Macrophage β3 Integrin Suppresses Hyperlipidemia-Induced Inflammation by Modulating TNFα Expression

Jochen G. Schneider, Yimin Zhu, Trey Coleman, Clay F. Semenkovich

Objective—High-fat, cholesterol-containing diets contribute to hyperlipidemia. Both high-fat diets and hyperlipidemia are associated with chronic inflammatory diseases like atherosclerosis. Integrins, heterodimeric mediators of inflammatory cell recruitment, are not generally thought to be affected by diet. However, high-fat feeding promotes inflammation, atherosclerosis, and death in hyperlipidemic mice with β3 integrin deficiency, and treatment of humans from Western populations with oral β3 integrin inhibitors increases mortality. The mechanisms responsible for these β3 integrin-associated events are unknown.

Methods and Results—Here we show that diet-induced death in β3 integrin-deficient mice is a TNFα-dependent process mediated by bone marrow–derived cells. In 2 different hyperlipidemic models, apoE-null and LDL receptor–null mice, β3-replete animals transplanted with β3-deficient marrow died with Western-type high-fat feeding whereas β3-deficient animals transplanted with β3-replete marrow were rescued from diet-induced death. Transplantation with β3-deficient marrow also increased atherosclerosis. TNFα expression was increased in β3-deficient macrophages and normalized by either retroviral or adenoviral reconstitution of β3 integrin expression. Treatment with the anti-TNFα antibody infliximab rescued β3 integrin–deficient mice from Western diet–induced death, directly implicating TNFα in the pathophysiology triggered by diet-induced hyperlipidemia.

Conclusions—These findings suggest that macrophage β3 integrin, acting through TNFα, suppresses inflammation caused by hyperlipidemia attributable to high-fat feeding. (Arterioscler Thromb Vasc Biol. 2007;27:2699-2706.)

Key Words: integrins ■ TNFα ■ diet ■ bone marrow transplantation ■ atherosclerosis ■ infliximab

Eating a high-fat diet, generally reflecting increased intake of both triglycerides and cholesterol, is increasingly common as Western culture is exported throughout the world. This practice contributes to atherosclerosis, obesity, and diabetes, disorders characterized by inflammation.1 Nutrient excess prompts changes in a host of inflammatory proteins associated with metabolic disease including TNFα, interleukin (IL)-6, NF-κB, JNK, PKC, C-reactive protein, matrix metalloproteinases, and others,2 but how diet is linked to specific inflammatory mediators is obscure.

Because the inflammatory response that occurs in atherosclerosis and obesity is characterized in part by macrophage infiltration,3,4 the interaction between these inflammatory cells and the endothelium could be affected by diet. Monocytes initially form a loose attachment to the vasculature, then roll along the endothelial surface until they adhere firmly, migrate between endothelial cells into tissues, and become macrophages. This process is mediated by integrins (heterodimers on the surface of monocytes), cellular adhesion molecules (including P-selectin, E-selectin, intercellular adhesion molecule-1 [ICAM-1], vascular cell adhesion molecule [VCAM]-1, and PECAM-1), and chemokines such as MCP-1. Reports of the effects of high-fat diets on these proteins are inconsistent and their role in vascular disease is still poorly defined. In mice, the absence of several types of adhesion molecules decreases diet-induced atherosclerosis5 but absence of CD11b, the α chain for the β2 integrin Mac-1 (found extensively in vascular lesions), does not.6 In humans, antibodies against ICAM-1,7 the β2 integrins,8,9 and P-selectin10 have failed to improve vascular outcomes.

Unlike antagonism of β2 integrins, antagonism of β3 integrin (at least in the short-term) benefits patients with atherosclerotic disease. There are 2 integrins in the β3 family, αIIbβ3 and αvβ3, each defined by the α subunit that partners with the regulatory β3 subunit. αIIbβ3, or glycoprotein IIb/IIIa, is found on platelets where its inhibition blocks platelet aggregation to decrease thrombosis in patients undergoing vascular interventions.11 αvβ3, originally identified as the vitronectin receptor, is present in blood vessels as well as inflammatory cells including macrophages.12 Short-term inhibition of αIIbβ3 reproducibly improves outcomes, but long-term inhibition using small molecules curiously increases mortality in humans,13 prompting speculation that defective β3 signaling could increase inflammatory mediators.
We generated data in support of this idea when we found that hyperlipidemic β3-deficient mice developed accelerated atherosclerosis and lethal pulmonary inflammation when fed a high-fat diet,\textsuperscript{14} a striking phenotype lacking a molecular mechanism. We pursued the mechanism by testing the hypothesis that the β3 integrin on bone marrow–derived cells affects the inflammatory response induced by high-fat cholesterol-containing diets. We found that transplanting β3 integrin–deficient marrow into β3 integrin–replete animals reproduces the diet-induced phenotype, that transplanting β3 integrin–replete marrow into β3 integrin–deficient animals rescues them from the diet-induced phenotype, that the β3 integrin on macrophages mediates expression of proinflammatory mediators including TNFα, and that antagonism of TNFα with the antibody infliximab rescues β3 integrin–deficient mice from the diet-induced phenotype. These results unexpectedly implicate the β3 integrin on macrophages in the suppression of an inflammatory cascade initiated by TNFα and linked to one of the most common conditions in developed countries, diet-induced hyperlipidemia.

