Evidence That the Initial Up-regulation of Phosphatidylcholine Biosynthesis in Free Cholesterol-loaded Macrophages Is an Adaptive Response That Prevents Cholesterol-induced Cellular Necrosis

PROPOSED ROLE OF AN EVENTUAL FAILURE OF THIS RESPONSE IN FOAM CELL NECROSIS IN ADVANCED ATHEROSCLEROSIS*

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Macrophages in atherosclerotic lesions accumulate free cholesterol (FC) as well as cholesteryl ester and appear to have high rates of phospholipid (PL) synthesis and increased PL mass. Previous short term (i.e. 24 h) studies with cultured macrophages have shown that these cells respond to FC loading by up-regulating phosphatidylcholine biosynthesis. We propose that this response is adaptive by keeping the FC:PL ratio in the macrophages from reaching toxic levels. We further propose that one cause of macrophage necrosis, a prominent and important event in atherosclerosis, is an eventual decrease of this adaptive response. To explore these ideas, cultured macrophages were loaded with FC for up to 4 days and assayed for phosphatidylcholine biosynthesis, FC and PL mass, and cytotoxicity. For the first 24 h, cellular phosphatidylcholine biosynthesis and FC and PL mass increased 3–4-fold, and thus the FC:PL molar ratio was prevented from reaching very high levels; at this point, there were no overt signs of cytotoxicity. Over the next 24–48 h, however, phosphatidylcholine biosynthesis, and then phosphatidylcholine mass, began to decrease. Initially, the macrophages remained healthy and continued to accumulate FC, but eventually these macrophages, but not unloaded macrophages, became necrotic (swollen organelles and disrupted membranes). Lipoprotein dose studies indicated a close relationship between the onset of macrophage necrosis and the FC:PL ratio. To test further the causal nature of these relationships, cellular FC and PL mass were independently manipulated by using high density lipoprotein₃ (HDL₃) to decrease cellular FC and choline depletion to decrease cellular PC. As predicted by our hypotheses, HDL₃ protected FC-loaded macrophages from necrosis, whereas choline depletion accelerated cytotoxic changes. These findings support the idea that the initial increase in phosphatidylcholine biosynthesis in FC-loaded macrophages is an adaptive response that prevents cholesterol-induced macrophage necrosis. We propose that an eventual failure of the PL response in foam cells may represent one cause of macrophage necrosis in advanced atherosclerotic lesions.

Cholesterol-loaded macrophages are prominent features of atherosclerotic lesions (1–3), and there is increasing evidence that these cells play an important role both in early atherogenesis and in the clinical progression of advanced lesions (4–6). Although cholesteryl ester accumulation in lesional macrophages (foam cells) is often emphasized, these cells also accumulate large amounts of FC, particularly in advanced atherosclerosis (7–10). In this light, we have been interested in elucidating biological responses of macrophages to FC loading. One such response is the post-translational activation of the phosphatidylcholine (PC) biosynthetic enzyme, CTP:phosphocholine cytidylyltransferase (CT), which leads to an increase in PC biosynthesis and in cellular PC mass (11, 12). This response is likely to be physiologically important, since increases in PC biosynthesis and mass have been noted to occur in lesional macrophages in vivo (13–17).

We have hypothesized that this PC response is initially adaptive (11, 12), since it would keep the cellular FC:PL ratio from getting too high and causing damage to cells (see Ref. 18). For example, membranes enriched with FC demonstrate inhibition of several membrane-bound enzymes (19–22), and cholesterol crystals may accumulate in such cells (14). A corollary of our hypothesis is that an eventual blunting of this PC response would lead to cellular necrosis, and this scenario may be one cause of the necrosis of macrophages that is known to occur in advanced lesions (5, 6, 23, 24). Macrophage necrosis has been proposed to play an important role in plaque destabilization and thus clinical progression of lesions (5, 6).

