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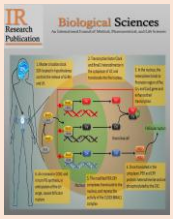

The effects of LDL cholesterol and pitavastatin treatment on fibroblast migration, SREBP-2, and LDLr expression

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ABSTRACT

Most non-healing wounds show a lack of cell migration contributing to chronic inflammation and infection. Cholesterol levels in the blood may have an impact on cell migration, as migrating cells demonstrate a need for cholesterol in order to synthesize additional cell membranes. Statins are a popular drug used for lowering blood cholesterol by competitive inhibition of HMG CoA reductase, a pivotal step in the cholesterol synthesis pathway. In this study, we examined the effects of low-density lipoprotein (LDL) and statin treatment on fibroblasts in vitro. A cholesterol ELISA was utilized to examine cholesterol levels in cultured fibroblasts following treatment with pitavastatin or LDL. A scratch-test assay was performed to examine fibroblast migration following treatment in addition to the MTT cell proliferation assay. Western blot analysis was used to examine the cholesterol signalling protein SREBP-2 and LDLr expression in treated cells. LDL treatment enhanced cell proliferation and migration, while both were inhibited by pitavastatin. Pitavastatin increased both SREBP-2 and LDLr expression in treated cells compared to LDL and vehicle control treatments. These results indicate pitavastatin inhibits cell migration and the cellular response is to increase cholesterol levels to make up for the inhibition of cholesterol synthesis though the mevalonate pathway. This could have significant effects on cell migration and wound healing in vivo for patients with inhibited wound healing responses on statin therapy.

Introduction

Chronic non-healing wounds are an important medical concern which can have a profound effect on the morbidity and mortality of the patient (1). Diabetes and obesity are two medical conditions on the rise that exhibit impairment in wound healing processes (2, 3). Wound healing is a complex process that requires coordinated events including haemostasis, inflammation, proliferation, re-epithelialization, and remodelling (4). For proper wound healing to occur dermal and epidermal cells (fibroblasts and keratinocytes) need to differentiate, proliferate, and migrate into the wound site. This

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Abbreviations: *HMG CoA Reductase: 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase; LDL: Low-density Lipoprotein; LDLr: Low-density Lipoprotein Receptor; ELISA: Enzyme Linked Immunosorbent Assay; MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; SREBP: Sterol Regulatory Element Binding Protein.*

migration requires the synthesis of additional plasma membrane phospholipids and cholesterol. Cholesterol is an essential component of the cell membrane as it maintains integrity and fluidity. Cholesterol can be synthesized inside the cells or taken up by receptors present on the cell surface (5). Cholesterol is transported in the blood via carrier proteins called lipoproteins. LDL receptors (LDLr) bind and regulate the cellular uptake of LDLs which are internalized via receptor-mediated endocytosis and hydrolyzed to free cholesterol in the lysosomes (6, 7). To maintain lipid homeostasis, each cell balances its internal and external cholesterol by a well-regulated feedback system of sterol regulatory element binding proteins (SREBPs). SREBP-2 monitors cellular cholesterol levels and responds by the transcription of genes and enzymes involved in the cholesterol synthesis pathway. SREBP-2 is a more potent inducer of LDLr and SR-BI gene expression than SREBP-1 (8).

Statins are drugs that lower cholesterol levels by blocking their production in the liver. They work by blocking HMG-CoA-Reductase (3-hydroxyl-3-methylglutaryl-coenzyme), an enzyme produced early in the mevalonate pathway of cholesterol synthesis (5). Statins are known to have broad-spectrum pleiotropic effects including anti-inflammatory, antioxidative, immunomodulatory, antibacterial, and improvement of microvascular function (4). Statins have been shown to improve wound healing processes in both animal and human trials (4, 9). The ability of pitavastatin to upregulate LDLr in normal human keratinocytes and HaCaT cells was confirmed by El-Latif et al. (10). Their study also showed pitavastatin increased proliferation in keratinocytes, but not on their ability to migrate.

While the importance of keratinocyte proliferation and migration in wound healing is well established, dermal fibroblast migration and proliferation have not been as extensively studied. Fibroblasts play a central role in the wound healing response through regeneration of the dermal matrix, as well as having a transient role as myofibroblasts in wound contraction (11, 12). The present study was conducted to examine how dermal fibroblasts respond to pitavastatin treatment and whether decreasing cholesterol synthesis through statin treatment would negatively affect the migratory response in these cells. We utilized dermal fibroblasts in vitro to examine the effects of LDL and pitavastatin treatment on cell proliferation, viability, migration, and expression of proteins associated with cholesterol signaling within the cell. Here we show that pitavastatin treatment, presumably through the inhibition of endogenous cholesterol production, significantly impairs cell migration in vitro.

