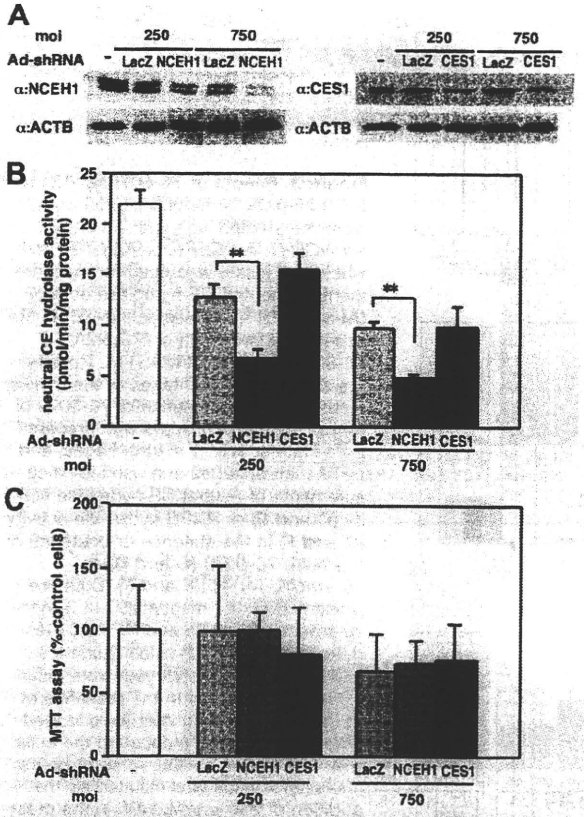

Figure 4. Effects of knockdown of NCEH1 or CES1 on the neutral CE hydrolase of HMMs. Recombinant adenoviruses coding for short hairpin RNA (shRNA) against LacZ, NCEH1, or CES1 were used to infect HMMs at 250 and 750 mois. Two days after infection, whole-cell lysate was subjected to Western blotting **(A)** and the measurement of neutral CE hydrolase activity **(B)**. **C,** MTT assay was performed to assess cell viability of HMMs infected with recombinant adenoviruses coding for shRNA against LacZ, NCEH1, or CES1. Data are expressed as percentages to control cells. Data are presented as the means±SD of 3 **(B)** or 4 **(C)** measurements. $^{*}P<0.05$, $^{**}P<0.01$ (determined by ANOVA followed by the Bonferroni post hoc analysis).

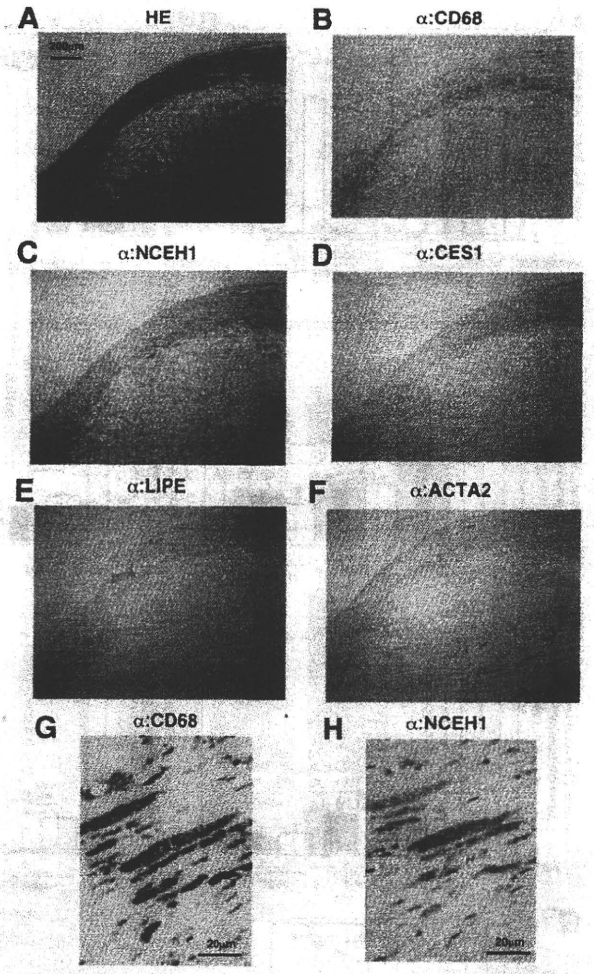

Figure 5. Expression of NCEH1 protein in human atherosclerotic lesions. Sections of aorta from case 1 or 7 that contained atheromatous plaques were used for histological examination. **A,** Hematoxylin/eosin (HA) staining. Immunohistochemistry for CD68 **(B and G)**, NCEH1 **(C and H)**, CES1 **(D)**, LIPE **(E)**, or ACTA2 **(F)** in the serial sections of A. Original magnifications: ×40 **(A through F)**; ×400 **(G and H)**.

