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CD98 regulates vascular smooth muscle cell proliferation in atherosclerosis

Yvonne Baumer^{1,*}, Sara McCurdy^{1,*}, Martin Alcala², Nehal Mehta³, Bog-Hieu Lee⁴, Mark H. Ginsberg⁵, and William A. Boisvert^{1,6}

¹Center for Cardiovascular Research, John A. Burns School of Medicine, University of Hawaii, Honolulu, HI

²Department of Chemistry and Biochemistry, Facultad de Farmacia, Universidad CEU San Pablo, Madrid, Spain

³Section of Inflammation and Cardiometabolic Diseases, National Heart, Lung and Blood Institute, National Institutes of Health, Bethesda, MD

⁴Department of Food and Nutrition, School of Food Science and Technology, Chung-Ang University, Seoul, Korea

⁵Department of Medicine, University of California San Diego, La Jolla, CA

⁶Kazan Federal University, Kazan, Russia

Abstract

Background and aims—Vascular smooth muscle cells (VSMC) migrate and proliferate to form a stabilizing fibrous cap that encapsulates atherosclerotic plaques. CD98 is a transmembrane protein made of two subunits, CD98 heavy chain (CD98hc) and one of six light chains, and is known to be involved in cell proliferation and survival. Because the influence of CD98hc on atherosclerosis development is unknown, our aim was to determine if CD98hc expressed on VSMC plays a role in shaping the morphology of atherosclerotic plaques by regulating VSMC function.

Methods—In addition to determining the role of CD98hc in VSMC proliferation and apoptosis, we utilized mice with SMC-specific deletion of CD98hc (*CD98hc^{fl/fl}SM22aCre⁺*) to determine the effects of CD98hc deficiency on VSMC function in atherosclerotic plaque.

Results—After culturing for 5 days *in vitro*, *CD98hc^{-/-}* VSMC displayed dramatically reduced cell counts, reduced proliferation, as well as reduced migration compared to control VSMC.

*Corresponding authors: Center for Cardiovascular Research, University of Hawaii John A. Burns School of Medicine, 651 Ilalo Street, Honolulu, HI 96813 (W. A. Boisvert); Department of Food and Nutrition, School of Food Science and Technology, Chung-Ang University, Seoul, Korea (B.-H. Lee), wab@hawaii.edu (W. A. Boisvert); lbheelb@cau.ac.kr (B.-H. Lee).

*These authors contributed equally to this work.

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Conflict of interest

The authors declared they do not have anything to disclose regarding conflict of interest with respect to this manuscript.

Analysis of aortic VSMC after 8 weeks of HFD showed a reduction in *CD98hc*^{-/-} VSMC proliferation as well as increased apoptosis compared to controls. A long-term atherosclerosis study using *SMC-CD98hc*^{-/-}/*ldlr*^{-/-} mice was performed. Although total plaque area was unchanged, *CD98hc*^{-/-} mice showed reduced presence of VSMC within the plaque ($2.1 \pm 0.4\%$ vs. $4.3 \pm 0.4\%$ SM22 α -positive area per plaque area, $p < 0.05$), decreased collagen content, as well as increased necrotic core area ($25.8 \pm 1.9\%$ vs. $10.9 \pm 1.6\%$, $p < 0.05$) compared to control *ldlr*^{-/-} mice.

Conclusions—We conclude that CD98hc is required for VSMC proliferation, and that its deficiency leads to significantly reduced presence of VSMC in the neointima. Thus, CD98hc expression in VSMC contributes to the formation of plaques that are morphologically more stable, and thereby protects against atherothrombosis.

Keywords

Vascular smooth muscle cell; CD98; Cell proliferation; Apoptosis; Atherosclerosis

Introduction

During progression of atherosclerosis, transformation of vascular smooth muscle cells (VSMC) from the quiescent contractile phenotype towards the proliferative migratory phenotype into the plaque area to form a fibrous cap is believed to be an essential step in the formation of stable plaques¹. The two major phenotypes of VSMC include fully differentiated, contractile cells responsible for vasodilation and vasoconstriction, and migratory, proliferative cells that are activated during growth or injury². During atherosclerosis development, VSMC respond to mediators such as platelet derived growth factor (PDGF)-BB, endothelin-1, thrombin, IFN- γ and IL-1 secreted by endothelial cells³ and leukocytes within the plaque^{4, 5}, leading to migration from the vessel media to the intima where they secrete extracellular matrix components⁶. Advanced atherosclerotic plaques are characterized by the presence of fibrous cap, composed of VSMC and collagen, especially collagen type VIII³, that surrounds and stabilizes the center core of the plaque, reducing the risk of plaque rupture and its consequences^{1, 4, 7}. On the other hand, VSMC apoptosis leads to drastic vessel remodeling, with increased inflammation and coagulation, and thinning of the fibrous cap, making the plaque more prone to rupture^{8, 9}.