The goal of the present study was to test these ideas using FC-loaded cultured macrophages. In our previous studies, the macrophages were FC-loaded for no longer than 24 h, at which point the FC biosynthetic response was still increasing, and the cells appeared healthy (11). In the present study, we have cultured these cells for longer periods, and we found that the

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1 The abbreviations used are: FC, free cholesterol; ACAT, acyl-CoA: cholesterol O-acyltransferase; CT, CTP:phosphocholine cytidylyltransferase; Con A, concanavalin A; DMEM, Dulbecco’s modified Eagle’s medium; FBS, fetal bovine serum; HDL₃, high density lipoprotein₃; LDL, lactate dehydrogenase; LDL, low density lipoprotein; LPDS, lipoprotein-deficient serum; PBS, phosphate-buffered saline; PC, phosphatidylcholine; PL, phospholipid; lyso-PC, lysophosphatidylcholine.
PC biosynthetic response, but not the accumulation of FC, began to decrease after 24 h of culture. As predicted by our hypotheses, this event caused an increase in the cellular FC:PL ratio, and the cells subsequently showed signs of necrosis. Furthermore, removal of cellular FC prevented cytotoxicity, whereas premature blunting of the PL response accelerated cytotoxicity. These findings support the idea that the initial increase in PC biosynthesis in response to FC loading in macrophages is adaptive and raise the possibility that an eventual blunting of this response may lead to form cell necrosis in advanced atherosclerotic lesions.

**EXPERIMENTAL PROCEDURES**

**Materials**—The Falcon tissue culture plasticware used in these studies was purchased from Fisher. Tissue culture media and reagents were obtained from Life Technologies, Inc., and fetal bovine serum (FBS) was purchased from Gemini Bioproducts (Calabasas, CA). Choline-deficient medium, generously provided by Dr. Martin Houweling (University of Alberta), was made by adding the following supplements to 5 liters of DMEM formula 79–5141: NaHCO3 (18.5 g), D-glucose (22.5 g), L-lysine (125 g), L-threonine (550 g), L-methionine (150 mg), and L-arginine (420 mg). Lipoprotein-deficient serum (LPDS) was prepared from fetal bovine serum by preparative ultracentrifugation (density, 1.21 g/ml) (25).<ref>

**RESULTS**

**Effects of Prolonged FC Loading of Macrophages on PC Biosynthesis, Cellular Lipid Content, and Cell Viability**—FC loading of cultured macrophages over a 24-h period leads to an increase in PC biosynthesis due to post-translational activation of the PC biosynthetic enzyme, CTP:phosphocholine cytidylyltransferase (CT) (11, 12). This response results in an increase in cellular PC mass (11). To explore the effects on PC biosynthesis of more prolonged FC loading, such as occurs in atherosoma (7–10), macrophages were incubated with 50 μg of acetyl-LDL/ml plus the ACAT inhibitor 58035 (26) for up to 80 h (Fig. 1). The data show that after the initial increase in PC biosynthesis, there is a decline in the rate of this reaction. We have hypothesized that the initial increase in PC biosynthesis is an adaptive response that prevents the cellular FC:PL ratio from reaching potentially toxic levels (11, 12, 18). Thus, in the context of this hypothesis, the data in Fig. 1 predicted two outcomes: first, that the FC:PL ratio would rise to high levels in macrophages loaded with FC for long periods; and second, that the macrophages would eventually show signs of cytotoxicity. The data in panel A of Fig. 2 show that cellular PL mass rose steeply during the first 24 h of FC loading, as described previously (11), but then increased at a slower rate and eventually declined, which is consistent with the PC biosynthesis data in Fig. 1. PL subclass analysis indicated that the major species contributing to this trend was FC (data not shown). FC mass, however, continued to rise at the same rate until the 3rd day of FC loading (panel B). These changes in cellular lipids resulted in a 3-fold increase in the FC:PL ratio by day 4 of FC loading (panel C).

