Hyaluronan Accumulation Is Elevated in Cultures of Low Density Lipoprotein Receptor-deficient Cells and Is Altered by Manipulation of Cell Cholesterol Content*

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The extracellular matrix molecule hyaluronan (HA) accumulates in human atherosclerotic lesions. Yet the reasons for this accumulation have not been adequately addressed. Because abnormalities in lipid metabolism promote atherosclerosis, we have asked whether disrupted cholesterol homeostasis alters HA accumulation in low density lipoprotein receptor-deficient cell cultures. Cultured aortic smooth muscle cells (ASMC) from Watanabe heritable hyperlipidemic (WHHL) rabbits and skin fibroblasts from homozygous patients with familial hypercholesterolemia accumulated 2–4-fold more HA than corresponding cells from age- and sex-matched normolipidemic rabbits and individuals. This occurred in both cell-associated and secreted HA fractions and was independent of cell density or cell number. Cultured aortic smooth muscle cells (ASMC) from WHHL ASM cultures synthesized twice the proportion of high molecular mass HA (>2 × 10⁶ Da) as normal rabbit ASMC but showed a lower capacity to degrade exogenous [³H]HA. Most importantly, cholesterol depletion or blocking cholesterol synthesis markedly reduced HA accumulation in WHHL ASM cultures, whereas cholesterol replenishment or stimulation of cholesterol synthesis restored elevated HA levels. We conclude the following: 1) maintaining normal HA levels in cell cultures requires normal cell cholesterol homeostasis; 2) HA degradation may contribute to but is not the predominant mechanism to increase high molecular mass HA accumulation in low density lipoprotein receptor-deficient WHHL ASM cultures; and 3) elevated accumulation of HA depends on cellular or membrane cholesterol content and, potentially, intact cholesterol-rich microdomains.

Hyaluronan (HA)² is a high molecular mass (1 × 10⁵–1 × 10⁷ Da) unbranched glycosaminoglycan (GAG), composed of repeating disaccharides of N-acetyl-d-glucosamine and d-glucuronic acid (1, 2). It is synthesized at the cytoplasmic surface of the plasma membrane by a family of HA synthases (HAS) (3, 4), and it is subsequently extruded through the plasma membrane into the pericellular matrix (3). HAS enzymes are encoded by three highly conserved mammalian genes, HAS1, HAS2, and HAS3, and are predicted integral plasma membrane proteins with multiple transmembrane domains (5, 6) that partially co-localize with membrane lipid microdomains (7). HA degradation occurs locally through at least two catabolic pathways as follows: internalization by an endocytic pathway that requires functional CD44 (8), a membrane microdomain-associated protein (9) and the principal cell-surface receptor for HA (10); and an extracellular catalysis mediated by hyaluronidase-2, a glycoprophosphinositol-anchored plasma membrane protein (11). The half-life of HA in tissues ranges from a few hours to several days. Interstitial HA is catabolized either locally or, very rapidly, in the lymphatics and by endothelial cells of the liver sinusoids (1, 12). Thus, in tissues where interstitial fluid normally drains into the lymphatic system, a rapid rate of turnover by local cells is not necessary to avoid excessive accumulation of HA.

Considerable work indicates that HA, a widely distributed component of the extracellular matrix (ECM) of vertebrate tissues (1, 2), accumulates in human atherosclerotic lesions (13–18) and in animal models of vascular injury and atherosclerosis (19–21). Furthermore, HAS2 transgene overexpression promotes atherosclerosis in apolipoprotein E knock-out mice (22). HA is frequently found in lipid-enriched areas of atherosclerotic lesions (19). It also co-isolates with lipoproteins from human atherosclerotic lesions (18) and promotes foam cell and cholesterol accumulation in skin xanthoma (23).

Although HA and cholesterol accumulation are both prominent features of atherosclerosis, the link between HA metabolism and cholesterol homeostasis in the cells of vascular lesions has not been adequately addressed. To explore this...
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relationship, we have used aortic smooth muscle cells (ASMC) cultured from low density lipoprotein (LDL) receptor-deficient Watanabe heritable hyperlipidemic (WHHL) rabbits that exhibit atherosclerosis because of high plasma LDL cholesterol levels (24–27). Disrupted cholesterol homeostasis in this model is because of a mutation in the LDL receptor gene (28) and is similar to the most common class of mutations in the human disease of familial hypercholesterolemia (FH) (29).

We report that cultured WHHL ASMC accumulate 2–4-fold more HA than ASMC from normolipidemic, sex- and age-matched New Zealand White (NZW) rabbits. In addition, the HA synthesized by WHHL ASMC is of a larger average hydrodynamic size. These changes appear to be due to a combination of increased synthesis and decreased degradation of HA in WHHL ASMC. Furthermore, we show that removing cholesterol from the WHHL ASMC returns HA accumulation by these cells to control levels, whereas replenishing cholesterol to these cells restores the elevated HA accumulation. These findings directly link cholesterol homeostasis in cells to the metabolism of HA, and may help to explain HA accumulation in atherosclerotic lesions.