CD98 is a transmembrane protein made up of a heavy chain (CD98hc) and one of several light chains that is involved in biological events and diseases that require proliferative and migratory processes, including cancer¹⁰ and several autoimmune diseases^{11, 12}. The protein has dual functions, including transporting large, neutral amino acids via its interaction with various light chains, and mediating integrin signaling via its cytoplasmic tail, specifically $\beta 1$ - or $\beta 3$ -integrins^{13, 14, 15}. Together, these functions serve to regulate cell survival, proliferation, migration and even malignant transformation^{10, 16}.

Our recent studies highlighting the importance of CD98 in VSMC survival during response to vascular injury¹⁷ led us to hypothesize that CD98hc in VSMC would affect atherosclerosis development and plaque remodeling. To study this, we generated CD98hc conditional knockout mice with Cre recombinase driven by the SM22 α promoter (to ensure the selective knockout of CD98hc in VSMC) crossed with the atherosclerosis-prone *ldlr*^{-/-}

mice, resulting in $CD98hc^{fl/fl}SM22\alpha Cre^{+}/ldlr^{-/-}$ ($CD98hc^{-/-}$) or $CD98hc^{fl/fl}SM22\alpha Cre^{-}/ldlr^{-/-18}$ mice (Fig. 1E). These $CD98hc^{-/-}$ and control mice were used to determine the role of CD98hc in VSMC function in atherosclerosis.

Materials and methods

Animal model

CD98 heavy chain (CD98hc) protein is encoded by the *SLC3A2* gene. Generation of vascular smooth muscle-specific deletion of CD98hc (on the C57BL/6 background) was previously published¹⁷. In brief, CD98hc was floxed with loxP sites and crossed with mice expressing Cre recombinase under the smooth muscle-specific SM22 α promoter to obtain $CD98hc^{fl/fl}SM22\alpha Cre^{+}$ knockout and $CD98hc^{fl/fl}SM22\alpha Cre^{-}$ control mice. For our studies, these mice were crossed with $ldlr^{-/-}$ mice to obtain CD98hc VSMC-specific knockout mice prone to atherosclerosis. Genotyping of $CD98hc^{fl/fl}SM22\alpha Cre^{+}/ldlr^{-/-}$ ($CD98hc^{-/-}$) or $CD98hc^{fl/fl}SM22\alpha Cre/ldlr^{-/-18}$ genomic DNA confirmed the double or single knockout, respectively (Supplemental Fig. 1A).

Cell culture

Whole aortas of control or $CD98hc^{-/-}$ mice were digested using 1.5 Wünsch units of Liberase TM (Roche, USA) for 1 h at 37°C. The resulting cell suspension was strained through a 40 μ m cell strainer, washed with PBS and plated in SMC medium (DMEM containing 20% FBS, 20 mM glutamine, 1% P/S, and NEAA) (all Gibco, USA). Cells were left unattended for 5 days at 5% CO₂/37°C. After 5 days in culture, cells were detached using 0.25 Trypsin/EDTA (Gibco, USA), replated at a concentration of 5×10^5 cells/cm² in SMC medium and used within 24 h for *in vitro* experiments.

In vitro proliferation

5×10^5 control or $CD98hc^{-/-}$ VSMC were treated over night with 10 μ M EdU $\pm$ PDGF BB (Peprotech, USA). Afterwards, cells were fixed using 2% PFA in PBS for 10 min at RT, washed with 2% BSA in PBS, permeabilized using 0.1% Triton X-100 in PBS for 5 min at RT. EdU incorporation was detected using an Alexa Fluor 594-labeled azide according to manufacturer's recommendation (Life Technologies, USA). SMC were subsequently stained using an FITC-labeled α SMA antibody (AbCam, USA). Nuclei were visualized using DAPI. Images were recorded using an Axiovert microscope (Zeiss, Germany).

