Liver X receptor agonist inhibits proliferation of ovarian carcinoma cells stimulated by oxidized low density lipoprotein

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Objectives. We previously observed an association between ovarian cancer outcome and statin use and hypothesized lipoproteins have direct effects on ovarian cancer proliferation. Here we investigate the direct effects of low density lipoprotein (LDL) and oxidized LDL (oxLDL) on proliferation and the inhibitory effects of fluvastatin and a liver X receptor (LXR) agonist.

Methods. The effects of LDL, oxLDL, the LXR agonist TO901317, fluvastatin and cisplatin on cellular proliferation were determined using MTT assays. LXR pathway proteins were assayed by immunoblotting. Cytokine expression was determined by antibody array.

Results. Concentrations of oxLDL as small as 0.1 μg/ml stimulated CAOV3 and SKOV3 proliferation, while LDL had no effect. TO901317 inhibited the proliferation of CAOV3, OVCAR3 and SKOV3 cells stimulated by oxLDL. Fluvastatin inhibited oxLDL mediated proliferation of CAOV3 and SKOV3. Cardiotrophin 1 (CT-1) was mitogenic to CAOV3 and SKOV3, was induced by oxLDL, and was reversed by TO901317. OxLDL increased cisplatin IC50s by 3.8 μM and > 60 μM for CAOV3 and SKOV3 cells, respectively. The LXR pathway proteins CD36, LXR, and ABCA1 were expressed in eight ovarian carcinoma cell lines (A2780, CAOV3, CP70, COScar82, ES2, OVCAR3, SKOV3).

Conclusions. OxLDL reduced ovarian carcinoma cell chemosensitivity and stimulated proliferation. These effects were reversed by LXR agonist or fluvastatin. The LXR agonist also inhibited expression of the ovarian cancer mitogen CT-1. These observations suggest a biologic mechanism for our clinical finding that ovarian cancer survival is associated with statin use. Targeting LXR and statin use may have a therapeutic role in ovarian cancer.

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Introduction

The association between serum cholesterol and cancer risk and survival is unclear. Attempts to correlate risk or outcome of numerous cancers with serum low density lipoprotein (LDL) cholesterol have yielded somewhat controversial results with evidence pointing towards improved outcome in patients with high serum cholesterol [1–3]. One study also demonstrated that women on a diet high in cholesterol had no increased risk of epithelial ovarian cancer [4]. However, recently it was reported that the levels of oxidized LDL (oxLDL) in the serum of ovarian cancer patients were positively associated with patient outcome [5]. Our group has also recently reported that epithelial ovarian cancer patients on statins had improved survival [6].

Levels of oxLDL are in part regulated by the available oxidizable LDL precursor and may be altered by cholesterol lowering drugs such as statins. Cholesterol homeostasis is maintained in part by cells expressing scavenger receptors (SRA and CD36) that internalize oxLDL which are then converted to oxysterol ligands of the nuclear liver X receptors α and β (LXRα and LXRβ), heterodimers of the retinoid X receptor (RXR) [7]. Activated LXR/RXR heterodimers activate target genes possessing the LXR element (LXRE) including the ATP binding cassette transporters ABCA1 and ABCG1 leading to cholesterol efflux to HDL or cholesterol excretion via biliary and intestinal cells [7]. LXR activation is also associated with increases in the expression of numerous proinflammatory cytokines which also promote cellular proliferation [7]. While statin therapy can lower...
serum oxLDL [8–11], statins also have lipid independent functions on oxidative pathways including regulation of scavenger receptors. In monocytes and foam cells, statins can alter scavenger receptor expression via regulation of PPAR, NF-κB, Rho and Ras [12], but these statin functions have not been studied in cancer cells.

One potential means for treating oxLDL related diseases is modulation of LXR by synthetic LXR agonists. Presently three such synthetic LXR ligands have been developed including TO901317, GW3965 and N,N-dimethyl-3β-hydroxycholenamide (DMHCA). LXR agonists upregulate expression of the ATP binding cassette transporters and cholesterol efflux while simultaneously downregulating oxysterol mediated activation of proinflammatory cytokines [13,14]. In mouse models of atherosclerosis LXR agonists reduce both inflammation in atherosclerotic plaques and serum cholesterol [15]. LXR agonists also inhibit the proliferation of endothelial cells [16]. oxLDL and LXR agonists have been little studied on cells other than endothelial, monocytes and macrophage foam cells in the context of cardiovascular diseases, but one study demonstrated oxLDL is a mitogen to cultured human fibroblasts [17].