The second prediction of our hypothesis, namely that the macrophages would show signs of cytotoxicity after prolonged FC loading, was investigated using both morphological and biochemical methods. Morphologically, we noted that the macrophages began to round and vacuolize and then began to detach from the plate at day 2 of FC loading. Phase micrographs of control and 3-day FC-loaded macrophages are shown in Fig. 3. Note that the macrophages incubated with 58035...
alone (panel B) or acetyl-LDL alone (panel C) showed similar density and morphology as control macrophages (panel A). In contrast, many of the macrophages that were FC-loaded by incubation with acetyl-LDL plus 58035 had detached from the plate, and most of those that remained were fragmented and rounded (panel D). Electron micrographs of control macrophages and those loaded with FC for 1 and 3 days are shown in Fig. 4. Two major points are revealed by these data. First, the 1-day FC-loaded cells (panel B) appear morphologically healthy compared with unloaded macrophages (panel A), although the loaded cells did contain intracellular membrane whorls, as described previously (11). These whorls most likely are major sites for the increased PL mass in FC-loaded cells. Apparently healthy cells were also noted at 30 h of FC loading (see also below), indicating no overt cytotoxicity at the time the PC biosynthesis response first began to decrease (see Fig. 1). Second, the 3-day FC-loaded cells that did remain attached to the plate (panel C) showed signs of necrosis, including swollen cytoplasm and disrupted organelles. Careful inspection of electron micrographs of many 2- and 3-day FC-loaded macrophages consistently showed these signs, whereas properties of apoptosis (e.g. fragmented and condensed chromatin, condensed organelles, and multiple plasma membrane blebs (37)) were not seen at these times or at 1 day of FC loading. In addition, a biochemical marker of apoptosis, cleavage of 116-kDa poly(ADP-ribose) polymerase to an 80-kDa signature fragment by an interleukin 1β-converting enzyme-like protease (38), was not observed at any stage during FC loading of macrophages (data not shown). Quantitative biochemical data supporting the morphological observations of both cell detachment and cytotoxicity are shown in Fig. 5. Contents of cellular protein (panel A) and DNA (panel B) decreased by more than 50% in 3-day FC-loaded macrophages, consistent with a loss of cells from the monolayer. In addition, there was 2–3-fold increase in the release of intracellular [3H]adenine (panel C) and LDH (panel D) from these cells, consistent with cellular necrosis. As with the morphological observations, these changes were not seen at 30 h of FC loading, the initial time of decrease of PC biosynthesis (Fig. 1). In summary, the data in Figs. 1–5 are consistent with the notion that a decline in the initial induction of PC biosynthesis in FC-loaded macrophages leads to a toxic FC:PL ratio, which, in turn, results in cellular necrosis.

To further establish the relationship between the cellular FC:PL ratio and cytotoxicity in FC-loaded macrophages, we incubated the cells with three different concentrations of acetyl-LDL, all in the presence of 58035 (Fig. 6). At various time points, we measured cellular FC and PL contents and looked for signs of necrosis using both morphological (phase microscopy) and biochemical (cellular protein) criteria, as described above. At 10 µg of acetyl-LDL/ml plus 58035, the cellular FC:PL ratio remained only slightly elevated for up to 4
days, and overt necrosis did not begin until after the 5th day (Fig. 6, panel A). At 25 mg of acetyl-LDL/ml plus 58035, the FC:PL ratio increased to a level greater than that seen with the lower lipoprotein concentration, and necrotic changes began approximately 2 days earlier, at day 3 (panel B). At the highest concentration of acetyl-LDL (50 µg/ml) plus 58035, the FC:PL ratio rose steeply, and necrosis began by day 2 (panel C).

Although the absolute FC:PL ratio at which necrosis was triggered in J774 macrophages varied somewhat between experiments, we usually found that elevation above 0.4 for >24 h led to these changes. Thus, there appears to be a close correlation between the FC:PL ratio in macrophages and the onset of cellular necrosis.