EXPERIMENTAL PROCEDURES

Materials—Skin fibroblasts from homozygous patients with FH and from matched normolipidemic individuals, and Dulbecco’s modified Eagle’s medium (DME) with Earle’s salts twice enriched with vitamins and amino acids were purchased from Coriell Cell Repositories (Camden, NJ). Coriell repository numbers for the normal cell lines were as follows: GM00408D, GM00500B, GM002674B, and GM002651E. Repository numbers for the FH cells were as follows: GM00283, GM00486C, GM00488C, GM00701C, GM00200G, and GM03040C. Rabbit SMC growth medium and rabbit SMC serum- and growth factor-free medium (SFM) were purchased from Cell Applications Inc. (San Diego, CA). Fetal bovine serum (FBS) was from Irvine Scientific (Santa Ana, CA). Phosphate-buffered saline (PBS) and trypsin/EDTA were from Invitrogen. Hepes-buffered saline, 0.025 mg/ml trypsin, 0.25 mM EDTA and trypsin/EDTA were from Invitrogen. Heps-buffered saline, 0.025 mg/ml trypsin, 0.25 mM EDTA and trypsin-neutralizing solutions were from Cambrex (Walkersville, MD). d-[3H]Glucosamine HCl (24 Ci/mmol), [3H]acetic acid sodium (4.1 Ci/mmol), and Streptomyces hyaluronidase were purchased from MP Biomedicals (Aurora, OH). Streptomyces griseus Pronase was from Roche Diagnostics. Hyaluronic acid, from human umbilical cord, and all other reagents were products of Sigma.

Rabbits—A WHHL rabbit colony was maintained by inbreeding as described previously (25). Breeding pairs of the WHHL rabbits were a generous gift of Drs. Daniel Steinberg and Joseph Witztum, University of California, San Diego. In addition, two 4-month-old WHHL rabbits were purchased from Covance Research Products Inc. (Denver, PA). The animals were 4–10-month-old males and females. Age- and sex-matched NZW rabbits were from Western Oregon Rabbit Co. (Philomath, OR). The rabbits were sacrificed by sodium pentobarbital injection (intravenous 0.5 ml/lb body weight), and the aortas were harvested. Animal care and procedures were conducted in accordance with applicable state and federal laws and under protocols approved by the University of Washington Institutional Animal Care and Use Committee.

Cell Culture—Primary ASMC were isolated from WHHL and NZW rabbit aortas by the explant outgrowth method (30) and grown in rabbit SMC growth medium. All cell cultures were maintained at 37 °C, in a humidified atmosphere containing 5% CO2, and used between the fourth and sixth passage. For cell proliferation experiments, 24 h after seeding in rabbit SMC growth medium, cells were growth-arrested in 0.1–0.5% FBS/SFM for 48 h. Fresh 10% FBS/SFM was then added to growth-arrested ASMC at day 0, and subsequently after 24 h (day 1) and then every 48 h (days 3, 5, 7, and 9) in 10% FBS/SFM. In cell density experiments, growth-arrested ASMC were incubated with fresh 0.1 or 10% FBS medium for 20 h.

Methyl-β-cyclodextrin (MeβCD) is commonly used as a cell cholesterol acceptor to deplete cells (31, 32) and their plasma membrane (33) of cholesterol. Over 50% cholesterol depletion can be achieved using MeβCD at concentrations as low as 5 mM during a 15-min incubation (33). Cholesterol-MeβCD complex is used as a cholesterol donor because it is a water-soluble form of cholesterol that is rapidly and efficiently taken up by cells, thus inducing cholesterol loading of cell membranes (32, 34). For cholesterol depletion and replenishing experiments, the indicated amounts of MeβCD were added to growth-arrested ASMC for 1 h in 0.5% FBS/SFM. Medium was removed, and the cells were gently washed with SFM. Fresh medium was then added with or without 0.25 mM cholesterol-MeβCD water-soluble complex in an 8:1 mol:mol ratio for 30 min in 0.5% FBS/SFM (32, 34). This complex is a cholesterol donor. The cholesterol complex was also added to control cells. To block cholesterol synthesis, 30 μM lovastatin was added for the last 24 h of the 48-h growth-arrest period. To counteract the lovastatin inhibition of cholesterol synthesis, 30 μM mevalonic acid was added simultaneously with the lovastatin or to controls in parallel dishes. Following the incubations with cyclodextrins or lovastatin (with or without mevalonic acid), the cells were washed gently one time with SFM and then incubated with 1% FBS/SFM for 2.5 h.

Skin fibroblasts were maintained in DMEM supplemented with 10% FBS. For experiments, fibroblasts were seeded at the indicated cell density in 10% FBS/DMEM. After 24 h, they were growth-arrested in 0.1% FBS/DMEM for 48 h. Fresh medium containing 10% FBS was then added for 24 h.

At the end of each incubation period, the medium was collected, incubated with 100–500 μg/ml Pronase at 37 °C for 2–18 h to release HA from its binding proteins, then heated at 100 °C for 20–30 min to inactivate the Pronase. Cell layers were washed with PBS, incubated in Tris-buffered Pronase (0.5 mM Tris, pH 6.5) at 37 °C for 18 h, scraped into the buffer, and heat-inactivated. After centrifugation at 1000 rpm for 5 min, HA in the supernatant was quantified and normalized to cell number.