**Methods**

**Bone Marrow Transplantation**

Experimental protocols were approved by the Washington University Animal Studies Committee. Animals were weaned at 3 weeks to mouse chow providing 6% calories as fat as described.\textsuperscript{14} Congenic animals were generated for experiments. Bone marrow was transplanted into lethally irradiated donors, and degree of engraftment was estimated in Kaplan–Meier plots that were analyzed by the log-rank method.\textsuperscript{27} Survival is represented in Kaplan–Meier plots that were analyzed by the log-rank method and confirmed by Cox regression. The assumption that the proportional hazard does not change was validated by showing that the logarithm of the estimated cumulative hazard function was constant over time.

**Inflammatory Stimuli**

To stimulate inflammation, littermates were injected with lipopolysaccharide (LPS) (10 mg/kg, serotype 0111:B4, Sigma), then plasma was collected and flash-frozen. For the in vivo treatment with anti-TNFα antibody, β3\textsuperscript{−/−} apoE\textsuperscript{−/−} littersmates were injected with a loading dose of either infliximab (10 mg/kg, Centocor) or an IgG kappa isotype control antibody at the same dose. The mice were then started on a Western-type diet containing 0.15% cholesterol and providing 42% calories as fat (TD 88137, Harlan). Injections with 10 mg/kg body weight of infliximab or isotype control were given twice weekly.

**Analytical Procedures**

Serum lipids were measured after a 4-hour fast and aortic sinus histology (supplemental Figure IA, available online at http://atvb.ahajournals.org), genotyping (supplemental Figure IB) and characterization of GFP-positive leukocytes in both apoE\textsuperscript{−/−} and LDLR\textsuperscript{−/−} mice as described in Methods.
Mean GFP positive leukocytes in several experiments 4 weeks after transplantation was 84.5% (range 66.3 to 94.3%). With Western diet feeding, only 3 of 18 apoE-deficient (β3 wild-type) mice transplanted with β3-deficient marrow survived compared with 19 of 19 mice receiving β3 wild-type marrow (Figure 1A, *p*<0.0001). LDLR-deficient (β3 wild-type) mice transplanted with β3-deficient marrow also had increased mortality with Western diet feeding compared with animals receiving β3 wild-type marrow (Figure 1B, *p*=0.002). In the converse experiments, apoE-deficient mice also deficient for β3 integrin were rescued from Western diet-induced death by transplantation with β3 wild-type marrow (apoE−/−) (Figure 1C, *p*<0.0001), and LDLR-deficient mice also deficient for β3 integrin were rescued from Western diet-induced death by transplantation with β3 wild-type (LDLR−/−) marrow (Figure 1D, *p*<0.0001). The phenotype appeared to be more pronounced in apoE-deficient macrophages, suggesting that macrophage apoE affects the interaction between integrin levels and diet-induced inflammation, a notion consistent with known effects of apoE on macrophage function.21 There was no effect on mortality in transplanted animals fed low fat chow instead of the Western diet (supplemental Figure II).

Autopsies revealed an extensive mononuclear infiltrate in the lungs of β3−/−apoE−/− mice transplanted with β3−/−apoE−/− marrow and β3−/−LDLR−/− mice transplanted with β3−/−LDLR−/− marrow found dead with Western diet feeding (supplemental Figure IIIA and IIIB). Stains and cultures of this infiltrate were negative for pathogens but infiltrates in both apoE−/− and LDLR−/− mice stained positive for macrophages (supplemental Figure III C and IIID). Lungs were histologically normal in β3+/+apoE−/− mice and β3+/+
LDLR−/− mice transplanted with β3+/+ apoE−/− or β3+/+ LDLR−/− bone marrow, respectively, and euthanized after six weeks of high fat feeding (supplemental Figure IIIA and IIIB).

**Accelerated Vascular Disease in Mice Transplanted With β3-Deficient Marrow**

Atherosclerosis, a macrophage-driven inflammatory process, was more extensive in β3+/+LDLR−/− mice transplanted with β3+/+LDLR−/− marrow as compared with β3−/−LDLR−/− marrow (Figure 2A and 2C), the experiment in which sufficient animals survived to allow vascular analysis (Figure 1B). Accelerated vascular disease occurred despite the absence of a macrophage β3 genotype effect on serum cholesterol (Figure 2B). For each of the experimental groups, genotype had no effect on body weight (supplemental Table I) or serum chemistries (supplemental Figure IV).