The experiments described up to this point were conducted with J774 macrophages, a dividing cell line. To examine these issues in a nondividing macrophage, we studied both resident and immunologically primed mouse peritoneal macrophages. The primed macrophages, which are defined as being able to respond to subsequent stimulation with lipopolysaccharide, were prepared by injecting the peritoneal cavities of mice with concanavalin A (Con A) 3 days before harvesting the peritoneal macrophages (29). Unforeseen differences in acetyl-LDL-induced cholesterol loading between these two states of nondividing macrophages provided us an opportunity to test our ideas. As shown in panel A of Fig. 7, the Con A-elicited macrophages (closed squares) accumulated more FC than the resident cells (open squares) when incubated with acetyl-LDL plus 58035. The increase in PL mass, however, was similar in both cell types (panel B), and so the FC:PL ratio increased substantially more in the Con A-elicited cells (panel C). Morphological observations (not shown) and cellular protein mass data (panel D) revealed that the Con A-elicited cells eventually showed signs of necrosis and loss of adherence, whereas the resident cells remained healthy and adherent throughout 3 days of observation. Con A-elicited macrophages that were not loaded with FC maintained a FC:PL ratio of 0.3 and showed no overt signs of cytotoxicity throughout the 3-day period. Note that peritoneal macrophages appear to be more resistant to FC-mediated toxicity than J774 macrophages, since a ratio of 0.7 in resident cells did not lead to necrotic changes during this time. Nonetheless, the overall principle of a relationship between the FC:PL ratio and cytotoxicity is established with these nondiv-
Effect of Separate Manipulations of Cellular FC Content and PL Response in FC-loaded Macrophages—To further define the causal relationships between PL metabolism, the FC:PL ratio, and cellular necrosis in FC-loaded macrophages, we conducted one set of experiments in which FC mass, but not PL mass, was altered, and another set of experiments in which the PL response, but not FC mass, was manipulated. In the first set of experiments, macrophages were loaded with FC for 36 h and then chased in medium in the absence or presence of HDL₃, an inducer of cellular cholesterol efflux (39). At the time of addition of the HDL₃ (36 h), the macrophages had accumulated both FC and PL as expected (Table I), and the cells showed no signs of necrosis (compare panel D of Fig. 8 with the control macrophages in panel A of Fig. 8). The macrophages chased in the absence of HDL₃ continued to accumulate a little more FC, presumably due to the uptake of residual surface-bound acetyl-LDL and the processing of prelysosomal acetyl-LDL, whereas those chased in the presence of HDL₃ lost substantial amounts of FC as expected (Table I). The PL content of these macrophages, however, was very similar (Table I). As shown in Fig. 8, the macrophages chased in the absence of HDL₃ showed signs of toxicity (compare panels E (2 days) and F (3 days) with the control macrophages in panels B and C), whereas those chased in the presence of HDL₃ remained healthy (panels G and H of Fig. 8). Thus, as predicted by our hypothesis, preventing the rise in the FC:PL ratio prevented cytotoxicity.

In the second set of experiments, our goal was to manipulate the PL response of FC-loaded macrophages. To accomplish this goal, we took advantage of the previous finding that incubating cells in choline-depleted medium partially inhibits PC biosynthesis (40). To establish this point in our system, we first analyzed the PL content of control and choline-depleted macrophages, both under cholesterol-deficient and FC-loading conditions. For these experiments, it was necessary to load the cells with a source of cholesterol that did not contain choline-phospholipids, and we chose nonlipoprotein cholesterol, which is known to modestly up-regulate PC biosynthesis in macrophages (11). As shown in Table II, choline deficiency resulted in a relative decrease in the PL content of both unladen and FC-loaded macrophages compared with the respective choline-replete cells. The phase micrographs of these cells are shown in Fig. 9. Importantly, choline depletion in the absence of FC loading did not result in cytotoxicity under the conditions of our experiment (compare the macrophages in panel B with the control cells in panel A). When loaded with nonlipoprotein cholesterol, the choline-replete macrophages remained healthy (panel C), whereas the choline-depleted cells showed definite signs of cytotoxicity, including detachment, rounding, and fragmentation (panel D); cellular protein measurements confirmed this finding (not shown). Similar data were obtained with macrophages loaded with cholesterol-phosphatidylserine liposomes as another source of cholesterol without choline (compare the choline-depleted cells in panel F with the choline-replete cells in panel E). Thus, partially blunting the PL response in FC-loaded macrophages promotes cytotoxicity, as predicted by our hypothesis.

DISCUSSION

As summarized in Fig. 10, the findings in this report support the hypothesis that the initial rise in PC biosynthesis in FC-loaded macrophages is an adaptive response that keeps the FC:PL ratio from rising to toxic levels. This idea clearly explains the lag in the onset of FC-mediated cytotoxicity observed in our study as well as that of Warner et al. (33), which did not look at cellular PL metabolism. With prolonged FC loading, however, this adaptive response fails, the FC:PL ratio rises to cytotoxic levels, and macrophage necrosis ensues. Although we did not...
study the exact cause of macrophage death in these studies, high cellular FC levels are known to inhibit several critical membrane enzymes, including Na+/K+-ATPase activity (19), Ca2+/Mg2+-ATPase activity (20), carnitine palmitoyltransferase activity (21), and alkaline phosphatase activity (22).