Materials and Methods

Cell culture of human fibroblasts

Dulbecco's Modified Eagle Medium (DMEM, Cascade Biologics) supplemented with 10% Newborn Calf Serum (NCS, Cascade Biologics) was used to culture adult human dermal fibroblasts (HDFa, Cascade Biologics) in monolayer cultures at 37 degree C, 5% CO2/95% air in a humidified cell culture incubator. The cells were sub-cultured in 6 well plates or 96 well plates for wounding assay, MTT assay, and subsequent treatments. The culture medium was changed 24 hours after establishing a culture and changed every day as the cultures approached 80% confluence. For all experiments, cells between passages 2 and 6 were used.

Wound Healing Assay

Six-well plates at 80-90% confluence were switched to serum free medium for 24 hours at 37 degree C to synchronize cell division. Cells at 100% confluence were then treated with mitomycin C (10 µg/mL in cell media) for one hour to arrest cell division. The cultures were wounded by scratching the monolayer with a sterile pipette tip. Cells were then treated with LDL (25 µg/ml; Biomedical Technologies), Pitavastatin (Pit, 0.1 mmol, Santa Cruz), vehicle (DMSO, no treatment), or Epidermal Growth Factor (EGF, 30 µg/ml). All treatments were based on cell migration and viability from previous studies. Each treatment was prepared as a stock solution in DMSO and diluted to the final concentration in culture media, with a final concentration of 0.01% DMSO in all treatments and vehicles. Each treatment was performed in triplicate. The wounded area was imaged using an Olympus BX41 inverted microscope with a Spot Flex cooled CCD camera and MiniSee image acquisition software at three points along the wound at 24 and 48 hours post-incubation. Treatment effects on migration were determined by the changes in wound surface area using ImageJ software (NIH).

MTT Assay

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay for cell viability and proliferation was performed on the cultured fibroblast cells grown in 96 well plates and treated as listed above minus the mitomycin. The treatment medium was removed from all the wells after 4 hours and 100 µl fresh medium was added for an additional 24 hours. The MTT assay was performed according to instructions in the Vybrant MTT Cell Proliferation Assay Kit (Sigma). The t-Test was used to examine differences between treatments.

Protein Extraction

Following treatments, the proteins were extracted with RIPA Lysis buffer (Sigma). 200 µl of cold RIPA buffer was placed in each well after washing cells with cold phosphate-buffered saline (PBS). Protease inhibitor (Roche Diagnostics) was added to the RIPA Buffer immediately before use to prevent proteolysis. The lysates were gathered by scraping the cells and were frozen at -80 degree C for protein analysis. The wells and the vials were kept on ice with occasional swirling the plates for uniform spreading throughout the process. Protein quantification was determined with the BCA protein assay kit (Pierce) in 96 well plates using 25 µl of each unknown sample. Standards and working solutions were prepared following the instruction in the BCA Assay kit. Three replicates of standards or unknowns were used. The absorbance was read at 562 nm using a SpectraMax 384 plate reader (Molecular Devices).

Cholesterol ELISA

Total cholesterol was examined in fibroblast cell cultures using a colorimetric kit (Biovision). Fibroblasts were cultured and treated as above with either no treatment, pitavastatin, wounding, or wounding with pitavastatin. After 24 hours of incubation with treatments, cells were washed with cold PBS and the wells were scraped with a cell scraper. The extracts were collected in Eppendorf tubes and lipids were extracted by adding 200µL of chloroform: isopropanol: NP-40 in a micro-homogenizer. The samples were then centrifuged, and the supernatant (organic phase) was decanted into a new tube. After incubation at 50°C to remove chloroform, samples were placed under a vacuum to remove all other organic solvent traces. Extracted samples were adjusted to 50 µL per well and a master mix of assay buffer, probe, enzyme mix, and cholesterol esterase was made as per Biovision protocol. Cholesterol standards were included in the assay plate at 0, 1, 2, 3, 4, and 5 µg/mL. After one-hour incubation at 37°C in the dark, absorbance was measured at 570 nm in a microplate reader. Absorbances were multiplied by values obtained by the standard curve, and concentrations of µg/mL were obtained. Three independent experiments were performed, and the averages and standard deviations of each treatment were plotted. The t-Test analysis was used to examine differences in treatments.