SMC wounding assay using ECIS

An electric cell substrate impedance-sensing set-up (ECISZ Θ , Applied BioPhysics Inc, USA) was used to detect impedance of proliferating SMC as well as their recovery potential after wounding. Electrode arrays were incubated with SMC medium to equilibrate for 1 h at 37°C before 5×10^5 control or $CD98hc^{-/-}$ VSMC were inoculated per well with immediate measurement initiation. By measuring the impedance, cell adherence and proliferation across the electrode surface can be determined over time. After 24 h of proliferation with or without the addition of 20 ng/ml PDGF BB (Peprotech, USA), VSMC were wounded using the ECIS wounding module for 30 sec at a current of 30,000 Hz and impedance measurement continued. The data obtained were analyzed using ECIS software and Excel.

Western Blot

VSMC were cultivated for 5 days after isolation from aortas. Cells were then kept in culture for an additional 24 h with or without the addition of 20 ng/ml PDGF BB (Peprotech, USA). VSMC lysates were obtained using RIPA buffer and 15 µg protein per sample examined by Western blot analysis for the presence of p38, p42, p44 and p46 (Phospho-MAPK Family Antibody Sampler Kit #9910, Cell Signalling). Western blots were analyzed using Licor's ImageStudio software and Prism.

Atherosclerosis model and analysis

All animal protocols were approved by the University of Hawaii Institutional Animal Care and Use Committee. Male *CD98hc^{fl/f}SM22αCre⁺/ldl^{-/-}* (*CD98hc^{-/-}*) or *CD98hc^{fl/f}SM22αCre⁻/ldl^{-/-}*¹⁸¹⁸ mice, 8–10 weeks of age were put on a high fat diet (HFD) containing 15.8% (wt/wt) fat and 1.25% cholesterol (94059; Harlan Teklad) for 16 weeks. Upon sacrifice, the mouse hearts and aortas were collected and processed for further analysis. The aortic sinus was cut into 5 µm sections and analyzed using Oil Red-O staining and immunohistochemistry. The aorta as well as the aortic root sections were stained for esterified lipids using Oil Red-O as described previously¹⁹. The staining was quantified using ImageJ software to determine the extent of atherosclerosis. Collagen in aortic root sections was stained with picrosirius red and analyzed by ImageJ (NIH) as well as polarized light microscopy as described previously²⁰. The presence and extent of macrophage as well as smooth muscle cell infiltration into plaque areas were determined by immunofluorescence with subsequent analysis using ImageJ. To visualize macrophages and smooth muscle cells, aortic root sections were stained with MOMA-2 and SM22α (both Abcam) antibodies, respectively. Serum cholesterol and triglyceride levels were detected in samples collected before HFD start and at harvest, subjected to colorimetric or fluorometric assays, respectively, according to the manufacturers recommendation (Cayman, USA). Circulating leukocyte populations were detected by flow cytometry using a panel of antibodies to detect relative populations of T cells, B cells, monocytes, and neutrophils between groups. Samples were run on a BD FACS Aria (BD, USA).

Immunohistochemistry of aortic root sections

Aortic root sections of atherosclerosis study were co-stained for SM22α (Proteintech Group, USA, 10493-1-AP) and MOMA-2 (AbCam, USA, ab33451). For this, sections underwent trypsin antigen retrieval for 15 min at 37°C, blocking and overnight primary antibody incubation at 4°C. At the next day, sections were incubated with cy2 and cy3 labeled secondary antibodies (AbCam, USA) for 1 h at RT. Primary antibodies against Ki67 (Abcam, 16667) or α-smooth muscle actin (Thermo Fisher, 17H19L35) were used to stain aortic root sections using donkey-anti-rabbit HRP conjugated secondary antibody and an AEC detection kit (both Thermo Fisher), according to manufacturer's directions.

Timeline sections of male *ldl^{-/-}* mice after 0, 4, 8, 12 and 16 weeks of HFD were stained for CD98hc (eBiosciences, USA, clone RL388), SM22α and MOMA-2, as described above. Nuclei were visualized using DAPI. Images were recorded using an Axiovert microscope (Zeiss, Germany). One-way ANOVA (with Tukey's post-hoc test) was performed for

comparison of SMC content and necrotic core area between groups from 0–16 weeks of HFD.