Furthermore, as Small (14) points out, excessive accumulation of cholesterol monohydrate crystals could lead to lysosomal rupture and cellular necrosis. In the case of membrane enzyme inhibition, the cause of FC-induced toxicity is probably related to perturbations of membrane fluidity, which could be compensated by increases in membrane phospholipid content (18). Cholesterol crystal formation would also be expected to be prevented by increases in cellular PL (14). Interestingly, mouse peritoneal macrophages appear to require a higher FC:PL ratio than J774 macrophages to trigger cytotoxic changes (Fig. 7). Investigation into the mechanism of this relative resistance may shed additional light on how cells adapt to excess FC.

The FC-loaded macrophages used in our studies are noted to have intracellular membrane whorls (see Ref. 11 and Fig. 4, panel B), which probably represent the sites where most of the increased PL mass in these cells accumulate. Furthermore, filipin-labeling studies have shown that much of the FC that accumulates in cultured macrophages incubated with acetyl-LDL plus 58035 is localized in perinuclear lysosomes, presumably in lysosomal membranes (12). These findings suggest that our experimental model may reflect physiological events, since lesion macrophages in vivo have both intracellular membrane whorls (10) and accumulate FC in lysosomes (41, 42). These observations raise questions, however, about how the increased phospholipid might protect the cells from FC-mediated toxicity. First of all, where in the cell does the accumulation of FC cause problems? Many of the enzymes inhibited by excess FC are localized in the plasma membrane (see above), and Warner et al. (33) have shown that FC export from the lysosomes is necessary for FC-mediated toxicity in macrophages. Thus, even though the bulk of FC appears to be in lysosomes, a critical amount of excess FC is probably in other cellular membranes, particularly the plasma membrane, and this localization is likely important for FC-mediated toxicity. Interestingly, we have shown that FC synthesis is still stimulated when lysosomal FC export is blocked (12), suggesting that lysosomal FC, while itself not initially toxic, may be the signal to “warn” the cell to protect other membranes from ensuing FC enrichment and damage. Eventually, massive lysosomal FC accumulation after very prolonged FC loading may result in cholesterol crystallization (43); consistent with this idea, cholesterol crystallization appears to be a relatively late effect of FC loading, since we have not observed it in our cells even after 3 days of incubation with acetyl-LDL plus 58035.

How does the presence of intracellular membrane whorls pertain to the proposed ability of increased cellular PL to protect macrophages from FC-mediated toxicity? One possibility is that the membrane whorls serve as a “sink” for excess cellular cholesterol; for example, a critical amount of excess cholesterol from the sites of sensitive membrane enzymes (e.g., plasma membrane) might be transferred to the whorls, thus preventing inhibition of these enzymes (see above). Another idea is that the whorls represent a storage form of “excess” phospholipid in FC-loaded macrophages; in this scenario, phospholipid would be transferred from the whorls to membranes in the cells that have a high FC:PL (cf. Ref. 44). If such cholesterol or phospholipid transfer reactions are, in fact, found to play a role in the adaptive response of macrophages to FC loading, a defect in these transfer reactions might contribute to or accelerate FC-mediated cytotoxicity.

Two important issues related to our studies but not addressed herein are the mechanisms of FC accumulation in vivo and the mechanism of blunting of the FC biosynthetic response with prolonged FC loading. Regarding the first issue, cells normally possess several mechanisms to prevent the accumulation of excess FC. These include cellular cholesterol efflux, cholesterol esterification, down-regulation of LDL receptors and of endogenous cholesterol biosynthesis, and cholesterol

![Diagram A](image1.png)  ![Diagram B](image2.png)  ![Diagram C](image3.png)  ![Diagram D](image4.png)

**FIG. 6. Relationship between FC:PL ratio and necrosis in FC-loaded macrophages.** Monolayers of J774 macrophages were incubated for the indicated times in DMEM, 10% LPDS containing 5 μg of 58035/ml plus 10 μg (panel A), 25 μg (panel B), or 50 μg (panel C) of acetyl-LDL/ml. The cells were then assayed for cellular PL and FC content, and the cellular FC:PL ratio was calculated from these values. At each time point, the cells were also examined for signs of necrosis by phase microscopy and cellular protein content; the arrow labeled with Necr indicates the time at which the cells started to become rounded, fragmented, and detached (see Figs. 3 and 5).