Hyaluronan Assay—We used a modification (35) of a previously described (36) competitive ELISA in which the samples were first mixed with HA-binding protein isolated from bovine cartilage that has been biotinylated (bPG) and then added to human umbilical cord HA-coated microtiter plates, the final signal being inversely proportional to the level of HA added to the bPG.
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Characterization of Hyaluronan by Hydrodynamic Size—WHHL and NZW rabbit ASMC, seeded and then growth-arrested as described above, were labeled for 6 h with 50 µCi/ml \[^{3}H\]glucosamine \(([^{3}H]GlcN)\) spiked at 0-, 6-, 12-, and 18-h time points in 0.5% or 10% FBS-containing medium. At the end of each 6-h pulse, the medium was collected, incubated with Pronase, and then heated as for the ELISAs. Heating HA to 100 °C did not change its size as determined by size exclusion chromatography on Sephacryl S-1000 (data not shown). HA and other GAGs were separated from unincorporated \[^{3}H\]GlcN by chromatography on Sephadex G-50 columns. Macromolecular fractions containing identical \(^{3}\)H counts were incubated with or without 0.5 unit/ml \(^{3}H\)glucosamine \(([^{3}H]GlcN)\) spiked at 0-, 6-, 12-, and 18-h time points in 0.5% or 10% FBS-containing medium. At the end of each 6-h pulse, the medium was collected, incubated with Pronase, and then heated as for the ELISAs. Heating HA to 100 °C did not change its size as determined by size exclusion chromatography on Sephacryl S-1000, column, under nondissociative conditions. Fractions (0.5 ml) were eluted in 0.5 m sodium acetate, 0.025% CHAPS, pH 7.0, and the radioactivity was measured by liquid scintillation counting. HA radioactivity for each fraction was determined as hyaluronidase-resistant radioactivity from that of the undigested standard. The percent of HA with molecular mass \(>2 \times 10^6\) Da was calculated and used to compare samples.

Hyaluronan Degradation—To examine HA degradation, \[^{3}H\]HA was prepared as described (37). Briefly, NZW ASMC were incubated in 135-mm dishes with growth medium for 3 days and then growth-arrested in 0.5% FBS/SFM. After 48 h, 62.5 µCi/ml \[^{3}H\]acetate (1.25 mCi/dish) was added with fresh 10% FBS/SFM for 3 days. The medium was collected and treated with Pronase and heated as above. After centrifugation at 1000 rpm for 5 min, the medium supernatant was extensively dialyzed against Ca\(^{2+}\)- and Mg\(^{2+}\)-free PBS and subsequently against distilled water at 4 °C. Solid NaCl was added to a final concentration of 0.03 M, and \[^{3}H\]GAGs were precipitated with 1% cetylpyridinium chloride (CPC) at room temperature. After centrifugation at 15,000 rpm for 15 min, the pellet was resuspended in 2 ml of 0.1% CPC, 0.4 M NaCl to dissolve HA. Dissolved HA was then precipitated with 4 volumes of 100% ethanol at \(-20\) °C for at least 18 h. The pellet was extensively washed with cold 100% ethanol, air-dried, and then resuspended in 1.5 ml of distilled water. Purity of the \[^{3}H\]HA preparation was checked by CPC precipitation of hyaluronidase-digested or undigested aliquots using a slot-blot assay as described (38). Ninety one percent of labeled GAGs were hyaluronidase-sensitive. To eliminate residual unincorporated \[^{3}H\]acetate, the labeled HA preparation was desalted on a Sephadex G-50 size exclusion column. Eluted \[^{3}H\]HA had a specific activity of 284 dpm/µg and contained at least 34% with a molecular mass \(>2 \times 10^6\) Da. To maximize the accessibility of the HA to its potential degradation pathways, the original \[^{3}H\]HA preparation was sonicated twice for 30 s at 4 °C (Soni- fier 450, output levels were set to the microtip limit, percent duty cycle was constant). This procedure reduced the proportion of HA with mass \(>2 \times 10^6\) Da to 2%. For degradation experiments, sonicated \[^{3}H\]HA \((2.58 \times 10^6\) dpm/ml) was added to growth-arrested ASMC in SFM. To determine background degradation, \[^{3}H\]HA was incubated in SFM in cell-free culture dishes. After 18 h, medium aliquots were ultrafiltered through a 50,000-Da cutoff filter (Centricon, Millipore Inc., Billerica, MA), and the radioactivity in the filtrate was taken as degraded HA. Control background counts, from medium not exposed to cells, were subtracted from each sample medium filtrate. In addition, the labeled HA in the retentate was assayed for hydrodynamic size by chromatography on Sephacryl S-1000, as described above.

Statistical Analysis—Data are reported as means of triplicate wells from one representative experiment. Error bars indicate S.D. or S.E. as described in the figure legends. Statistically significant differences between WHHL and NZW or within a cell strain were determined using an unpaired Student’s t test, with \(p < 0.05\) considered significant. To quantify the differences in HA accumulation between the WHHL and NZW cells as demonstrated in Figs. 2 and 3, a likelihood ratio test was performed using the R Project for Statistical Computing (Free Software Foundation). This procedure simultaneously evaluates both the slope and intercept estimates. If the results of this test are significant, then it can be concluded that the two groups are statistically different. To achieve a linear relationship between cell density and HA production per cell, the log transform of cell density was performed.