In the present study we determined that ovarian carcinoma cells possess CD36 scavenger receptor and are stimulated to proliferate by oxLDL. The LXR agonist TO901317 and fluvastatin reversed oxLDL mediated proliferation. We also demonstrated that oxLDL reduced the sensitivity of ovarian carcinoma cells to cisplatin. Our study demonstrated that increases in oxidized LDL cholesterol may negatively impact ovarian cancer outcome and suggests that LXR ligands and statins may be an effective strategy for treating ovarian cancer patients.

Materials and methods

Tissue culture

CAOV3, ES2, OVCAR3, PA1, and SKOV3 were cultured as recommended by American Type Culture Collection. CSOC882 and CSOC909 were cultured as previously described [18], A2780 and CP70 were cultured in RPMI 1640 and 10% fetal calf serum (FCS) supplemented with 2 mM l-glutamine and 0.2 U/ml insulin. OVCA432 was cultured in modified Eagle’s medium (MEM) and 10% FBS + 2 mM l-glutamine. All reagents were purchased from Gibco.

RNA interference

RNA interference (RNAi) was accomplished by suspension transfection of CAOV3 cells using Metafectene (Biontex Laboratories) and 30 nM of the indicated siRNA oligos. When two siRNAs were used they were 15 nM each. Transfected cells were plated in 96 well plates in quadruplicate, 10,000 cells/well. siRNAs included LXRα (sc-38828), LXRβ (sc-45316) and Control siRNA-A (sc-37007) (Santa Cruz Biotechnology). The next day cells were serum starved for 4 h and treated with the indicated amounts of TO901317 overnight. Cell abundances were determined by MTT assay as described below.

Immunoblotting

Proteins were separated on precast polyacrylamide gels (Bio-Rad), transferred to Hybrid ECL (Amersham), and detected by enzymatic chemiluminescence (ECL) (Amersham). Antibodies included rabbit anti-CD36 scavenger receptor (Santa Cruz #sc-9154, used at 2 μg/ml), goat anti-LXRα/β (Novus Biologicals #NB100-1465, used at 2 μg/ml), rabbit anti-ABCA1 (Novus Biologicals #NB400-105, used at 2 μg/ml), rabbit anti-NAP2 (Abcam Inc. #ab9554, used at 0.25 μg/ml), mouse anti-actin monoclonal antibody (Sigma-Aldrich #AC-40, used at 1:1000), and rabbit anti-CT1 (Abcam #ab9837, used at 0.5 μg/ml). Secondary antibodies conjugated to horseradish peroxidase were purchased from Jackson ImmunoResearch Laboratories, Inc. Images were processed densitometrically using ImageJ [19].

Proliferation assays

Ovarian carcinoma cell lines were seeded in 96 well plates at a density of 3000 cells per well. The next day cells were treated in reduced serum media (0.1% FBS) for 4 h then were treated with the indicated concentrations of cisplatin (Sigma), oxLDL (Biomedical Technologies), TO901317 (Cayman Chemicals), or CT-1 (Cell Sciences) for 24 or 48 h. Cell abundances were determined using the CellTiter kit (Promega). MTT concentration was determined by OD 490.

Antibody array analysis

SKOV3 cells were cultured in 10 cm dishes overnight. The next day cells were serum starved for 4 h then were treated with combinations of oxLDL (25 μg/ml), 25 μg/ml TO901317 and diluent as indicated for 12 h. Media were then collected and filtered to exclude cells. This conditioned media was then used without dilution to probe a human cytokine antibody array (RayBiotech, Inc. #AAH-CYT-8) following the vendor’s recommended protocol using kit reagents. The process involved detection of cytokines from media bound to antibody arrays using a cocktail of corresponding biotin conjugated anti-cytokine antibodies subsequently labeled by HRP-conjugated streptavidin, revealed by enzymatic chemiluminescence. Films exposed to ECL were photographed at high resolution using a UV gel imager's system (UVP, LLC). Images were then processed using ImageJ [19]. Fold inductions were calculated relative to arrayed control proteins.

Results

oxLDL stimulated proliferation of ovarian carcinoma cell lines, but LDL had no effect

We determined the effects of LDL and ox-LDL on proliferation of SKOV3 and CAOV3 ovarian carcinoma cell lines by MTT assays. Increasing doses of LDL did not significantly alter the growth of either of these cell lines (Fig. 1A). However, the proliferation of each of these cell lines was significantly increased upon treatment with oxLDL (Fig. 1B). Increased proliferation was also evident visually by examination of cells treated for 24 h with oxLDL by standard phase microscopy (Fig. 1C).

