: 2918030]
27. Dybdahl B, Wahba A, Lien E, et al. Inflammatory response after open heart surgery: release of heat-shock protein 70 and signaling through toll-like receptor-4. *Circulation* 2002;105:685–90. [PubMed: 11839622]
28. Hunter-Lavin C, Davies EL, Bacelar MM, et al. Hsp70 release from peripheral blood mononuclear cells. *Biochem Biophys Res Commun* 2004;324:511–7. [PubMed: 15474457]
29. Crisby M, Kallin B, Thyberg J, et al. Cell death in human atherosclerotic plaques involves both oncosis and apoptosis. *Atherosclerosis* 1997;130:17–27. [PubMed: 9126644]
30. Ball RY, Stowers EC, Burton JH, et al. Evidence that the death of macrophage foam cells contributes to the lipid core of atheroma. *Atherosclerosis* 1995;114:45–54. [PubMed: 7605375]
31. Gao B, Tsan MF. Induction of cytokines by heat shock proteins and endotoxin in murine macrophages. *Biochem Biophys Res Commun* 2004;317:1149–54. [PubMed: 15094389]
32. MacAry PA, Javid B, Floto RA, et al. HSP70 peptide binding mutants separate antigen delivery from dendritic cell stimulation. *Immunity* 2004;20:95–106. [PubMed: 14738768]
33. Millar DG, Garza KM, Odermatt B, et al. Hsp70 promotes antigen-presenting cell function and converts T-cell tolerance to autoimmunity in vivo. *Nat Med* 2003;9:1469–76. [PubMed: 14625545]

Fig. 1.

Expression of HSP genes in human macrophages treated with oxLDL. Human macrophages were treated with oxLDL (50 $\mu\text{g/mL}$) or control media without oxLDL for up to 24 h. Gene expression was analyzed by DNA microarrays or real-time-PCR. HSP genes classified as regulated using the change call algorithm after 6 or 24 h of oxLDL treatment (A). Gene expression is presented as fold change compared to untreated control. HSP70-1A/1B gene expression in oxLDL treated macrophages as analyzed by real-time-PCR (B). Data are presented as mean $\pm$ S.E.M. from four donors. The signal was normalized to the endogenous reference gene RPLP0. * $P < 0.05$ vs. control (ANOVA).

Fig. 2.

Effect of oxLDL on HSP70 release from human macrophages. Human macrophages were treated with oxLDL (50 $\mu\text{g/mL}$) or control media without oxLDL for up to 24 h. Concentration of HSP70 protein in the macrophage culture media (supernatant) was measured using HSP70 ELISA. Time course of oxLDL-induced HSP70 release (A). Data are presented as mean $\pm$ S.E.M. from four donors. Comparison of HSP70 concentrations in supernatants from 24 h oxLDL treated macrophages and 24 h untreated control macrophages (B). Data are presented as mean $\pm$ S.E.M. from eight donors. * $P < 0.05$ vs. control (Students t -test).

Fig. 3.

Effect of oxLDL supernatants on IL-1 β production in human macrophages. Human macrophages were treated with oxLDL (50 μ g/mL) or control media without oxLDL for 24 h. OxLDLsup and control super-natants was either pre-treated with anti-HSP70 neutralizing antibody (Ab48; 10 μ g/mL) or control antibody (10 μ g/mL IgG) at 37°C for 1 h before the supernatants were admixed with naïve macrophages for 24 h. Intracellular IL-1 β was measured by flow cytometry. Data is presented as mean $\pm$ S.E.M. from three independently performed experiments. * $P < 0.05$ vs. control (Student's t -test).

Fig. 4.

Effect of oxLDL supernatants on IL-1 β release from human macrophages. Macrophages were mock transfected or transfected with a plasmid expressing HSF1mut that inhibits HSP production. Twenty-four hours after transfection the macrophages were treated with oxLDL (50 μ g/mL) or control media without oxLDL for 24 h. The supernatants from the macrophages were either pre-treated with anti-HSP70 neutralizing antibody (Ab48; 10 μ g/mL) or control antibody (10 μ g/mL IgG) at 37°C for 1 h before admixing with naïve macrophages for 24 h at 37°C. Concentrations of IL-1 β in culture media was measured by ELISA. Data are presented as mean $\pm$ S.E.M. from three independently performed experiments. * $P < 0.05$ vs. control (Student's t -test).

Fig. 5.

Effect of oxLDL supernatants on IL-12 production in human macrophages. Human macrophages were treated with oxLDL (50 µg/mL) or control media without oxLDL for 24 h. OxLDLsup and control super-natants was either pre-treated with anti-HSP70 neutralizing antibody (Ab48; 10 µg/mL) or control antibody (10 µg/mL IgG) at 37°C for 1 h before the supernatants were admixed with naïve macrophages for 24 h. Intracellular IL-12 was measured by flow cytometry. Data is presented as mean ± S.E.M. from three independently performed experiments. * $P < 0.05$ vs. control (Student's *t*-test).

Fig. 6.

Effect of cholesterol depletion on oxLDL supernatant-induced IL-12 release. Macrophages were incubated with or without MβD (5.0 mmol/L) for 6 h at 37°C then treated with oxLDL (50 µg/mL) or control media without oxLDL for a further 24 h at 37°C. Supernatants were admixed with naïve macrophages for 24 h and IL-12 concentrations in the culture media was measured using IL-12 ELISA. Data are presented mean ± S.E.M. from four independently performed experiments. * $P < 0.05$ vs. control (Student's t -test).