RESULTS

WHHL ASMC Cultures Accumulate Elevated Levels of Hyaluronan and Grow More Slowly than Controls—In vivo accumulation of HA is a feature of atherosclerosis (16), a disease that commonly occurs in LDL receptor-deficient WHHL rabbits (24, 26, 39). To explore the source of HA deposition in the atherosclerotic wall, we isolated ASMC from the aortic arch of WHHL rabbits, quantified HA accumulation in low passage cultures, and compared it with HA production by NZW ASMC. As shown in Fig. 1A, during 2-day incubations, HA accumulates in the culture medium of WHHL ASMC to a significantly greater extent than in that of NZW ASMC, both on a per cell basis and at all time points studied. The maximal increase in HA accumulation in the cultures of WHHL as compared with NZW ASMC was \(\sim 2.5\)-fold, reached after 3 days of culture, and remained approximately the same over the 11 days tested. In 10% FBS-containing medium, the doubling time for WHHL ASMC was \(\sim 5\) days, but only \(\sim 1\) day for NZW ASMC (Fig. 1B). Interestingly, WHHL ASMC cultures had higher HA levels than those of NZW ASMC even in the absence of high serum (Fig. 1A, day 0), indicating that this difference is not dependent upon serum stimulation of the cells. These results showed for the first time that lacking a functional LDL receptor can be associated with enhanced HA accumulation in cell cultures over a wide range of incubation periods.

Elevated Hyaluronan Accumulation by WHHL ASMC Cultures Is Independent of Cell Density and Serum Level—Because the amount of HA produced in cell culture is inversely proportional to the cell density (40) and WHHL cells grow more slowly than NZW cells (41–43) (Fig. 1B), we tested the effect of cell density on HA levels. As shown in Fig. 2, in all cases, increasing the cell density significantly reduced the amount of HA secreted on a per cell basis in the medium, as expected (40). At each cell density tested, either in the presence (Fig. 2A)
absence (Fig. 2B) of high serum, HA accumulation was significantly elevated in WHHL ASMC medium. Similar results were found in the cell layer (Fig. 2A, inset), which typically contains ~10% of total HA. These findings were consistent with increased extracellular HA staining observed in the cell layers of WHHL ASMC cultures (data not shown). The higher HA accumulation in WHHL ASMC cultures in comparison with those of NZW ASMC was greatest in low serum (Fig. 2B). For this reason, we conducted most of the following experiments in low serum conditions.

**Hyaluronan Accumulation Is Elevated in Cultures of Skin Fibroblasts from Familial Hypercholesterolemic Patients**—As in LDL receptor-deficient WHHL rabbit ASMC, skin fibroblast cultures from homozygous FH patients also accumulate 2–3-fold more HA than fibroblast cultures from normal individuals, independent of cell density (Fig. 3). This statistically significant
result was reproduced in five cell strains from five different FH patients, and indicates that elevated HA accumulation in LDL receptor-deficient cell cultures is not unique to one species or cell type.

Hyaluronan Secretion by WHHL ASMC Cultures Is Significantly Elevated over NZW Controls at Early Time Points—Fresh 0.1% FBS-containing medium was added to growth-arrested cells, and HA accumulation in the medium was measured by ELISA at 2.5 and 6 h. HA accumulation on a per-cell basis in WHHL ASMC cultures was 3–4 times greater than those for NZW ASMC at both time points (Fig. 4). This experiment was repeated with two different cell lines each for NZW and WHHL rabbits, and similar results were obtained at 6, 9, and 12 h (data not shown).

WHHL ASMC Synthesize Hyaluronan with Greater Mass than NZW ASMC, and High Serum Induces a Hyaluronan Size Increase in NZW ASMC Cultures—The physical and biological properties of HA polymers are remarkably affected by their molecular size as well as their concentration (44, 45). Therefore, we determined the hydrodynamic size distribution of HA synthesized by WHHL and NZW ASMC after a 6-h pulse with [3H]GlCN, spiked either from 0–6 h or 18–24 h after addition of fresh media containing 0.5 or 10% FBS. Fig. 5A shows a typical size distribution profile of labeled medium collected after a 6-h incubation of WHHL ASMC with low serum, and then eluted on a size exclusion chromatography column (S-1000) before and after digestion with hyaluronidase. Fig. 5B shows a complete size profile of NZW ASMC HA obtained after similar culture conditions as WHHL ASMC. In Fig. 5A, in the absence of hyaluronidase, a prominent peak consisting of labeled material of high molecular mass (>2 × 10^6 Da) eluted near the void volume, followed by a broad shoulder of lower mass. Digestion with hyaluronidase eliminated the very large HA peak. The radioactivity remaining after subtraction of the hyaluronidase-sensitive material for each fraction was plotted as the hyaluronidase-sensitive material, or HA. Graphs of the hyaluronidase-sensitive material taken from Fig. 5, A and B, are presented together in Fig. 5C. In Fig. 5D, we expressed the amount of radioactivity eluting above 2 × 10^6 Da as a percent of total and used this population of high mass HA to compare the different cell lines. The NZW ASMC HA contained 24% high mass material in contrast to 46% for WHHL ASMC HA after 6 h in low serum (taken from Fig. 5C). Similar calculations were made for HA synthesized by WHHL and NZW ASMC at different times and in different serum concentrations (Fig. 5D). At 6 h, the percent of high mass HA synthesized by WHHL ASMC was twice that found with NZW ASMC, regardless of the serum level (Fig. 5D). At 24 h, this difference continued to exist in low serum, but not in high serum because the size of HA synthesized by NZW ASMC had doubled (Fig. 5D). The size of HA synthesized by WHHL ASMC also increased, but to a lesser extent than NZW ASMC (Fig. 5D). These results demonstrate that HA size is altered by the lack of a functional LDL receptor and the presence of high serum.