the neutral CE hydrolase activity further from that attained with Ad-sh*LacZ*. Infection with increasingly higher doses of Ad-sh*LacZ* nonspecifically reduced the protein expression (Figure 4A) and neutral CE hydrolase activity (Figure 4B). To rule out the possibility that infection of Ad-sh*LacZ*, Ad-sh*NCEH1*, or Ad-sh*CES1* is cytotoxic, we performed MTT assay (Figure 4C). There was no significant difference in MTT activity among the cells infected with 3 viruses. Viability of cells infected 750 mois of adenovirus slightly decreased compared with no treatment cells, but the difference was not significant. Although there is a nonspecific effect of adenovirus infection, these results support that neutral CE hydrolase activity in HMMs is primarily mediated by NCEH1, not by CES1 or LIPE, in combination with the results of inhibition of human NCEH1 in HMMs by AS115 (Figure 3).

Finally, we investigated the expression of NCEH1 in human aortas (Table). We stained sections of aortas from 20 cases of autopsy whose clinical characteristics are summarized in Table. The lesions were morphologically classified into 3 types: diffuse intimal thickening, fatty streak lesions, and atheromatous plaques.[35] Atheromatous plaques were rich in cells positive for CD68, a pan-macrophage/dendritic cell marker, as well as NCEH1-positive cells compared with fatty streak lesions or diffuse intimal thickening. NCEH1-positive cells were absent in diffuse intimal thickening. Figure 5 shows representative sections from atheromatous plaques. This region contains acellular necrotic core (Figure 5A). The subintimal area between the intima and the necrotic core contained many CD68-positive cells (Figure 5B). A majority of the CD68-positve cells were also positive for NCEH1 (Figure 5C and the Table). The colocalizing characteristics of CD68 and NCEH1 were more obvious at a higher magnification (Figure 5G and 5H). In contrast, CES1 was weakly positive for some CD68-positive cells (Figure 5D). LIPE protein was barely detectable in HMMs in the same condition (Figure 5E). ACTA2 was strongly positive in most of the

cells in the media and weakly positive for some of the cells in the intima (Figure 5F). Furthermore, we show representative sections from fatty streak lesions (Online Figure VII, A through C). Although the expression of CD68 and NCEH1 in fatty streak lesions decrease compared with atherosclerotic plaque, many of the CD68-positve cells were also positive for NCEH1 (Online Figure VII, B and C). CES1 was weakly expressed and LIPE was not expressed (data not shown). Thus, NCEH1 is the major enzyme that is specifically expressed in macrophages of human atherosclerotic lesions.

Discussion

Previously, we have reported that both Lipe and Nceh1 are involved in the hydrolysis of CE to a comparable degree in mice.[25] Circumstantial evidence, however, suggests that there are great differences in the hydrolysis of CE between human and mouse macrophages.[15,26,27] To understand the pathogenesis of atherosclerosis in humans, therefore, it is essential to determine the major enzyme that mediates the hydrolysis of CE hydrolysis in foam cell macrophages of human atherosclerotic lesions. In the present study, we show for the first time that NCEH1 is primarily involved in the hydrolysis of CE in human macrophages, thereby constituting the initial step toward reverse cholesterol transport in atherosclerotic lesions. We also show that contribution of CES1 and LIPE is trivial. Involvement of NCEH1 in atherosclerosis is further supported by its expression in situ in CD68-positive macrophages, which are abundant in human atheromatous plaques with cholesterol crystal in its necrotic core.