Western Blotting

Western Blot analysis was performed according to Protein Methods (13). Briefly, Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) with an 8% separating gel (30% acrylamide, 0.8% bis-acrylamide, Tric-HCl pH 8.8, 10% SDS, 10% Ammonium persulfate and TEMED) and 5% stacking gel (30% acrylamide, 0.8% bis-acrylamide, Tris-HCl pH 6.8, 10% SDS, H20, 10% Ammonium persulfate and TEMED) were prepared and allowed to polymerize. Equal aliquots of proteins determined from the BCA assay were used for protein analysis. 5X Loading buffer (Tris-HCl pH 6.8, 50% glycerol, 10% SDS, 2-mercaptoethanol, 1% bromophenol blue) was added to the protein samples, heated at 100 degree C for 5 minutes, and loaded into the wells. The electrophoresis buffer (Tris Base, glycine, SDS, and H20) was added to the inner and outer reservoir and the gel was electrophoresed at 200 V for one hour. The proteins were then transferred to a nitrocellulose membrane for an hour in cold transfer buffer (Tris Base, glycine, methanol, and H20) for blotting. Blots were blocked in 3% Bovine Serum Albumin (BSA) in TBS with 1% Tween-20 (TBST) for 2 hours to prevent the non-specific binding. Primary mouse anti-SREBP-2 antibody (Santa Cruz, 1:1000 in TBST), rabbit anti-LDLr antibody (Santa Cruz, 1:200), or mouse anti-β-actin antibody (abcam, 1:1000) was applied and the blots were incubated overnight at 4-degree C. The blots were washed three times for five minutes each with 0.2% BSA in TBST after primary and secondary antibody incubation. The secondary goat anti-mouse IgG-HRP or mouse anti-rabbit IgG HRP (Santa Cruz, 1:1000) was added to the blots and incubated for an hour at room temperature followed by three washes as above. ECL detection reagent (Pierce) was used to detect the secondary antibody. The results were quantified using the Gel Logic 1500 imaging system (Kodak) and Kodak Molecular Imaging software. The t-Test analysis was used to examine differences in treatments.

Statistical Analysis

Results are presented as the mean of triplicate runs of each experiment +/- 1 standard deviation of the mean. Data were analyzed using the two-tailed unpaired t-Test with the level of significance set at $p < 0.05$.

Results

Migration and Proliferation Assay

Effects of pitavastatin and LDL on fibroblast scratch test assays at 24 and 48 hours showed migration of cells into the scratch test area progressed in the vehicle control over the 48 hours and was improved by epidermal growth factor (EGF, 30 µg/ml) and low-density lipoprotein (LDL, 25 µg/ml) treatments (**Figure 1A**).