In vivo proliferation and apoptosis

All animal protocols were approved by the University of Hawaii Institutional Animal Care and Use Committee. 8–10 week-old male control or *CD98hc*^{-/-} mice were put on a high fat diet (HFD) containing 15.8% (wt/wt) fat and 1.25% cholesterol (94059; Harlan Teklad) for 8 weeks. 3 days before sacrifice, the mice were i.p. injected with 100 µg EdU per mouse. The mice were then sacrificed using CO₂, perfused with PBS, and the aortas were thoroughly cleaned, cut in 1 mm rings and digested with 1.5 Wünsch Units Liberase TM (Roche, USA) for 1 h at 37°C. The resulting cell suspension was divided in two parts. Both parts were stained for VSCM using αSMA-FITC antibody (AbCam, USA). Half of the cells were used to detect proliferation by staining for EdU using the Alexa Fluor 647 conjugated azide at 5 µM, according to manufacturer's recommendation (Life Technologies, USA). The remaining cells were used to detect apoptosis using a flow cytometry cleaved Casp3-PE antibody (BD, USA) according to manufacturer's recommendation. Proliferating as well as apoptotic stained cells were analyzed using a BD FACS Calibur flow cytometer. Data were analyzed by FlowJo, Microsoft Excel and Prism. Gating strategy is shown in Supplemental Fig. 2.

Transmission electron microscopy (TEM)

Specimens of *ldlr*^{-/-} mouse aortas on HFD for 0, 4, 8 and 12 weeks were fixed with 4% glutaraldehyde and 0.1 M calcium chloride in 0.1 M sodium cacodylate buffer, pH 7.2 for 24 h and washed in 0.1 M cacodylate for 3 × 10 min, followed by post-fixation with 1% OsO₄ in 0.1 M cacodylate buffer for 1 h. The tissue samples were dehydrated in a graded ethanol series (30%, 50%, 70%, 85%, 95%, 100%), substituted with propylene oxide, and embedded in LX112 epoxy resin. Ultrathin (60–80 nm) sections were obtained with a diamond knife on an RMC Powertome ultramicrotome, and double stained with uranyl acetate and lead citrate. Grids were viewed and photographed on a Hitachi HT7700 transmission electron microscope operating at 100 kV with an AMT XR41 4 megapixel camera (Advanced Microscopy Techniques, Corp.).

Statistical analysis

Comparison of means between groups was performed using PRISM software (GraphPad) with the Student's t-test unless otherwise stated. Data are presented as mean ± the standard error of the mean. Statistical significance was accepted at the level of $p < 0.05$.

Results

Smooth muscle cell proliferation and CD98 expression in atherosclerotic plaque

Ultrastructural detail of plaque progression in the upper thoracic aorta is depicted in Fig. 1A. While the typical layers of medial vascular smooth muscle cells (mVSMC) and collagen (C), alternating with an elastin fibrous layer (E) are intact even at 4 weeks post-HFD, these structures are in disarray, with breaks in the elastin layer through which SMC can migrate into the plaque and the first intimal VSMC (iVSMC) visible at 8 weeks post-HFD. After 12

weeks of HFD, plaques become very complex, with a clearly visible necrotic core covered by a compact, thick layer of iVSMC, which stains positive for SM22 α (Fig. 1B), a marker of quiescent VSMC that increases with time (Fig. 1C).

Aortic root sections of *ldlr*^{-/-} mouse hearts stained for CD98 and SM22 α show that the first substantial presence of VSMC within the plaque fibrous cap is seen after 8 weeks of HFD, which coincides with peak CD98 expression within the plaque (Fig. 1E). At 4 weeks HFD, very few VSMC can be detected within the plaque, however, macrophages in these early plaques are CD98-positive (Fig. 1E). After 12 or 16 weeks of HFD, a compact layer of CD98-negative VSMC can be detected covering the necrotic core (Fig. 1E), acting to stabilize the advanced atherosclerotic plaque. RNA from whole aortas of *ldlr*^{-/-} mice fed a high fat diet for 0, 4, 8, 12, or 16 weeks analyzed by RT-qPCR showed that CD98 transcript levels increase significantly over time, peaking after 8 weeks, but still significantly upregulated after 12 weeks (Fig. 1D).