**FIG. 7. PL and FC mass and cell monolayer protein content in FC-loaded resident and concanavalin A-elicited mouse peritoneal macrophages.** Monolayers of resident (open squares) and concanavalin A-elicited (closed squares) mouse peritoneal macrophages were incubated for the indicated times in DMEM, 10% LPDS containing 50 μg of acetyl-LDL/ml plus 5 μg of 58035/ml. The cells were then assayed for cellular FC content (panel A) and cellular PL content (panel B); from these data, the cellular FC:PL ratio was calculated (panel C). The cells remaining on the monolayer were then assayed for protein content (panel D) as in Fig. 5; the 100% values for the resident and Con A-elicited cells were 0.29 and 0.13 μg/well, respectively. As mentioned in the text, Con A-elicited macrophages that were not loaded with FC remained morphologically healthy throughout the 3-day period and showed much less loss of cells from the monolayer.

**Fig. 6.** Relationship between FC:PL ratio and necrosis in FC-loaded macrophages. Monolayers of J774 macrophages were incubated for the indicated times in DMEM, 10% LPDS containing 5 μg of 58035/ml plus 10 μg (panel A), 25 μg (panel B), or 50 μg (panel C) of acetyl-LDL/ml. The cells were then assayed for cellular PL and FC content, and the cellular FC:PL ratio was calculated from these values. At each time point, the cells were also examined for signs of necrosis by phase microscopy and cellular protein content; the arrow labeled with Necr indicates the time at which the cells started to become rounded, fragmented, and detached (see Figs. 3 and 5).

**Fig. 7.** PL and FC mass and cell monolayer protein content in FC-loaded resident and concanavalin A-elicited mouse peritoneal macrophages. Monolayers of resident (open squares) and concanavalin A-elicited (closed squares) mouse peritoneal macrophages were incubated for the indicated times in DMEM, 10% LPDS containing 50 μg of acetyl-LDL/ml plus 5 μg of 58035/ml. The cells were then assayed for cellular FC content (panel A) and cellular PL content (panel B); from these data, the cellular FC:PL ratio was calculated (panel C). The cells remaining on the monolayer were then assayed for protein content (panel D) as in Fig. 5; the 100% values for the resident and Con A-elicited cells were 0.29 and 0.13 μg/well, respectively. As mentioned in the text, Con A-elicited macrophages that were not loaded with FC remained morphologically healthy throughout the 3-day period and showed much less loss of cells from the monolayer.
metabolism (e.g. bile acid synthesis in hepatocytes) (39, 45, 46).
For macrophages internalizing large amounts of lipoprotein-cholesterol by means other than the LDL receptor (e.g. via the scavenger receptor), several of these mechanisms are irrelevant, including receptor down-regulation, down-regulation of cholesterol biosynthesis, and metabolism of cholesterol into bile. In lesions, cholesterol efflux may be impeded due to inaccessibility of the cells to inducers of efflux, or it may be overwhelmed by the large amount of cholesterol in the cells. Similarly, the esterification pathway may be saturated or inhibited; for example, oxysterols in oxidized LDL may prevent trafficking of lipoprotein-cholesterol to ACAT (47), or ACAT itself may become dysfunctional in advanced foam cells.