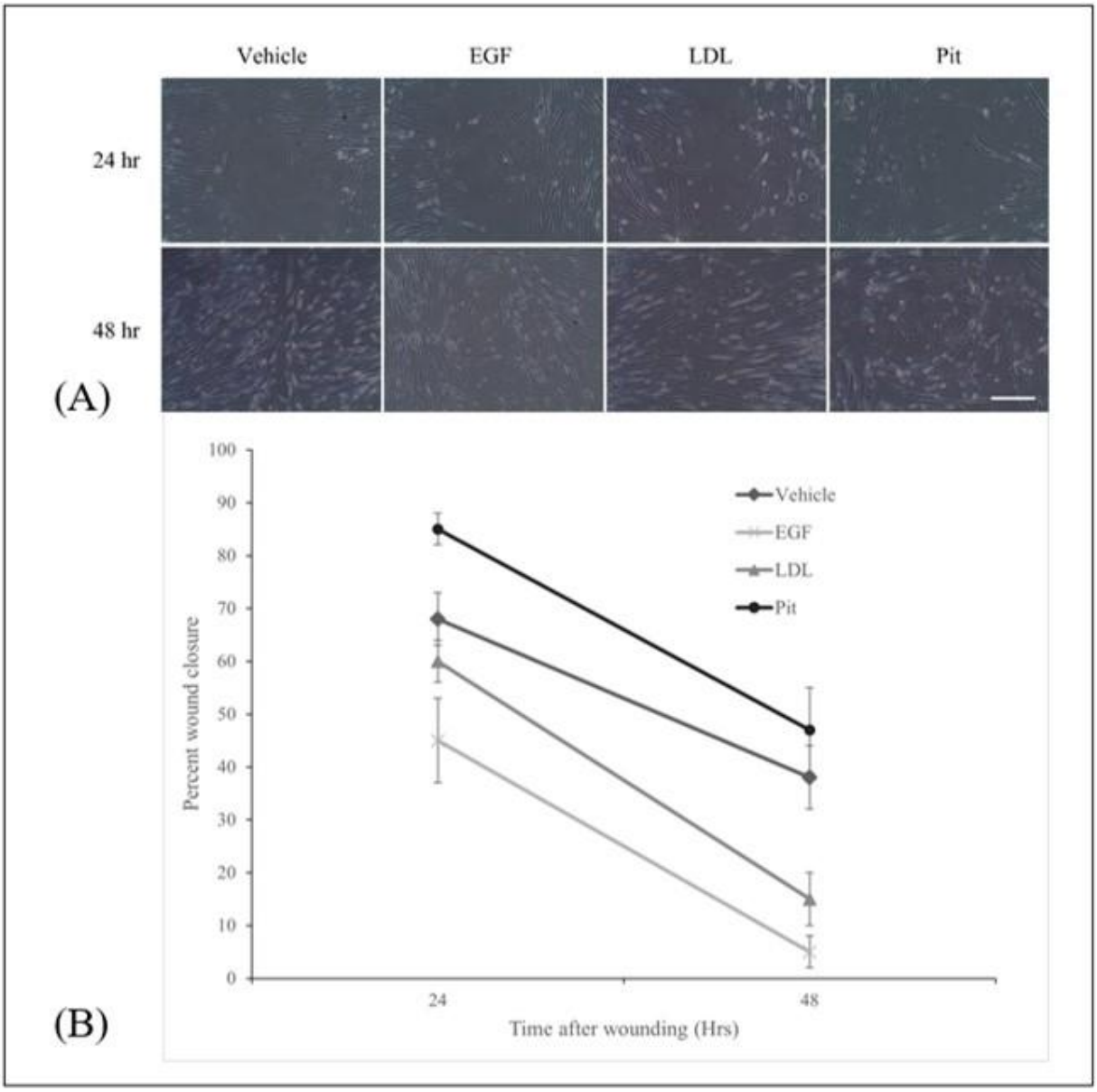

Figure 1. Effects of pitavastatin and LDL on fibroblast scratch test assays at 24 and 48 hours. Migration of cells into scratch test area progressed in the vehicle control, and was improved by epidermal growth factor (EGF, 30 µg/ml) and low-density lipoprotein (LDL, 25 µg/ml) treatments (A). Pitavastatin (0.1 mmol) inhibited cell migration compared to vehicle control. Quantification based on ImageJ analysis of open surface area is based on triplicate cultures of the above treatments (B). Error bars indicate +/-1 standard deviation form the mean. Bar = 100 µm

Pitavastatin (0.1 mmol) inhibited cell migration compared to vehicle control. Quantification based on ImageJ analysis of the open surface area is based on triplicate cultures of the above treatments (Figure 1B).

Based on this information the MTT assay was performed to measure cell viability following treatment. The cells treated with pitavastatin migrated least in the scratch test assay, however, viability was not significantly affected compared to the vehicle control (Figure 2).

Interestingly the LDL treatment increased cell viability significantly compared to both vehicle ($p=0.009$) and pitavastatin ($p=0.02$) treatments. Epidermal growth factor had the greatest effect on cell proliferation/viability, nearly double that seen in the vehicle-treated cells ($p<0.001$) compared to all other treatments.

Cholesterol ELISA

Total cholesterol was measured in adherent fibroblast cells to examine the effect of treatments