WHHL ASMC Have a Reduced Capacity to Degrade High Molecular Mass Hyaluronan—HA accumulation may result in part from decreased HA catabolism. Therefore, a series of metabolic experiments was performed to assess if WHHL ASMC degrade HA less efficiently than NZW. For this purpose, growth-arrested WHHL and NZW ASMC were exposed to exogenous [3H]HA for 18 h. SFM was used to avoid serum hyaluronidase activity. In initial experiments, unmodified [3H]HA that was prepared from [3H]acetic acid-labeled NZW ASMC (30% >2 × 10^6 Da) was not degraded by either cell strain (data not shown). These observations are consistent with findings that HA size is a limiting factor in its degradation (46). Therefore, we sonicated the original [3H]HA preparation and produced lower molecular mass HA containing less than 2% [3H]HA >2 × 10^6 Da (Fig. 6A, dashed line). This was then added to WHHL and NZW ASMC cultures. After 18 h, the remaining [3H]HA in the medium was subjected to molecular sieve chromatography on S-1000 (Fig. 6A). The original sonicated HA preparation had a Kav of ~0.48 on S-1000. Hyaluronan incubated with WHHL cells had a profile that was indistinguishable from the original material, but for HA incubated with NZW cells the Kav was ~0.63. These observations were replicated in two separate experiments.

To examine the production of small radiolabeled fragments by WHHL and NZW cells, aliquots of conditioned medium, prepared as above, were centrifuged in a Centricon 50,000-Da cutoff filter unit. As found previously in normal rat ASMC (46), NZW ASMC degrade exogenous [3H]HA but with low efficiency (only ~4%). Production of small (<50,000 Da) material was significantly less (25%) in WHHL cultures in comparison with NZW (Fig. 6B). These results demonstrate that in the absence of a functional LDL receptor, ASMC are defective in catabolism of high molecular mass HA. This difference may result from altered uptake as well as extracellular and/or intracellular breakdown of HA molecules and may contribute to the greater accumulation of HA in WHHL ASMC.
Cholesterol Depletion Reduces Hyaluronan Accumulation in Rabbit ASMC Cultures—Because WHHL ASMC have twice as much unesterified cholesterol (UC) as NZW ASMC (47), we depleted WHHL and NZW ASMC of cholesterol using cyclodextrins, which are water-soluble cyclic oligosaccharides that have the capacity to sequester cholesterol in their hydrophobic cavity, thereby removing cholesterol from cells rapidly and efficiently (34, 48). MeH9252CD significantly decreased HA secretion by WHHL rabbit ASMC in a dose-dependent manner relative to cells incubated without MeH9252CD (Fig. 7). Incubation with 20 mM MeH9252CD also resulted in lower HA accumulation in NZW ASMC cultures but to a lesser extent (43%) than in WHHL ASMC (61%). Inhibition of HA accumulation can be attributed to effects of cholesterol modulation, as opposed to nonspecific effects of MeH9252CD, because the ability to accumulate HA is restored to previously cholesterol-depleted cells upon replenishment.

**FIGURE 5.** WHHL rabbit ASMC synthesize larger hyaluronan molecules than NZW ASMC, but high serum stimulates an increase in hyaluronan size in NZW ASMC. Growth-arrested rabbit ASMC were incubated with fresh media and labeled with [3H]GlcN for 6-h periods, either 0–6 h (A–D) or 18–24 h (D) after addition of the fresh media. [3H]-Radiolabeled macromolecules from WHHL (A and D) and NZW (B and D) ASMC culture media were then subjected to Sephacryl-1000 size exclusion chromatography. A–C, (void (Vo) and total (Vt) volumes (Vt, large arrows), before (Δ) and after (▲) Streptomyces hyaluronidase digestion. Dextran blue was used to mark the elution position of molecular mass = 2 × 10^6 Da (small arrow, dotted line). C, Sephacryl S-1000-derived profile of [3H]HA was calculated from the proportion of hyaluronidase-sensitive material for each eluted fraction in WHHL (▲) and NZW (○) cultures in 0.5% serum at 0–6 h (profiles A and B, respectively). The amount of radioactivity eluting above the 2 × 10^6 Da marker position was determined and expressed as a percent of the total. C, these were 46% for WHHL (slashed area) and 24% for NZW (cross-hatched area) cells. D, percentage of total hyaluronidase-sensitive material with a molecular mass > 2 × 10^6 Da secreted by WHHL (▲) and NZW (○) cells in 0.5% FBS or 10% FBS at 0–6 and 18–24 h. This experiment was performed one time but with four different 6-h windows (0–6, 6–12, 12–18, 18–24 h) (data not shown). Results for percentage of high molecular mass HA in WHHL ASMC were similar in all four windows, ranging from 46 to 53% in low serum and 57–68% in high serum. Also, results were similar at all times for NZW ASMC in low serum medium, ranging from 24 to 33%. The percentage of large HA in NZW ASMC in high serum medium gradually increased with time changing from 28 to 47% and 59 and 63% at successive times (data not shown).

**FIGURE 6.** WHHL rabbit ASMC produce lower levels of [3H]hyaluronan degradation products. [3H]HA (284 dpm/ng), prepared from [3H]acetate-labeled NZW ASMC (HA Prep), was added to growth-arrested rabbit ASMC at 2.58 × 10^6 dpm/dish in serum-free medium. After 18 h, the medium was analyzed by size exclusion chromatography on SephacrylS-1000 (A), with the profile of the original [3H]HA preparation (dotted line) compared with medium incubated with WHHL (▲) and NZW (○) cultures. Medium aliquots were also filtered through a 50,000-Da cutoff Centricon filter unit, and the radioactivity of the filtrate was determined (B). Error bars are S.E. of triplicate wells; *, p < 0.02. This experiment was performed two times with similar results.