With respect to PC biosynthesis, the initial up-regulation in response to FC loading is due to post-translational activation of the enzyme CTP:phosphocholine cytidylyltransferase (CTP) (12); specifically, FC-mediated induction appears to involve a cell-signaling event involving the dephosphorylation of CT and probably other cellular proteins (12). The cause of the eventual blunting of the PC biosynthesis response in our cell-culture model and whether it is related to a decline in CT activity (e.g. through changes in CT phosphorylation state) require further

**TABLE I**

| Incubation time | −HDL₃ (h 36–96) | Cellular FC content (nmol/mg cell protein) | Cellular PL content (nmol/mg cell protein) | Cellular FC:PL ratio |
|----------------|-----------------|------------------------------------------|------------------------------------------|-------------------|
| 0 h            | −               | 23.7 ± 1.1                               | 79.1 ± 14.0                              | 0.3               |
| 36 h           | −               | 134.3 ± 4.0                              | 264.7 ± 6.8                              | 0.5               |
| 96 h           | −               | 176.6 ± 28.3                             | 179.9 ± 16.5                             | 1.0               |
| 96 h           | +               | 33.3 ± 3.9                               | 211.0 ± 11.3                             | 0.2               |

**FIG. 8.** Phase micrographs of control and FC-loaded macrophages subsequently incubated in the absence or presence of HDL₃. Panels A–C, monolayers of J774 macrophages were incubated in DMEM, 10% LPDS alone and viewed by phase microscopy at day 1 (A), day 2 (B), and day 3 (C). Panels D–H, other monolayers of J774 macrophages were incubated for 36 h in DMEM, 10% LPDS containing 50 µg of acetyl-LDL/ml plus 5 µg of 58035/ml. The cells were then washed with PBS and incubated an additional 60 h with DMEM, 10% LPDS containing 58035 in the absence or presence of 300 µg HDL₃/ml (see Fig. 8). The cells were viewed at day 1 before the differential chase (D), at day 2 (minus HDL₃ = E; plus HDL₃ = G), and at day 3 (minus HDL₃ = F; plus HDL₃ = H). The original microscopy magnification was × 40. Bar, 10 µm.
investigation. According to our working hypothesis, blunting of the PC biosynthesis response is an early event that subsequently causes a rise in the FC:PL ratio and FC-induced macrophage necrosis. We base this idea on the finding that PC biosynthesis started to decline at 24 h of FC loading (Fig. 1), while the first signs of necrosis were seen 24 h later. It is possible, however, that the blunting of the PC biosynthesis response itself is a very early result of subtle FC-induced toxicity, which then causes an escalation in the rise of the cellular FC:PL ratio. We hypothesize that this response is adaptive by keeping the FC:PL ratio from reaching cytotoxic levels. We further propose that this initially adaptive response eventually diminishes, perhaps due to deactivation of CT (e.g., by cytokines), leading to an increase in the cellular FC:PL ratio and, ultimately, cholesterol-induced cellular necrosis. Refer to text and Refs. 11, 12 for details.
at least one plausible explanation for macrophage necrosis in atherosclerotic lesions. Necrosis of macrophages in advanced atherosclerosis is thought to be an important event in lesion progression (5, 6, 23, 24). For example, degradative enzymes released from these cells might contribute to plaque rupture and eventual acute thrombosis (5, 6). The mechanism of cellular necrosis in atheromatous, however, is not known. In addition to our hypothesis, other possibilities include nutrient deprivation, loss of growth factors, free radical and oxidized lipid injury, and toxic cytokines (for example, see Ref. 53). Heine1 (54) attempted to test this hypothesis in a study of the vessels of rats on a partially choline-deficient diet. Necrosis and progression. Investigators recently showed that the decrease in the PL content of lesional cells might accelerate the atherosclerosis in macrophage necrosis and lesion progression. The goal of our laboratory is to test our hypotheses by using the aorta and coronary arteries, including increased lesion rates of fat, there was evidence of accelerated atherosclerosis in recently available mouse models of atherosclerosis, we hope to be able to show the importance of macrophage PL metabolism in macrophage necrosis and lesion progression.

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Our hypotheses predict that manipulations resulting in a decrease in the PL content of lesional cells might accelerate lesion necrosis and progression. Investigators several decades ago studied the vessels of rats on a partially choline-deficient diet, which is known to partially decrease the phosphatidyicholine (PC) content of cells in culture (40) and in liver in vivo (60). Interestingly, when these rats were fed cholesterol and saturated fat, there was evidence of accelerated atherosclerosis in the aorta and coronary arteries, including increased lesion rates. In lesional cell necrosis, this situation is not known and may be multifactorial, it is tempting to speculate that at least part of the mechanism may be related to our hypothesis about the cytotoxicity of increased FC:PL ratios. The current goal of our laboratory is to test our hypotheses in vivo by genetically manipulating arterial wall PL metabolism using induced-mutant mouse models. By crossing these mice to currently available models of atherosclerosis, we hope to be able to show the important role of macrophage PL metabolism in macrophage necrosis and lesion progression.