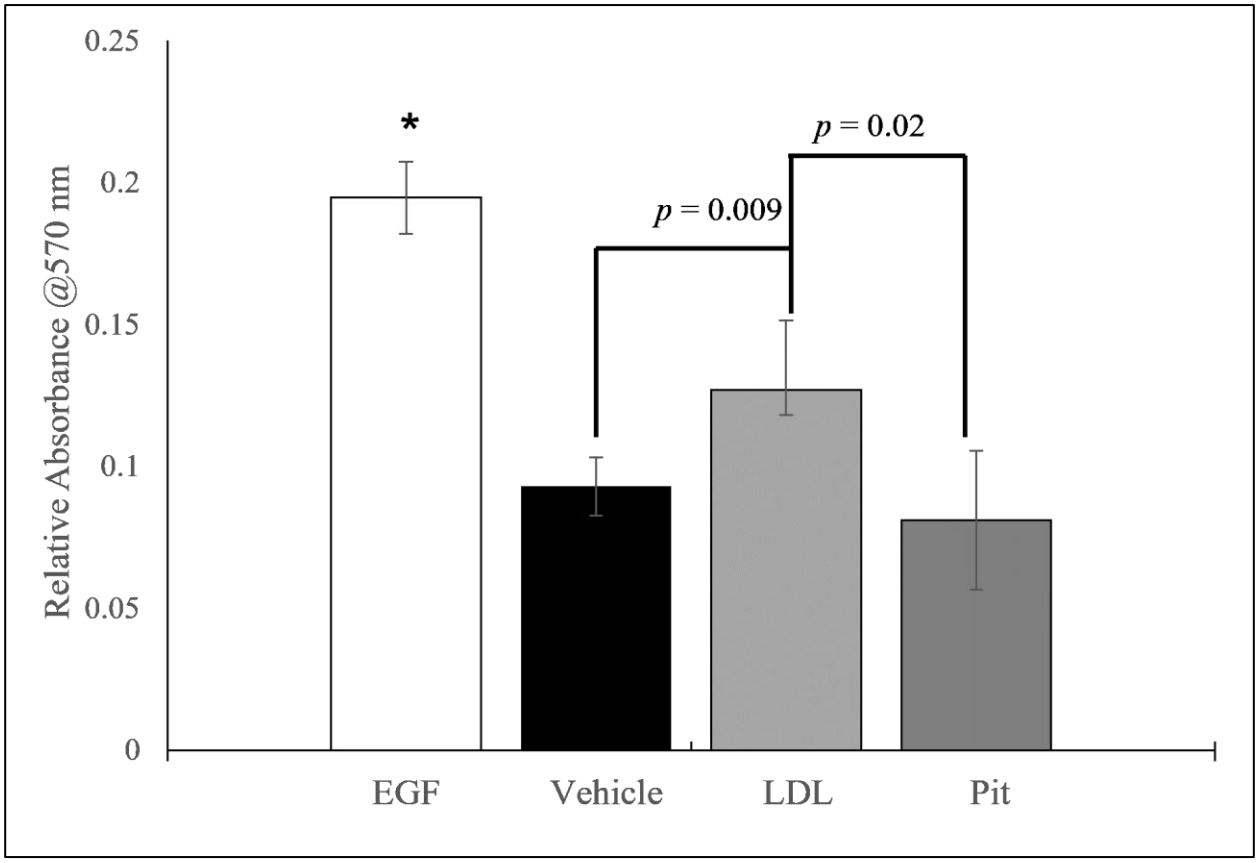

Figure 2. MTT assay for cell viability following treatment. The viability of cells treated with pitavastatin were not significantly affected compared to the vehicle control. The LDL treatment increased cell viability significantly compared to both vehicle (p=0.009) and pitavastatin (p=0.02) treatments. Epidermal growth factor had the greatest effect on cell proliferation/viability, nearly double absorbance seen in the vehicle treated cells (*p<0.001 compared to all other treatments). Error bars indicate +/-1 standard deviation form the mean.

on cholesterol levels in vitro. Cholesterol esterase was added to the master mix to convert cholesterol esters in the cell extracts into free cholesterol, therefore all measurements report total cholesterol in the adherent cells. Compared to the untreated control (2.30 µg/mL), there was a significant decrease (p = 0.04) in cholesterol in the pitavastatin treated cells (1.34 µg/mL, Figure 3).

While wounding increased the amount of cholesterol in the cells (2.87 µg/mL), this was not significant compared to no treatment. Wounding the cultures did produce a significant increase in total cholesterol in the pitavastatin-treated cells (2.26 µg/mL, p = 0.002). The total cholesterol in the wounded pitavastatin-treated cells was still significantly less than the wounded cells that were not exposed to pitavastatin (p=0.03).

SREBP-2 and LDLr Expression

SREBP-2 is one of the sets of SRE-binding proteins in the smooth endoplasmic reticulum and is associated with the SREBP cleavage-activating protein (SCAP) and the insulin-induced gene protein (INSIG). In the case of low cellular cholesterol SREBP-2 is cleaved and binds to the sterol regulatory element (SRE) in the nucleus to increase the transcription of genes associated with increased cholesterol levels in the cell. Here we show SREBP-2 levels are increased in the fibroblast following EGF and pitavastatin treatment (Figure 4).

Both treatments increase LDLr expression in the cell. LDL does not increase SREBP-2 levels in the fibroblast; however, it does increase LDLr expression. Quantification of the blots was performed, and the results were shown for SREBP-2 (Figure 4B) and LDLr (Figure 4C). SREBP-2 levels were significantly increased in the EGF (p=0.002) and pitavastatin (p=0.03) treatments compared to vehicle and compared to LDL treatment (p=0.0008 and p=0.03, respectively). LDLr was significantly increased (p<0.001) for all treatments compared to the vehicle control (*).

Discussion

Cholesterol is an essential component of the cell membrane and is obtained by two main mechanisms, internal synthesis via the mevalonate pathway and uptake through cell surface receptors.