LXR pathway proteins are expressed in ovarian carcinoma cell lines

We compared the expression of the LXR pathway proteins in seven ovarian carcinoma cell lines including CD36 scavenger receptor, LXRα/β, and ABCA1. The cell lines included A2780, CAOV3, CP70, CSOC882, ES2, OVCAR3, SKOV3. Each of these proteins were present in ovarian carcinoma cell lines but in variable amounts (Figs. 1D and E). We concluded from these results that the CD36 → LXR pathway is intact in most ovarian carcinoma cell lines.

LXR agonist TO901317 reversed oxLDL mediated proliferation

We hypothesized that activating the LXR pathway would inhibit proliferation of ovarian carcinoma cell lines expressing LXR pathway proteins. Treatment of each of OVCAR3, SKOV3, and CAOV3 with increasing doses of TO901317 inhibited proliferation in a dose-dependent manner (P < 0.01, t-test). These data demonstrate that LXR agonist can inhibit proliferation without oxLDL stimulation (Fig. 2A). TO901317 treatment also completely reversed the proliferation of each of these three cell lines stimulated by simultaneous oxLDL.
treatment (Fig. 2B). The proliferation of CAOV3 cells treated with siRNAs against LXRα, LXRβ or a combination of both was not inhibited by TO901317. Slopes were not significantly different from zero (P > 0.05) unless a control siRNA was used (P = 0.05; Fig. 2C).

Additionally, any LXR siRNA significantly inhibited proliferation compared to control siRNA at any dose of TO901317 (Fig. 2C). Note that Western blotting was used to show siRNA treatment achieved partial reduction of LXR protein.

OxLDL reduced sensitivity to fluvastatin

Treatments of oxLDL significantly reduced the sensitivity of each of CAOV3 and SKOV3 to fluvastatin. For CAOV3, the fluvastatin IC50s were 248 μM (−oxLDL) and 341 μM (+ oxLDL), and oxLDL treatment resulted in an increase of the fluvastatin IC50 by 93 μM (Fig. 3A). The curves corresponding to CAOV3 treatments with and without oxLDL were significantly different by two-way ANOVA (P < 0.001). For SKOV3, the fluvastatin IC50s were 134 μM (−oxLDL) and 373 μM (+ oxLDL), and oxLDL treatment resulted in an increase of the fluvastatin IC50 by 239 μM (Fig. 3B). The curves corresponding to SKOV3 treatments with and without oxLDL were significantly different by two-way ANOVA (P < 0.0001).

Cytokine array screening

Screening of a cytokine antibody array of 54 cytokines with the media of cultured SKOV3 cells conditioned with diluent, oxLDL, or oxLDL and TO901317 revealed numerous changes in the expression of various cytokines. Supplementary Table 1S presents average fold changes of the individual cytokines on the array determined by ImageJ analysis. With 2-fold as a threshold for induction, some cytokines were induced by oxLDL but not reversed by TO901317 (IL-1ra, IL-2, NAP-2), while others were not induced by oxLDL but reductions were observed upon treatment with TO901317 (IL-2Rβ and IL-2Rγ). For cardiotropin-1 (CT-1) we observed both an average 2.3 fold induction by oxLDL and an average 2.5 fold reversal by TO901317 by ImageJ analysis, that was also clearly evident by visual inspection (Fig. 4A). We used immunoblotting to verify that CT-1 expression was indeed induced upon treatment with oxLDL in both SKOV3 cells and also CAOV3 cells (Figs. 4B, C).
Cardiotropin-1 stimulated ovarian carcinoma cell proliferation

We determined whether CT-1 could induce proliferation of ovarian carcinoma cells since CT-1 was the only cytokine in our analysis whose expression was stimulated by oxLDL greater than two-fold that was reversed by TO901317. We treated both SKOV3 and CAOV3 cells with increasing doses of CT-1 demonstrating enhanced proliferation for both cell lines (Fig. 4D). The increases in proliferation were significantly different by one-way ANOVA ($P<0.0001$ and $P<0.05$ for CAOV3 and SKOV3, respectively). The highest dose of CT-1 used, 160 ng/ml, increased proliferation in a 24-h period by 1.34 fold for CAOV3 and 1.15 fold for SKOV3 compared to diluent treated controls.