Recently, we have identified Nceh1 as a microsomal enzyme that mediates the hydrolysis of CE in mouse macrophages.[15,32] In that report, we showed that the mRNA of a human ortholog of *Nceh1* was markedly increased during the differentiation from human monocytes to mature macrophages. The present results confirm that the expression of NCEH1 protein was also markedly increased (Figure 1C). This level of induction was similar to the pattern of increase in neutral CE hydrolase activity (Figure 1A). Adenovirus-mediated overexpression of human NCEH1 in CE-loaded THP-1 macrophages decreased the cellular CE content by stimulating cholesterol efflux along with the expression of ABCA1 (Figure 2D; Online Figure V). On the other hand, inhibition of human NCEH1 by AS115, a KIAA1363 inhibitor, or RNA interference in HMMs significantly decreased neutral CE hydrolase activity (Figure 3E and 4B). Furthermore AS115 significantly decreased cholesterol efflux from HMMs which had been loaded with CE (Figure 3G). Thus, NCEH1 primarily regulates the hydrolysis of CE in HMMs.

Is NCEH1 the only enzyme with neutral CE hydrolase activity in human macrophages? This is a pressing question, because at least 2 other enzymes have been proposed to regulate neutral CE hydrolase activity in macrophages: LIPE and CES1. LIPE was the first enzyme proven to contribute to neutral CE hydrolase activity in macrophages. LIPE is indeed expressed in several macrophage cell lines including MPMs and preferentially catalyzes the hydrolysis of CE.[36,37] Although some groups reported the expression of LIPE in human THP-1 macrophages,[27,38] other groups doubted its role as a neutral CE hydrolase because of an extremely low level of expression as compared with that in adipose tissues.[31] The negligible expression of LIPE in HMMs was supported by the finding that efflux of cholesterol was not stimulated by cAMP,[31] which is known to stimulate lipolysis by activating LIPE in adipocytes.[39] Consistent with the claim of the latter groups, we failed to detect significant expression of LIPE in HMMs (Figure 1C). The negligible role of LIPE as a neutral CE hydrolase in human macrophages is also supported by the finding that 76-0079, a reportedly specific inhibitor of LIPE, did not significantly inhibit neutral CE hydrolase activity in HMMs (Figure 3C) and cholesterol efflux from HMMs which had been loaded with CE (Figure 3G). This can be extrapolated to other immortal cell lines of human macrophages: THP-1 and U937 macrophages. In mice, on the other hand, Lipe contributes to neutral CE hydrolase activity in MPMs.[25] Thus, there seems to be a species difference in terms of the relative role of LIPE in the hydrolysis of CE in macrophages.

The rediscovery of CES1 in human macrophages provided a second twist in the history of the investigation of neutral CE hydrolase in macrophages. CES1 was originally identified as a human carboxylesterase in both liver[12] and macrophages.[13] The use of different names for CES1 has complicated matters. Ghosh found neutral CE hydrolase activity in CES1.[11] Gosh and colleagues subsequently reported that overexpression of CES1 inhibits the accumulation of CE in macrophages.[21,22] We tried to reproduce their findings in our model. So far, we have been unable to detect neutral CE hydrolase activity when overexpressed either in HEK293 cells (Online Figure III) or in THP-1 macrophages (Figure 2B). Overexpression of CES1 did not reduce the amounts of CE in the cells even in the presence of the SOAT inhibitor (Online Figure V, B). Thus, it is unlikely that CES1 stimulate reesterification of cholesterol directly or indirectly, thereby mitigating the effect of increased hydrolysis of CE on the cholesterol efflux. Although CES1 expression was recognized to a level comparable to that in the liver or WAT (Figure 1C), its silencing did not reduce the neutral CE hydrolase activity of HMMs (Figure 4B). Crow et al recently reported similar results: a recombinant CES1 protein did not possess neutral CE hydrolase activity.[40] Interestingly, they found that treatment of cholesterol-loaded THP-1 macrophages with ether paraoxon (a nonspecific CES inhibitor) or benzil (a specific CES inhibitor) caused enhanced retention of intracellular CE. This puzzling finding led them to speculate that the retention of CE is due in part to inhibition of enzymes other than CES1. Indeed, KIAA1363, an ortholog of *NCEH1*, is potently inhibited by many of organophosphates (OP) including paraoxon.[16,41]