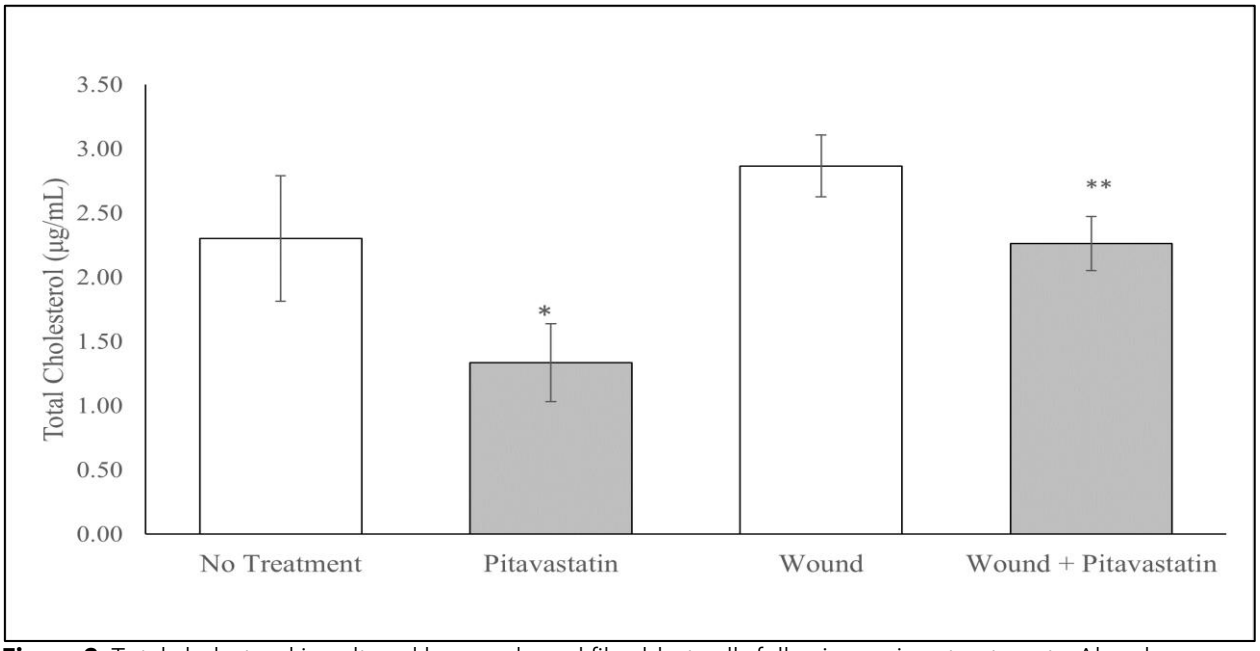

Figure 3. Total cholesterol in cultured human dermal fibroblast cells following various treatments. Absorbance was measured in the colorimetric total cholesterol ELISA at 570 nm for cholesterol standards ranging from 0-5 µg/mL. Unknown sample absorbance was used to quantify cholesterol levels compared to the standards. Unwounded cultures had an average cholesterol of 2.3 µg/mL. This was decreased significantly (*p=0.002) following treatment with pitavastatin (1.34 µg/mL) for 24 hours. Wounding the cultures showed a slight but not significant increase in total cholesterol (2.87 µg/mL) compared to the control. Wounding also significantly (p=0.03) increased the total cholesterol in the pitavastatin treated cells (**2.26 µg/mL) compared to the pitavastatin cultures that were unwounded, but still significantly less than wounding alone (p=0.03).