To study specifically the role of CD98hc in VSMC in the context of atherosclerosis, we crossed CD98hc conditional knockout mice with Cre recombinase driven by the SM22 α promoter (to ensure the selective knockout of CD98hc in VSMC) with the atherosclerosis-prone *ldlr*^{-/-} mice, resulting in *CD98hc^{fl/fl}SM22 α Cre⁺/ldlr^{-/-}* (*CD98hc*^{-/-}) or *CD98hc^{fl/fl}SM22 α Cre⁻/ldlr^{-/-}*¹⁸ mice (Supplemental Fig. 1). These *CD98hc*^{-/-} and control mice were used to determine the role of CD98hc in VSMC function in atherosclerosis.

CD98hc regulates VSMC proliferation and migration

VSMC isolated from aortas of *CD98hc*^{-/-} animals displayed approximately 60% reduced cell counts ($41 \pm 8.2\%$ of control) after 5 days of culture (Fig. 2A). *In vitro* proliferation assay using EdU incorporation method combined with SM22 α staining resulted in $11 \pm 1.7\%$ and $19 \pm 1.2\%$ EdU-positive proliferating cells for *CD98hc*^{-/-} and control VSMC, respectively (Fig. 2B). When PDGF-BB was used to activate VSMC isolated from *CD98hc*^{-/-} or control mouse aortas, the difference in proliferative capacity was even more stark, with *CD98hc*^{-/-} VSMC displaying approximately 70% less proliferating cells compared to controls ($13 \pm 2.3\%$ *CD98hc*^{-/-} vs. $46 \pm 3.5\%$ control). These data strongly indicate that CD98hc is essential for VSMC proliferation, especially under stimulatory conditions.

To verify the VSMC proliferation data, we utilized electric cell-substrate impedance sensing (ECIS) which can accurately measure cell confluence and proliferation over time. Control VSMC isolated from *CD98hc*^{-/-} mice displayed about 40% lower impedance 24 h after plating (Figures 2C and D). After electrically wounding the cells, the control cells immediately started to proliferate and re-populate the electrodes surface, which was even more pronounced after PDGF-BB treatment (Fig. 2E). In contrast, *CD98hc*^{-/-} VSMC showed impaired regeneration potential with or without PDGF-BB treatment, clearly demonstrating the importance of CD98hc for proliferation and migration, important processes relevant to atherosclerosis and wound healing.

To confirm our *in vitro* data, we conducted an *in vivo* experiment in which male *CD98hc*^{-/-} or control mice were put on HFD for 8 weeks (the time point at which we previously observed peak CD98 expression in SM22 α -positive plaque VSMC) and 3 days prior to

sacrifice were injected with EdU to label proliferating cells. Using flow cytometry, cells from whole aorta were first gated for SMC population, and then analyzed for the presence of EdU or cleaved-Casp3 to identify proliferating and apoptotic populations, respectively. There was a 25% reduction in proliferating VSMC from *CD98hc*^{-/-} animals ($2.3 \pm 0.2\%$ vs. $3.0 \pm 0.2\%$), and a 41% increase in apoptosis in *CD98hc*^{-/-} VSMC compared to controls ($7.9 \pm 0.6\%$ vs. $5.6 \pm 0.5\%$) (Fig. 2F and G). These *in vivo* data show that under atherosclerotic conditions, CD98hc is required for effective proliferation as well as survival of VSMC, suggesting an important role in atherosclerosis plaque development and progression.

CD98hc loss alters atherosclerotic plaque morphology towards an unstable plaque type

To investigate whether CD98 in VSMC affects atherosclerosis development, an atherosclerosis study was performed using *CD98hc*^{fl/fl}*SM22aCre*⁺ mice crossed with atherosclerosis-prone *ldlr*^{-/-} mice to obtain either *CD98hc*^{-/-} (*CD98hc*^{fl/fl}*SM22aCre*⁺/*ldlr*^{-/-}) or control (*CD98hc*^{fl/fl}*SM22aCre*⁻/*ldlr*^{-/-}) mice. Male mice of each genotype, 8–10 weeks old, were put on HFD for 16 weeks to allow the establishment of VSMC-rich atherosclerotic plaque.