**Cholesterol Depletion Reduces Hyaluronan Accumulation in Rabbit ASMC Cultures**—Because WHHL ASMC have twice as much unesterified cholesterol (UC) as NZW ASMC (47), we depleted WHHL and NZW ASMC of cholesterol using cyclodextrins, which are water-soluble cyclic oligosaccharides that have the capacity to sequester cholesterol in their hydrophobic cavity, thereby removing cholesterol from cells rapidly and efficiently (34, 48). MeβCD significantly decreased HA secretion by WHHL rabbit ASMC in a dose-dependent manner relative to cells incubated without MeβCD (Fig. 7). Incubation with 20 mM MeβCD also resulted in lower HA accumulation in NZW ASMC cultures but to a lesser extent (43%) than in WHHL ASMC (61%). Inhibition of HA accumulation can be attributed to effects of cholesterol modulation, as opposed to nonspecific effects of MeβCD, because the ability to accumulate HA is restored to previously cholesterol-depleted cells upon replenishment.
In increased HA accumulation in LDL receptor-deficient cells

**Increased HA Accumulation in LDL Receptor-deficient Cells**

In this study, we have demonstrated that HA accumulation is regulated by cell cholesterol content, and may require intact membrane lipid microdomains. Cultures of LDL receptor-deficient ASMC from WHHL rabbits and skin fibroblasts from FH patients accumulate 2-4-fold more HA than corresponding normal cells. This difference results from a combination of increased synthesis and decreased degradation of HA in WHHL ASMC. NZW cells, which degrade radiolabeled HA more rapidly than WHHL ASMC, only remove about 4% of HA in 18 h. Therefore, differences in degradation cannot, by themselves, be sufficient to explain the difference in HA accumulation.

Cholesterol levels in mammalian cells are tightly controlled by regulation of cholesterol uptake through LDL receptor-mediated endocytosis of plasma LDL (50), cholesterol biosynthesis (51), and cholesterol efflux (48). A negative feedback system controls cholesterol uptake and biosynthesis through sterol regulatory element-binding proteins (SREBPs) and HMG-CoA reductase, the rate-limiting enzyme in cholesterol biosynthesis (51), but this mechanism does not function properly in LDL receptor-deficient cells. UC accumulates abnormally in WHHL ASMC (47), and cholesterol ester content declines in FH fibroblasts (52) because of a failure to suppress HMG-CoA reductase activity (39, 49, 53) and to induce the activity of a cholesterol-esterifying enzyme (50). Cholesterol is an essential component of lipid bilayers in eukaryotic membranes and is thought to influence the function of membrane proteins through regulation of membrane fluidity and thickness (54-56) and through the assembly and function of heterogeneous and dynamic membrane microdomains (rafts). The most common specialized rafts are enriched in cholesterol and sphingolipids and act as platforms upon which proteins can preferentially segregate and regulate their activity (54, 55, 57).

The elevated cholesterol content of WHHL ASMC (47) most likely results in UC enrichment of the plasma membrane, where newly synthesized UC is distributed (55) and the majority of cell cholesterol is located (58). Consistent with this assumption, diet-induced hypercholesterolemia, which saturates and suppresses LDL receptors (50), results in elevated UC and sphingomyelin in plasma membranes from rabbit ASMC (59). Because the enzymes responsible for HA synthesis reside in the plasma membrane (3, 4) we have

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**Inhibition of Cholesterol Synthesis Decreases Hyaluronan Accumulation**—Because WHHL cells exhibit dramatically enhanced hydroxymethylglutaryl-coenzyme A (HMG-CoA) reductase activity (39, 49), a limiting step in cholesterol biosynthesis (50), we examined the role of cholesterol synthesis in HA accumulation. Blocking HMG-CoA reductase activity with 30 μM lovastatin for 24 h had no effect on HA accumulation by NZW ASMC cultures, but reduced it by 26% in WHHL ASMC cultures (Fig. 8). Reduction of HA accumulation varied between experiments from 25 to 50%. Similar observations were obtained with 50 μM mevastatin (data not shown). Inhibition of HA production can be attributed to effects of HMG-CoA reductase activity as opposed to nonspecific effects of the statin, because the ability to accumulate HA is restored to previously statin-incubated cultures upon stimulating HMG-CoA reductase activity with its substrate, mevalonic acid (Fig. 8). Adding mevalonic acid alone had no significant effect upon accumulation of HA in NZW or WHHL ASMC (data not shown). These results indicate that enhanced cholesterol synthesis may contribute to the elevated HA levels in cultures of LDL receptor-deficient cells.

**DISCUSSION**

In this study, we have demonstrated that HA accumulation is regulated by cell cholesterol content, and may require intact membrane lipid microdomains. Cultures of LDL receptor-deficient ASMC from WHHL rabbits and skin fibroblasts from FH patients accumulate 2-4-fold more HA than corresponding normal cells. This difference results from a combination of increased synthesis and decreased degradation of HA in WHHL ASMC. NZW cells, which degrade radiolabeled HA more rapidly than WHHL ASMC, only remove about 4% of HA in 18 h. Therefore, differences in degradation cannot, by themselves, be sufficient to explain the difference in HA accumulation.