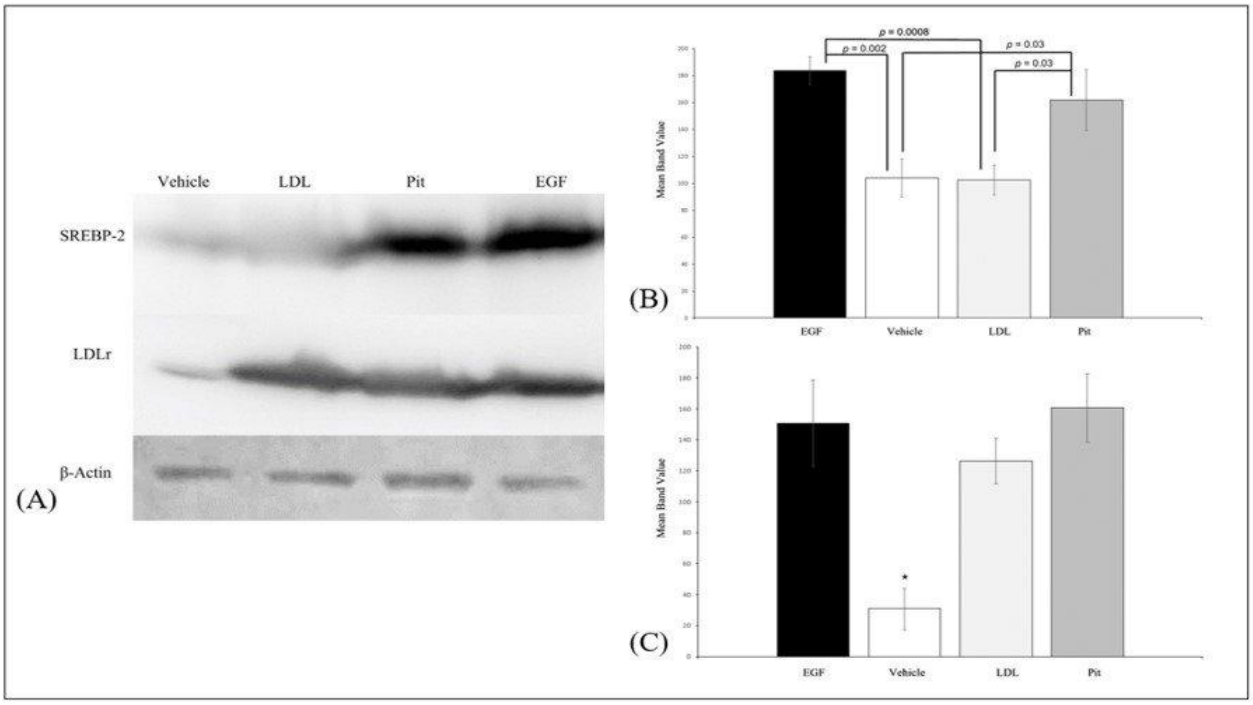

Figure 4. Western blot for SREBP-2 and LDLr in cultured human dermal fibroblast cells. SREBP-2 levels are increased in the fibroblast following EGF and pitavastatin treatment (A). Both treatments increase LDLr expression in the cell. LDL does not increase SREBP-2 levels in the fibroblast; however, it does increase LDLr expression. Quantification of the blots was performed, and the results shown for SREBP-2 (B) and LDLr (C). SREBP-2 levels were significantly increased in the EGF (p=0.002) and pitavastatin (p=0.03) treatments compared to vehicle and compared to LDL treatment (p=0.0008 and p=0.03, respectively). LDLr was significantly increased (p<0.001) for all treatments compared to the vehicle control (*).

Cholesterol carrier proteins play important roles in delivering the required cholesterol needed for the cell. Previous studies have shown that cholesterol improves the proliferation and migration of keratinocytes (10). In the present work, we have examined the role of LDL and pitavastatin on migration and cholesterol-mediated protein expression in fibroblasts. Statins are competitive drugs that lower cholesterol biosynthesis by inhibiting the HMG-CoA reductase enzyme, at the same time increasing the uptake of cholesterol from LDL via upregulation of LDLr (14). Low-density lipoprotein (LDL) is associated with cholesterol deposition in peripheral vessels and plaque formation, resulting in a high risk for atherosclerosis and heart disease (15, 16), and elevated LDL is a common reason for statin therapy. We chose pitavastatin in this study as it was shown to upregulate LDLr in cultured keratinocytes as well as mouse epidermis (10).

In the current study, we were able to show increased migration in cells treated with LDL compared to untreated controls. Pitavastatin showed a decrease in migration compared to all other treatments. This was presumed to be due to the need for fibroblast cells to generate a new cell membrane, a process requiring cholesterol. This can be seen in the total cholesterol assay, where cholesterol levels were increased following wounding. Pitavastatin significantly decreased cholesterol levels in cultured fibroblasts, and the inhibition of cholesterol synthesis in these cells may have caused cell migration to be inhibited. Pitavastatin had no significant effect on cell viability compared to the non-treated control as shown in the MTT assay, while LDL and EGF treatments significantly increased cell viability. Based on these results we can conclude that LDL has positive effects on fibroblast migration and viability, whereas pitavastatin had no effect on cell viability and inhibited cell migration. Interestingly wounding the cells treated with pitavastatin significantly increased cholesterol levels, but not to the level seen with wounding alone.