Analysis of atherosclerotic plaque area in aortas and aortic root sections revealed no significant differences (Fig. 3A and B). However, Oil Red-O and H&E staining of the aortic root areas from *CD98hc*^{-/-} mouse hearts displayed a more diffuse plaque morphology compared to the more compact, organized plaques of control mice. Further analysis of the plaque revealed a 2.5-fold increase in necrotic core area within *CD98hc*^{-/-} plaques ($25.8 \pm 1.9\%$ vs. $10.9 \pm 1.6\%$ necrotic core area per plaque area) (Fig. 3C). VSMC content was significantly reduced within the plaque area of *CD98hc*^{-/-} mice ($2.1 \pm 0.4\%$ vs. $4.3 \pm 0.4\%$ SM22α⁺ area per plaque area), as shown by red fluorescence in Fig. 3D (quantified in Fig. 3E). The reduced VSMC plaque content observed in *CD98hc*^{-/-} is supported by the *in vitro* findings of both reduced proliferation as well as increased apoptosis compared to control VSMC. Macrophage content in the aortic sinus lesion areas was similar between the *CD98hc*^{-/-} and control mice (Fig. 3D and F). There were no differences in weight, cholesterol and triglyceride levels, or blood cell composition between the two groups before and after HFD (Supplemental Fig. 3).

The increase in necrotic core in *CD98hc*^{-/-} mice indicates a switch from a compact, stable plaque to a diffuse and vulnerable plaque as a result of the loss of CD98hc in VSMC. To further characterize the plaque composition, aortic root sections from *CD98hc*^{-/-} and control mice were stained with Sirius red (Fig. 4A and B) and Masson Trichrome (Fig. 4C). Sirius red-positive plaque area was used to quantify collagen content, and these sections were also analyzed by circular polarized light to detect collagen fibers of varying size. Thick fibers are shown in red and orange, while thinner fibers are shown in yellow and green (Fig. 4B). Masson Trichrome staining also shows differences in collagen content (blue) between *CD98hc*^{-/-} and control mice, with less intense blue staining visible in *CD98hc*^{-/-} plaque (Fig. 4C). Aortic root serial sections were stained with αSMA to detect smooth muscle cells, and Ki67 to detect proliferating cells (Fig. 4D). Ki67⁺ iVSMC were quantified for control and *CD98hc*^{-/-} mice, revealing a significant decrease in proliferating VSMC in *CD98hc*^{-/-} mice

compared to controls (Fig. 4E). α SMA⁺ area was also quantified and is expressed as a percentage of total plaque area (Fig. 4F), confirming our earlier finding of reduced iVSMC content of *CD98hc*^{-/-} using the marker SM22 α to detect VSMC (Fig. 3E).

Ultrastructural details of the plaque were analyzed via TEM in the aortic sinus sections of both WT and *CD98hc*^{-/-} fed the HFD for 8 or 16 weeks (Fig. 5). The overview sections were stained with Richardson stain which clearly shows the integrity of the elastin layers with inter-dispersed SMC layers adjacent to the neointima. The 8-week elastin-SMC area shows an interrupted elastin layer and the first signs of SMC migration in the control section (Fig. 5A), whereas the *CD98hc*^{-/-} section shows no signs of elastin interruption or SMC migration. The 16-week sections show a similar trend but more robust migration of SMC in the control section (Fig. 5B). Quantification of elastin breaks was performed by counting the number of breaks (Fig. 5C, yellow arrowheads) in aorta sections from control and *CD98hc*^{-/-} mice. *CD98hc*^{-/-} mice show significantly less breaks in the elastin layers of the aorta compared to controls (Fig. 5D), which agrees with our findings of reduced iVSMC content of *CD98hc*^{-/-} atherosclerotic plaque (Fig. 3E and 4F).

Discussion

The importance of VSMC in the stability of plaque is illustrated by the prevailing paradigm that formation of fibrous cap by proliferating and migrating VSMC lessens the risk of plaque rupture and the subsequent acute events such as myocardial infarction or stroke. At the stage of plaque development, when VSMC proliferate and migrate into the plaque (beginning after about 8 weeks of HFD in our *ldlr*^{-/-} mice), we observe that VSMC are also expressing high levels of CD98 (Fig. 1C and D). Furthermore, CD98hc deficiency in VSMC limits their capacity to proliferate under steady state and stimulatory conditions *in vitro* (Fig. 2A and B). Rapid proliferation of other cell types such as T cells²¹ and B cells¹² is dependent on CD98hc. We show *in vivo* that after 8 weeks of HFD, *CD98hc*^{-/-} VSMC display significantly reduced proliferation and increased apoptosis (Fig. 2F and G), supporting our *in vitro* data. We are the first to investigate the dependence of VSMC proliferation/migration on CD98hc in the context of atherosclerosis, using a complex mouse model of the disease.