Cholesterol levels in mammalian cells are tightly controlled by regulation of cholesterol uptake through LDL receptor-mediated endocytosis of plasma LDL (50), cholesterol biosynthesis (51), and cholesterol efflux (48). A negative feedback system controls cholesterol uptake and biosynthesis through sterol regulatory element-binding proteins (SREBPs) and HMG-CoA reductase, the rate-limiting enzyme in cholesterol biosynthesis (51), but this mechanism does not function properly in LDL receptor-deficient cells. UC accumulates abnormally in WHHL ASMC (47), and cholesterol ester content declines in FH fibroblasts (52) because of a failure to suppress HMG-CoA reductase activity (39, 49, 53) and to induce the activity of a cholesterol-esterifying enzyme (50). Cholesterol is an essential component of lipid bilayers in eukaryotic membranes and is thought to influence the function of membrane proteins through regulation of membrane fluidity and thickness (54-56) and through the assembly and function of heterogeneous and dynamic membrane microdomains (rafts). The most common specialized rafts are enriched in cholesterol and sphingolipids and act as platforms upon which proteins can preferentially segregate and regulate their activity (54, 55, 57).

The elevated cholesterol content of WHHL ASMC (47) most likely results in UC enrichment of the plasma membrane, where newly synthesized UC is distributed (55) and the majority of cell cholesterol is located (58). Consistent with this assumption, diet-induced hypercholesterolemia, which saturates and suppresses LDL receptors (50), results in elevated UC and sphingomyelin in plasma membranes from rabbit ASMC (59). Because the enzymes responsible for HA synthesis reside in the plasma membrane (3, 4) we have

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**FIGURE 7.** Cholesterol depletion reduces hyaluronan accumulation in WHHL and NZW rabbit ASMC cultures and is reversed by cholesterol replenishment. The indicated amounts of MeβCD were added to WHHL (■) and NZW (□) growth-arrested rabbit ASMC for 1 h at 37 °C in 0.5% FBS. Then to replace membrane cholesterol, fresh medium either without or containing 0.25 mM cholesterol (Chol) in 0.5% FBS was added for 30 min at 37 °C. Finally, the cells were incubated for 2.5 h at 37 °C in fresh medium with 1% FBS. Error bars are S.D. of triplicate wells; * and ** are p < 0.05 and p < 0.01, respectively. This experiment was performed two times with similar results.

**FIGURE 8.** Lovastatin partially decreases hyaluronan accumulation in WHHL ASMC cultures. 24-Hour growth-arrested WHHL (■) and NZW (□) rabbit ASMC were incubated with or without 30 μM lovastatin, added simultaneously with or without 30 μM mevalonic acid. After 24 h, fresh medium containing 1% FBS was added for 2.5 h. Error bars are S.D. of triplicate wells; * and ** are p < 0.05 and p < 0.01, respectively. This experiment was performed two times with similar results.

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**Inhibition of Cholesterol Synthesis Decreases Hyaluronan Accumulation**—Because WHHL cells exhibit dramatically enhanced hydroxymethylglutaryl-coenzyme A (HMG-CoA) reductase activity (39, 49), a limiting step in cholesterol biosynthesis (50), we examined the role of cholesterol synthesis in HA accumulation. Blocking HMG-CoA reductase activity with 30 μM lovastatin for 24 h had no effect on HA accumulation by NZW ASMC cultures, but reduced it by 26% in WHHL ASMC cultures (Fig. 8). Reduction of HA accumulation varied between experiments from 25 to 50%. Similar observations were obtained with 50 μM mevastatin (data not shown). Inhibition of HA production can be attributed to effects of HMG-CoA reductase activity as opposed to nonspecific effects of the statin, because the ability to accumulate HA is restored to previously statin-incubated cultures upon stimulating HMG-CoA reductase activity with its substrate, mevalonic acid (Fig. 8). Adding mevalonic acid alone had no significant effect upon accumulation of HA in NZW or WHHL ASMC (data not shown). These results indicate that enhanced cholesterol synthesis may contribute to the elevated HA levels in cultures of LDL receptor-deficient cells.

**DISCUSSION**

In this study, we have demonstrated that HA accumulation is regulated by cell cholesterol content, and may require intact membrane lipid microdomains. Cultures of LDL receptor-deficient ASMC from WHHL rabbits and skin fibroblasts from FH patients accumulate 2-4-fold more HA than corresponding normal cells. This difference results from a combination of increased synthesis and decreased degradation of HA in WHHL ASMC. NZW cells, which degrade radiolabeled HA more rapidly than WHHL ASMC, only remove about 4% of HA in 18 h. Therefore, differences in degradation cannot, by themselves, be sufficient to explain the difference in HA accumulation.

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hypothesized that conditions that alter the cholesterol content of the plasma membrane influence the synthesis and accumulation of HA by ASMC. It is of interest that MeβCD removes HAS from the plasma membrane of a human breast adenocarcinoma line, indicating a likelihood of HAS localization in lipid rafts (7). This manipulation also disrupts microdomains in the plasma membrane (57) and leads to dissociation of raft proteins from lipids (55). Our results show that cholesterol depletion of the WHHL ASMC dramatically decreases HA production by these cells within 2.5 h. This suggests that the regulation of HA, an important ECM molecule, is tied to cholesterol homeostasis of the cell and that intact membrane microdomains may be key regulators of HA levels. In addition, we found that inhibition of cholesterol biosynthesis by statins also reduces HA accumulation in WHHL ASMC cultures. Statins are competitive inhibitors of HMG-CoA reductase (50) but have pleiotropic effects. However, reversal of the statin effect by mevalonic acid (Fig. 8) suggests a specific effect on cholesterol synthesis. HA accumulation was only partially inhibited by statins, and this may be related to the reported resistance of LDL receptor-deficient cells to statins (50).