To better understand the mechanism behind the increased migration and proliferation of cells with LDL, we investigated the signal transduction pathways involved in cholesterol biosynthesis. LDLr in cell membranes mediates the uptake of LDL and provides cells with additional cholesterol. In previous studies, LDLr was upregulated in wounded cells in scratch test assays and was further increased following LDL treatment (7, 10). Transcription of LDLr is controlled by SRE found in the promoter of the gene. SREBPs are membrane-bound transcription factors that regulate the expression of many genes involved in cholesterol biosynthesis (17). SREBPs are of the basic helix-loop-helix leucine zipper (bHLH) family of DNA binding proteins that sense the amount of cholesterol in cell membranes and help maintain the negative feedback control of cholesterol homeostasis (18). Normally in the presence of excess cholesterol SREBPs are sequestered in the endoplasmic reticulum where they are bound to an escort protein: SREP cleavage-activating protein (SCAP) and the ER-resident protein Insig. If internal cholesterol is depleted, SREBPs binding to Insig become disrupted and SCAP transports SREBP to the Golgi (19). Inside the Golgi, two proteolytic cleavages occur by site 1 and site 2 proteases. The new transcription activation domain of the SREBP protein translocates to the nucleus, binds to sterol regulatory elements (SRE), and activates the transcription of genes associated with cholesterol synthesis and uptake. Some of the SREBP-regulated genes activated are LDL receptors (LDLr), SREBP-2, SREBP-1, Scavenger reporter B1 (SR-BI), Insulin-induced gene 1 (INSIG-1), ATP binding cassette transporter A1 (ABCA1), SCAP and HMG-CoA reductase (HMGCR). Transcriptional activation results in increased LDL internalization via LDLr and the increased cytosolic cholesterol inhibits activation of the SREBP through negative feedback. Many studies have shown the importance of the SREBP-pathway for cellular cholesterol homeostasis in diseases such as Familial hypercholesterolemia, Niemann-Pick disease C, Tangier disease, and nonalcoholic steatohepatitis (6, 18). Two different genes encode three SREBP isoforms; they are SREBP-1a, SREBP-1c, and SREBP-2 (20). SREBP-1a activates all SREBP-responsive genes, including the synthesis of cholesterol, fatty acids, and triglycerides. SREBP-1c mainly enhances the transcription of genes required for fatty acid synthesis, while SREBP-2 preferentially activates cholesterol synthesis (21).

Since LDL treatment increased the regulation of LDLr we were interested in the effects of LDL on SREBP-2. LDLr and SREBP-2 proteins were increased significantly following EGF and pitavastatin treatment. LDL treatment did not significantly increase SREBP-2 levels; however, it did increase LDLr expression. These results indicate that while pitavastatin and EGF directly upregulate LDLr expression through the SREBP-2 pathway, LDL utilizes an alternative pathway for increasing LDLr in fibroblast cells. Statins are used clinically to reduce the risk of cardiovascular disease by decreasing plasma LDL cholesterol. In the liver, SREBP-2 is activated in response to the reduction of hepatic cholesterol content due to statin therapy. As a result, ABCA1 gene expression is enhanced which increases HDL cholesterol, which is then able to scavenge cholesterol from the periphery and reduce plaque deposition (22). In our study, pitavastatin showed increased SREBP-2 and LDLr expression in fibroblast cells, and yet cell migration was decreased. Presumably, cell migration requires

external cholesterol intake when cellular stores are limited due to inhibition of the cholesterol synthesis pathway. Statin therapy has also been shown to decrease inflammatory biomarkers such as C-reactive protein and interleukin-18 (IL-18) in hypertensive patients (23). IL-18 promotes fibroblast migration and myofibroblast transition, and its inhibition by statin therapy may be an additional cause of decreased cellular migration (24, 25).

Conclusion

In this study, we have shown that fibroblasts in culture exhibit a decreased migratory response when treated with pitavastatin compared to vehicle control, and increased migration in response to treatment with LDL. Adding LDL to pitavastatin-treated cells did not increase migration beyond pitavastatin alone (data not shown). Pitavastatin had no effect on cell viability but did significantly decrease cholesterol levels in the cells. Pitavastatin-treated cells also showed an increase in SREBP-2 and LDLr, indicating a need for the cells to increase intracellular cholesterol levels. Taken together these results indicate pitavastatin inhibits cell migration, presumably due to a decrease in cholesterol synthesis affecting cell membrane turnover. Further, the cellular response is to increase cholesterol levels to make up for the inhibition of cholesterol synthesis through stimulation of the mevalonate pathway and increasing cellular uptake, both of which are stimulated by the transcription factor SREBP-2. This could have significant effects on cell migration and wound healing in vivo for patients with inhibited wound healing responses to statin therapy.

Contribution of authors

Both authors listed in this manuscript were responsible for the study design, data collection, interpretation, and writing the article.

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Conflict of interest

The authors have no conflicts of interest to declare.

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