REFERENCES
1. Schaffner, T., Taylor, K., Bartucci, E. J., Fischer-Drago, K., Beeson, J. H., Glagov, S., and Wissler, R. W. (1980) Am. J. Pathol. 100, 57–73
2. Gerrity, R. G. (1981) Am. J. Pathol. 103, 181–190
3. Faggionato, A., Ross, R., and Harker, L. (1984) Arteriosclerosis 4, 323–340
4. Smith, J. D., Trogn, E., Ginsberg, M., Grigaux, C. Tian, J., and Miyata, M. (1995) Proc. Natl. Acad. Sci. U.S.A. 92, 8624–8628
5. Libby, P., and Clinton, S. K. (1993) Curr. Opin. Lipidol. 4, 355–363
6. Fuster, V., Badimon, L., Badimon, J. J., and Then, P. (1984) J. Biol. Chem. 259, 242–250
7. Katz, S. S., Shipley, G. G., and Small, D. M. (1976) J. Clin. Invest. 58, 200–211
8. Landberg, B. (1985) Atherosclerosis 56, 93–110
9. Rapp, J. H., Connor, W. E., Lin, D. S., Inahara, T., and Porter, J. M. (1983) J. Lipid Res. 24, 1329–1338
10. Fowler, S. (1980) Acta Med. Scand. Suppl. 642, 151–158
11. Shiratori, Y., Okwu, A. K., and Tabas, I. (1994) J. Biol. Chem. 269, 11327–11348
12. Shiratori, Y., Houweling, M., Zha, X., and Tabas, I. (1995) J. Biol. Chem. 270, 28994–29003
13. Buck, R. J., and Ross, S. R. (1986) J. Biol. Chem. 231, 103–129
14. Small, D. M. (1988) Arteriosclerosis 8, 103–129
15. Labarca, C., and Paigen, K. (1980) Proc. Natl. Acad. Sci. U.S.A. 77, 1709–1713
16. chocolate, and toxic cytokines (for example, see Ref. 53). Heinle (1987) showed that lyso-PC levels are only slightly elevated in before the onset of necrosis, but they are markedly elevated in at 5 percent of the control level. This lyso-PC may then accelerate the necrosis of neighboring cells or contribute to specific atherogenic effects thought to be signaled by this lipid (cf. Refs. 57–59).

Our hypotheses predict that manipulations resulting in a decrease in the PL content of lesional cells might accelerate lesion necrosis and progression. Investigators several decades ago studied the vessels of rats on a partially choline-deficient diet, which is known to partially decrease the phosphatidyicholine (PC) content of cells in culture (40) and in liver in vivo (60). Interestingly, when these rats were fed cholesterol and saturated fat, there was evidence of accelerated atherosclerosis in the aorta and coronary arteries, including increased lesion necrosis. This conclusion is consistent with our hypothesis as well as with hypotheses implicating other toxic lipids. In this regard, we did consider the possibility that the generation of lyso-phosphatidyicholine (lyso-PC), which can be cytotoxic (55, 56), contributed to the necrosis of FC-loaded, PL-rich macrophages. In preliminary studies, we found that lyso-PC levels are only slightly elevated before the onset of necrosis, but they are markedly elevated in at 5 percent of the control level. This lyso-PC may then accelerate the necrosis of neighboring cells or contribute to specific atherogenic effects thought to be signaled by this lipid (cf. Refs. 57–59).

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Evidence That the Initial Up-regulation of Phosphatidylcholine Biosynthesis in Free Cholesterol-loaded Macrophages Is an Adaptive Response That Prevents Cholesterol-induced Cellular Necrosis: PROPOSED ROLE OF AN EVENTUAL FAILURE OF THIS RESPONSE IN FOAM CELL NECROSIS IN ADVANCED ATHEROSCLEROSIS

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