OxLDL reduced cisplatin chemosensitivity

Treatments with oxLDL significantly reduced the chemosensitivity of each of CAOV3 or SKOV3. For CAOV3, the cisplatin IC50s were 4.5 μM (−oxLDL) and 7.4 μM (+oxLDL), and oxLDL treatment resulted in an increase of the cisplatin IC50 by 2.9 μM (Fig. 5A). For SKOV3, the cisplatin IC50s were 42 μM (−oxLDL) and 111 μM (−oxLDL, determined by extrapolation since cells treated with...
highest cisplatin dose (100 μM) had not quite reached a 50% reduction) and oxLDL treatment resulted in an increase of the cisplatin IC50 by 69 μM (Fig. 5B). For each of SKOV3 and CAOV3, the curves corresponding to treatments with and without oxLDL were significantly different by two-way ANOVA (P < 0.0001 for each cell line).

**Discussion**

Ovarian cancer outcome may be influenced by genetic, epigenetic, and clinical determinates as well as metabolic conditions that alter cancer proliferation. Recently we demonstrated that ovarian cancer patients taking statins had improved survival [6]. In addition, a recent meta-analysis of 37,248 individuals found reduced cancer incidence for statin users [20]. While not all studies have demonstrated positive associations between serum cholesterol levels and outcome in solid malignancies [1–4], one study demonstrated a direct correlation between serum oxLDL and ovarian cancer outcome [5]. Prompted by these clinical observations, the present study sought to investigate the hypothesis that oxLDL, TO901317 and fluvastatin may have direct effects on the
proliferation of ovarian cancer cells, and expression of proinflammatory cytokines.

Oxidized LDLs are mitogenic to ovarian cancer cells

Oxidized LDL stimulated ovarian carcinoma cells to proliferate while generic LDL did not. We investigated SKOV3 and CAOV3 ovarian cancer cell lines for their proliferative response to LDL and observed no significant changes in cellular growth over the full range of doses tested. Contrary to expectations, all doses of LDL elicited decreases in proliferation for both cell lines compared to no treatment at all, but no further decreases were observed with higher doses (Fig. 1A). However, oxidized LDL potently stimulated both the SKOV3 and CAOV3 cell lines to proliferate (Fig. 1B). Our data support the hypothesis that elevated oxLDL may worsen ovarian cancer outcome by stimulating cancer proliferation and are consistent with results from a clinical study showing significantly greater serum oxLDL levels in ovarian cancer patients compared to a healthy controls [5]. Our initial findings on LDL and oxLDL mediated proliferation directed our attention to modulation of the liver X receptor pathway as a regulator of oxLDL.

LXR agonists reverse oxLDL mitogenesis of ovarian cancer cells

LXR agonists activate LXR more potently than oxysterols resulting in elevated cholesterol efflux by genes possessing LXR elements (LXREs), and reduction of the expression of proinflammatory cytokines by genes lacking LXREs (Reviewed by [14]). Because some studies have shown lipid independent actions of LXR agonists [21,22] we investigated whether TO901317 could inhibit proliferation of ovarian cancer cells both in the absence and presence of oxLDL stimulation. We demonstrated that TO901317 alone significantly reduced cell abundances in proliferation assays, and that TO901317 fully reversed the growth stimulated by oxLDL. Immunoblotting demonstrated that each of seven ovarian cancer cell lines expressed LXR pathway proteins CD36, LXR, and ABCA1, and that the three that we further investigated, OVCAR3, SKOV3, and CAOV3, expressed the highest levels of LXR. We also demonstrated that LXR agonist did not inhibit proliferation of CAOV3 cells treated with each of three combinations of LXR siRNAs, and the effects of LXR siRNAs were significantly different from that observed using a control siRNA. Indeed when both LXRα and LXRβ siRNAs were used in combination a slight increase of proliferation was observed which might be due to multiple actions of the agonist. While we consistently found that the potency of TO901317 was reduced when transfecting siRNAs, we also observed that treatment of CAOV3 cells with any combination of LXR siRNAs significantly reduced proliferation compared to the control. This observation is entirely consistent with LXR as a mediator of proliferation by activation of proinflammatory cytokines, which is suppressed by LXR activation by agonists. These data demonstrate that the observed TO901317 effects were mediated by LXR. We are aware of only one other group of investigators that demonstrated the effects of any LXR agonist on the proliferation of cancer cells. Their studies demonstrated that treatment of LNCaP prostate cancer cells with TO901317 significantly reduced proliferation of cells cultured in vitro as well as in xenografts [23,24].