After performing a full 16-week atherosclerosis study, we observed no significant difference in total plaque area. However, CD98hc deficiency led to altered plaque morphology, with reduced VSMC presence, decreased collagen content, and increased necrotic core formation. In general, we observed a more diffuse, less compact plaque morphology in *CD98hc*^{-/-} mice compared to controls, which is most likely due to the reduced presence of VSMC and lack of adequate matrix deposition, leaving the plaques less organized and more vulnerable. These findings are important since about 75% of coronary thrombosis events are caused by rupture of weakened atherosclerotic plaques²². VSMC apoptosis is linked to weakening of the plaque²², leading to plaque rupture and thrombus formation. Thus, our observation that CD98hc deficiency in VSMC results in reduced plaque stability is supported by the finding that a decrease in proliferating VSMC as well as an increase in necrotic core area was detected in plaques of *CD98hc*^{-/-} compared to control mice. Furthermore, CD98hc is reported to exhibit its survival-promoting functions via its integrin-binding domain¹⁷, and deletion of CD98hc in embryonic stem cells has been shown to impair integrin-dependent

signaling such as FAK, Rac or Akt and lead to increased anchorage-independent apoptosis²³. These potential mechanisms may explain how CD98 functions within VSMC to prevent apoptosis and to promote proliferation and migration, but requires further investigation. Nonetheless, our study provides solid evidence that CD98 is an important part of overall atherosclerosis development through its effects on VSMC function.

It is commonly believed that necrotic core in the atherosclerotic plaque is formed when lipid-laden macrophages die either through apoptosis or necrosis and deposit cellular debris as well as lipid material in the core of the plaque²⁴. In our study, we observed a significantly larger necrotic core in the aortic sinus sections of *CD98hc*^{-/-} mice compared to controls, despite macrophage numbers being similar between the two groups of mice. VSMC apoptosis is known to enhance necrotic core formation²⁵, therefore, it is likely that the increase in apoptosis that we observed in *CD98hc*^{-/-} aortic VSMC contributed to the growth of the necrotic core. It is also possible that there may have been an increase in macrophage infiltration into the plaques of *CD98hc*^{-/-} mice perhaps in lieu of a reduced VSMC number, which resulted in increased apoptosis of macrophages that ultimately caused the necrotic core to be more prominent. However, further studies will be needed to determine exactly what contributed to the growth of the necrotic core in our *CD98hc*^{-/-} mouse plaque.

Our observation of reduced elastin breaks in *CD98hc*^{-/-} aortas indicates that there is impaired degradation of the matrix proteins, notably elastin, needed for typical migration of VSMC into the developing plaque. It has been shown that matrix-remodeling enzymes are expressed preferentially in the synthetic phenotype, with very little expression in contractile VSMC²⁶. Because CD98hc deficiency in VSMC inhibits proliferation and survival, their phenotype more closely resembles that of quiescent, contractile cells rather than synthetic, proliferative cells. Although this hypothesis would be in agreement with our findings, further studies examining the expression of matrix remodeling genes and proteins in control vs. *CD98hc*^{-/-} VSMC need to be performed to elucidate the specific mechanisms involved.

In conclusion, our findings indicate that CD98hc is required for VSMC proliferation since its deficiency leads to significantly reduced presence of VSMC and increased areas of necrotic core in the neointima. Thus, CD98hc is an important component of plaque stability that confers resistance to atherothrombotic events.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- CD98 deficiency severely limits vascular smooth muscle cell proliferation
- Atherosclerotic mice lacking CD98 in their smooth muscle cells have reduced vascular smooth muscle cells in their plaque
- Plaques from CD98-deficient mice have significantly larger necrotic core
- Breaks in elastin layer of atherosclerotic plaques are reduced in CD98 deficiency
- CD98 may play an important role in plaque stability