There is a great difference in the ability to mobilize cholesterol between different types of macrophages. For example, THP-1 cells are known to be relatively ineffective at mobilizing cholesterol, primarily owing to slow hydrolysis of CE.[8,42] In addition to the weak expression of LIPE, the absence of NCEH1 may explain the characteristics of THP-1 macrophages (Figure 1C). There are many other examples of differences in neutral CE hydrolase activity.[9,43–45] It is tempting to speculate that these differences are attributable to the difference in the expression level of NCEH1. Furthermore, we examined the difference of the expression of NCEH1, CES1 and LIPE by M-CSF or GM-CSF treatment. NCEH1

protein and neutral CE hydrolase activity proportionally increased (Online Figure II). In those M-CSF– or GM-CSF–treated HMMs, it is thought that NCEH1 greatly contributes to neutral CE hydrolase. It is further study to investigate difference of the contribution of NCEH1, CES1, and LIPE in macrophages stimulated by other cytokines or in different macrophages subclasses.

Finally, the expression of NCEH1 in CD68-positive macrophages in human atherosclerotic lesions in situ substantiates the role of NCEH1 in the development of atherosclerosis proposed above (Figure 5). NCEH1 was expressed in CD68-positive macrophages, but not in other cells such as smooth muscle cells and endothelial cells. This pattern of expression is very similar to that of SOAT1.[35] The number of NCEH1-positive cells was generally larger in atheromatous plaques, which contain a necrotic center with a fibrous cap (Table). In contrast, samples with diffuse intimal thickening lacked NCEH1-positive cells. The abundance of these cells was moderate in fatty streak lesions (Online Figure VII, A through C). In spleen as representative of other organs, NCEH1 was abundantly expressed in CD68-positive cells in the marginal zone around white pulp (Online Figure VII, D and E). Interestingly, the expression level of NCEH1 was not homogeneous in CD68-positive macrophages in those atherosclerotic lesions and spleen. Some CD68-positive macrophages weakly expressed NCEH1. Does this heterogeneity represent the presence of distinct subpopulations of macrophages? Does it just reflect the different stages of differentiation? These questions need to be answered to elucidate the precise pathogenesis of atherosclerosis.

In conclusion, we demonstrate for the first time that NCEH1 is quantitatively the most important neutral CE hydrolase in human macrophages and atherosclerosis. We also clearly show that the contribution of LIPE and CES1 is negligible compared with that of NCEH1, implying that we should be careful when findings in cholesterol metabolism of mice are extrapolated to those of humans. These findings indicate that NCEH is the only enzyme that requires attention when dealing with neutral CE hydrolase activity in human macrophages. Given its high levels in CD68-positive macrophages in initial fatty streaks, as well as in more advanced atheromatous plaques of human aortas, NCEH1 is a promising target for the treatment of atherosclerosis.

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Disclosures

None.

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Novelty and Significance

What Is Known?

- Intracellular hydrolysis of cholesterol ester (CE) is the initial and rate-limiting step for removal of cholesterol from macrophage foam cells that predominate in fatty streak atherosclerotic lesions.
- Hormone-sensitive lipase (LIPE) and neutral cholesterol ester hydrolase (NCEH1) have comparable roles in CE hydrolysis in mouse macrophages, but the relative contribution of the hydrolytic enzymes in human macrophages is unknown.

What New Information Does This Article Contribute?

- NCEH1 is the principal enzyme that performs CE hydrolysis in human monocyte-derived macrophages (HMMs).
- The contributions of both cholesterol ester hydrolase (CES1) and LIPE to CE hydrolysis are trivial compared to NCEH1.
- NCEH1 is abundantly expressed in CD68-positive macrophages in cholesterol crystal–rich regions of human atherosclerotic lesions.

There is circumstantial evidence of differing contributions of NCEH1, LIPE, and CES1 to CE hydrolysis in macrophages of differing species. As we have reported previously, in mice, both LIPE and NCEH1 are involved in CE hydrolysis in macrophages. Although at least 3 enzymes are able to hydrolyze CE in nonlysosomal compartments in human macrophages, the relative contribution of these enzymes is controversial. The present study established the predominant role of NCEH1 compared with CES1 and LIPE in CE hydrolysis in human macrophages. Furthermore, we also demonstrated the expression of NCEH1 in macrophages in human atherosclerotic lesions. These findings facilitate the focus on NCEH1, a promising therapeutic target for promoting reverse cholesterol transport, and provide a novel paradigm for understanding the pathogenesis of human atherosclerosis.

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