Fluvastatin inhibits proliferation of ovarian cancer cells

Statins are pleiotrophic compounds with inhibitory actions on cellular proliferation that are unrelated to cholesterol lowering. We tested only fluvastatin on platinum-sensitive and -resistant ovarian cancer cell lines to determine whether statins might have direct inhibitory effects on the proliferation of ovarian cancer cells. Our study demonstrated that fluvastatin inhibited the proliferation of both CAOV3 and SKOV3 ovarian carcinoma cell lines, and was unique among other studies in that we demonstrated fluvastatin could also inhibit proliferation mediated by oxLDL. Simvastatin, lovastatin and mevastatin were all effective for reducing proliferation of TOV112D ovarian carcinoma cells in the only other study to investigate an ovarian carcinoma cell line [25]. There are now a number of studies that have investigated statin effects on the proliferation of other cancer cell types demonstrating statins are generally effective for
reducing cancer proliferation [26]. For breast cancer cells in culture, Cambell et al. [27] showed that lipophilic statins were most potent and that these statins best inhibited cells with constitutively active MAP-kinase pathways.

Cardiotrophin 1 expression was induced by oxLDL and reversed by LXR agonist

Our study also identified cardiotrophin 1 as a cytokine that was induced by oxLDL but reversed by TO901317. We made this observation by screening a cytokine antibody array with the media of SKOV3 cells treated with or without oxLDL and TO901317. The purpose of this experiment was to demonstrate in principle that the LXR agonist could reverse the expression of proinflammatory cytokines for proliferation induced by oxLDL treatment. Of all the cytokines on the array, CT-1 was the only one induced by oxLDL by greater than 2 fold and fully reversed by TO901317 (see Supplementary Tables 1S and 2S for the complete list).

CT-1 is a member of the IL-6 superfamily and activates the IL-6 receptor gp130 [28]. CT-1 expression is anti-apoptotic and loss of CT-1 in cardiomyocytes is associated with increased cell death related to heart failure (Lopez et al., 2007). CT-1 has also been characterized as a hepatocyte stimulator stimulating factor and has been used therapeutically in cirrhotic rats to stimulate angiogenesis and liver regeneration in a manner not requiring gp130 expression suggesting that another CT-1 receptor may exist [29]. CT-1 is also expressed in adipose tissue and upregulated in metabolic syndrome [30]. Therefore, elevated CT-1 levels due to obesity could contribute to reduced ovarian cancer survival, as previously observed by our group. Since PPAR, NFkB and Ras support CD36 expression [12], CT-1 expression might also promote oxLDL signaling as PPAR and NFkB were induced in various cell types including adipocytes, monocytes and hepatocytes [30,31] and gp130 activates Ras in numerous cell types [32].

oxLDL reduces chemosensitivity of ovarian cancer cells

The observation that oxLDL potently stimulated the proliferation of ovarian cancer cells raised the question whether oxLDL could alter the sensitivity of ovarian cancer cells to chemotherapy. We tested this hypothesis by treating both CAOV3 and SKOV3 cells with or without oxLDL and increasing doses of cisplatin and observed that oxLDL treatment of both cell lines significantly reduced the cisplatin IC50s. Our study suggests that therapies that reduce oxLDL effects might have a role in augmenting chemoresponse and/or overcoming chemoresistance.

LXR agonists and statins as therapeutics for ovarian cancer

Our data suggest further investigation into the use of statins and LXR agonists as adjunctive therapies for ovarian cancer is merited. Statins may be particularly effective since they might reduce the negative effects of high serum cholesterol, while simultaneously inhibiting proliferation of cancer cells stimulated by oxLDL and other mitogens. Like statins, LXR agonists are multifunctional, enhancing cholesterol efflux stimulated by oxLDL while simultaneously reversing the expression of proinflammatory cytokines. We have now demonstrated that both fluvastatin and TO901317 are direct inhibitors of ovarian cancer cell proliferation in the absence of oxLDL and that each can reverse ovarian cancer cell proliferation mediated by oxLDL. Furthermore, TO901317 reduces ovarian cancer expression of the mitogen CT-1 that is stimulated by oxLDL. In Fig. 6, we present a model for how LXR agonists and statins might have a role as adjuvant therapies or as primary or secondary prevention strategies for ovarian cancer. Further investigation of statins, oxLDL, and the LXR pathway in ovarian cancer are warranted.

Conflict of interest statement

The authors have no conflicts of interest to declare.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found in the online version, at doi:10.1016/j.ygyno.2009.09.034.

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