Fig. 1. VSMC and CD98 expression in various stages of atherosclerotic plaque
 (A) TEM analysis of *ldlr*^{-/-} mouse aorta sections showing elastin layers (E), medial VSMC (mVSMC) and intimal VSMC (iVSMC) at areas of breaks (*) in the elastin. (B) Immunofluorescence staining for SM22 α in aortic root sections showing the distribution of mVSMC and iVSMC covering the necrotic core (NC) and their quantification in plaque over time. (C) Quantification of SM22 α staining in aortic root sections (shown in E, n=4/time point). (D) RT-qPCR data showing expression pattern of CD98 in aortas of *ldlr*^{-/-} mice on HFD from 0–16 weeks (n=4/time point). (E) Aortic root sections showing expression of

CD98 (red), SM22 α (red, SMC), and MOMA-2 (green, macrophages) at different stages of atherosclerosis. CD98 is expressed robustly in macrophages (*) after 4 weeks and in VSMC after 8 weeks of HFD. iVSMC and mVSMC are shown by white arrows and arrow heads respectively.

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Fig. 2. CD98hc controls VSMC proliferation and migration

(A) After 5 days of culture, the number of proliferating cells is significantly reduced in *CD98hc*^{-/-} VSMC. (B) Proliferation of VSMC (SMA positive), measured ± PDGF-BB using EdU assay, shows significantly impaired proliferation of *CD98hc*^{-/-} VSMC compared to control cells, especially with added PDGF-BB. (red=EdU, blue=DAPI). (C) Defective proliferation of *CD98hc*^{-/-} VSMC is shown by ECIS measurement of impedance across an electrode as cells adhere and proliferate (D) and after wounding and subsequent recovery (E). (F and G) *CD98hc*^{-/-} and control mice were put on HFD for 8 weeks and injected with

EdU (100 µg/mouse) 3 days before sacrifice. The aorta of each mouse was digested analyzed for EdU⁺ (proliferating) (F) and cleaved-Casp3⁺ (apoptotic). (G) VSMC using flow cytometry. * $p < 0.05$.

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Fig. 3. VSMC-CD98hc deficiency alters atherosclerotic plaque morphology

After 16 weeks of HFD measurement of atherosclerosis in whole aorta (A) and aortic root sections (B), using Oil Red-O staining revealed no significant differences in plaque content between *CD98hc*^{-/-} and control mice. Quantification of necrotic core area (C) revealed significantly increased necrotic core in *CD98hc*^{-/-} animals compared to controls. (D) Immunofluorescence staining for SM22α (red) to visualize VSMC and MOMA-2 (green) to visualize macrophages in aortic root sections. Nuclei are stained with DAPI and shown in

blue. SM22 α and MOMA-2 positive areas were analyzed with ImageJ as a percent of total plaque area and are quantified in E and F respectively. * $p < 0.05$, n=15.

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Fig. 4. Characterization of plaque composition and VSMC proliferation

(A) Aortic root sections from *CD98hc^{-/-}* and control mice were stained with Sirius red for quantification of collagen content. (B) Sirius red was also analyzed by circular polarized light to detect collagen fibers of varying size with thick fibers shown in red and orange, while thinner fibers are shown in yellow and green. (C) Masson Trichrome staining reveals keratin and muscle in red, collagen in blue, cytoplasm in pink, and black nuclei. (D) Aortic root serial sections were stained with αSMA to detect smooth muscle cells, and Ki67 to detect proliferating cells. Ki67⁺ proliferating VSMC were quantified for *CD98hc^{-/-}* and

control mice (E). α SMA⁺ area was also quantified and is expressed as a percentage of total plaque area (F). * p <0.05, *** p <0.001, n=8.

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Fig. 5. VSMC content and ultrastructural details of atherosclerotic plaque from control and *CD98hc^{-/-}* mice

TEM images display thoracic aorta sections stained with Richardson stain showing the neointima and elastin layers interspersed within VSMC layers (top row) after 8 weeks (A) or 16 weeks (B) of HFD in control or *CD98hc^{-/-}* mouse aortas. The bottom row for both time points and genotypes depicts TEM photos of junctions between the last elastin layer and neointima. (C and D) TEM analysis of breaks in the elastin layers of the thoracic aorta (yellow arrowheads) was performed by counting the number of breaks in at least 3 images taken for each mouse aorta. * $p < 0.05$, $n = 3$.