We propose that in LDL receptor-deficient cells the enrichment of plasma membrane in UC and sphingomyelin promotes the assembly of microdomains that are thicker, more lipid-ordered, and stable (55, 57) into which HAS may preferentially partition. This may in turn result in higher HAS activity/stability within the expanded rafts. This possibility is supported by recent findings that vertebrate HAS1, HAS2, and HAS3 are targeted to plasma membrane domains that are either yet-uncharacterized sites (60) or microvillus-like cell surface protrusions in which HA synthesis is concentrated (7, 61). Others have found that detergent solubilization of mammalian HAS influences the activity of the enzyme(s), suggesting that lipids may be involved in regulating mammalian HAS activity (62). Also, Weigel et al. (63) have demonstrated the phospholipid dependence of class 1 HAS activity, suggesting that changes in membrane fluidity because of altered cell cholesterol homeostasis may activate HA biosynthetic pathways, which would partly account for enhanced HA accumulation in WHHL and FH cell cultures.

Previous studies have uncovered a link between cholesterol homeostasis and HA synthesis and accumulation. For example, diet-induced hypercholesterolemia in rabbits increases HA content of atherosclerotic lesions (20, 64) and leads to elevated mRNA levels for HAS1 in experimental vein grafts (65). Wiczorek and Zollner (52) have reported increased GAG synthesis by FH skin fibroblasts, accompanied by levels of microsomal glucuronyltransferase and HMG-CoA reductase which increased reciprocally with the microsomal cholesterol ester content. The mechanism underlying the regulation of HA levels and cell cholesterol content in LDL receptor-deficient cells is unknown. Genes involved in maintaining cholesterol homeostasis are plausible candidates. The disproportionate increase in HMG-CoA reductase activity in the LDL receptor deficiency state may reflect high nuclear SREBP levels (51), which may also target HAS1 and HAS2 genes that have SREBP binding sequences in the proximal promotor region, according to Matinspector software (Genomatix, Munich, Germany). Interestingly, induction of endoplasmic reticulum stress has also been shown to activate SREBPs and may thus be an additional upstream regulator for activation of HAS1 and HAS2 genes (66, 67).

As others have found, absence of functional LDL receptors results in reduced proliferation (41–43). High mass HA has been shown to inhibit smooth muscle cell proliferation (21). Consistent with this observation, WHHL cells synthesize a significantly higher proportion of high molecular mass (≥2 × 10^6 Da) HA than normal controls. High molecular mass HA is accumulated in atherosclerosis (68). The difference in HA size regulation between the two cell strains may be explained by reduced HA degradation in WHHL ASMC, different expression levels of HAS isoforms that produce HA chains of different average length (5, 69), up-regulation of HAS activity by partition into lipid rafts (7), or direct regulation of HA synthesis by the LDL receptors. Because the strength of the interaction between mammalian HAS and a growing HA chain has been suggested to dictate HA chain size (70), it is possible that the high UC content of the plasma membrane concentrates and stabilizes HAS proteins in microdomains, thereby supporting a strong interaction between HAS and the growing HA chain, and high molecular mass HA production in WHHL ASMC.

Reduced HA degradation by the high molecular mass HA-producing cells supports findings that high molecular mass HA has a lower degradation rate than low molecular mass HA (46), and suggests that the initial extracellular degradation of high molecular mass HA to low molecular mass is a prerequisite to its uptake and intracellular degradation. Accordingly, a limiting factor in HA degradation is HA internalization by many cell types, including ASMC (46). In WHHL ASMC, HA internalization could be limited by the relatively smaller amount of low molecular mass HA available.

Increased HA synthesis, uncompensated by enhanced HA degradation, may result in an unbalanced turnover of HA in WHHL ASMC, promoting elevated amounts of HA and the formation of high molecular mass HA-rich ECM. This would result in vivo in matrix expansion and neointimal thickening. Our results may help to explain in vivo HA accumulation in atherosclerosis in humans (13, 15, 17, 18), in diet-induced hypercholesterolemia in rabbits (20, 64), in WHHL rabbits, and in other animal models (14, 19, 21, 71). Increases in vascular HA may contribute to a number of pro-atherosclerotic functions. For example, we and others (reviewed in Ref. 72) have shown that increases in pericellular ASMC HA promote their proliferation and migration (73, 74). Long term exposure of macrophages to HA promotes apoptosis of these cells (75). It is also clear that HA-enriched ECM that is generated by ASMC can serve as a substrate for monocyte/macrophage adhesion and retention (76), and the presence of HA at the plaque thrombus interface (17) argues for a role for HA in thrombosis. Finally, fragments of HA induce the release from macrophages of inflammatory cytokines (77), which may be key inflammatory mediators in atherosclerosis.

3. T. N. Wight, S. Sakr, and C.-Y. Han, unpublished data.
Increased HA Accumulation in LDL Receptor-deficient Cells

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