**Inflammatory Stressors Increase Macrophage β3 Expression**

Increased inflammation in the absence of the β3 gene in macrophages raises the possibility that β3 in this cell type suppresses inflammation. If this were physiologically relevant, β3 expression might be induced by inflammatory stressors. Consistent with this notion, β3 mRNA was increased (Figure 3A) when bone marrow macrophages from β3+/+apoE−/− mice were treated for 1 hour with either of 2
classic initiators of inflammatory cascades, TNFα (50 ng/mL) or LPS (1 μg/mL). Palmitate, a proinflammatory fatty acid, increased β3 mRNA (Figure 3B) as well as β3 protein (not shown), in these cells. To determine whether in vivo exposure to cholesterol-containing lipoproteins associated with diet-induced hyperlipidemia affects β3 expression, peritoneal macrophages elicited from mice fed chow were compared with elicited macrophages from mice fed the Western diet (Figure 3C). β3 expression was increased in cells obtained from Western diet–fed mice (Figure 3C).

**β3 Suppresses TNFα**

Message levels for TNFα, a central mediator of inflammatory responses under many conditions, were increased in unstimulated bone marrow macrophages from β3−/−apoE−/− as compared with β3+/−apoE−/− mice (Figure 3D). Restoration of β3 expression in these cells using a human β3 retrovirus decreased TNFα expression (Figure 3F) but control virus treatment did not (Figure 3E). Normalization of TNFα was also demonstrated in LPS-stimulated bone marrow macrophages. Both TNFα message and protein were increased in LPS-stimulated cells from β3−/−apoE−/− mice treated with a control adenovirus (Figure 3G) as compared with β3+/−apoE−/− mice treated with a control adenovirus (Figure 3G with protein shown in inset). After reconstitution of β3 expression using a human β3 adenovirus, TNFα mRNA (main figure) and TNFα protein (inset) were normalized (Figure 3H).

**Increased Cytokines in the Absence of β3 Integrin**

The increased TNFα phenotype in unstimulated and stimulated β3-deficient macrophages was also present systemically in mice with β3 deficiency. Administration of LPS (10

**Figure 3.** Induction of β3 integrin mRNA in macrophages (A–C) and increased TNFα in β3-deficient macrophages (D–H). A, β3 expression in bone marrow–derived macrophages treated with TNFα (50 ng/mL), LPS (1 μg/mL), or vehicle for 1 hour (n=3 for each condition). *P<0.01. B, β3 expression in bone marrow–derived macrophages after treatment with 500 μm/L palmitate with BSA (open bars) or BSA alone (solid bars) (n=3 dishes per condition). *P<0.01, #P<0.05. C, β3 expression in thioglycollate-elicited peritoneal macrophages from apoE−/− mice fed a chow diet (solid bar) or Western diet (open bar) for 4 weeks (n=3 animals per condition). *P<0.05. D, TNFα expression in unstimulated bone marrow–derived macrophages from β3+/−apoE−/− mice (solid bar) and β3−/−apoE−/− mice (open bar). *P<0.05. E, TNFα expression in cells from mice with the same genotypes after transduction with a control retrovirus. *P<0.01. F, Expression in cells transduced with a human β3 integrin retrovirus (Rvβ3). G, TNFα message and supernatant protein levels (inset) of LPS-stimulated bone marrow–derived macrophages from β3+/−apoE−/− mice (solid bar) and β3−/−apoE−/− mice (open bar) after transduction with a control adenovirus (n=3). *P<0.01 for mRNA and *P<0.05 for protein. H, The same parameters in LPS-stimulated bone marrow–derived macrophages from β3+/−apoE−/− mice (solid bar) and β3−/−apoE−/− mice (open bar) after reconstitution of β3 expression using an adenovirus (Adβ3). Results are presented as mean±SEM.
mg/kg) resulted in higher circulating levels of TNFα protein at 1 hour in β3−/−apoE−/− as compared with β3+/−apoE−/− mice, an effect also seen 4 and 6 hours after treatment (Figure 4A). Consistent with the known stimulation of IL-6 by TNFα, IL-6 protein levels peaked at 4 hours after LPS in the circulation of these same mice and were also elevated in β3−/−apoE−/− mice after LPS stimulation was quantified in nuclear extracts at various times after treatment. *P<0.01, **P<0.05. Results are presented as mean ± SEM. B, Measurement of IL-6 concentration in the mice of Panel A. *P<0.05. C, Bone marrow–derived macrophages from β3−/−LDLR−/− (solid bars) or β3+/−LDLR−/− mice (open bars) were stimulated with LPS then p65 activation was measured in serum at various times after treatment. *P<0.01, **P<0.05. Results are presented as mean ± SEM. D, β3−/−apoE−/− mice were started on a Western diet and treated with infliximab, an anti-TNFα antibody (solid symbols), or an isotype-specific control antibody (open symbols). The significant difference in survival rate was assessed by log-rank comparison.

**Discussion**

These current data suggest that the β3 integrin on macrophages is an antiinflammatory molecule that suppresses TNFα. Inflammatory stressors such as LPS, Western diet, and TNFα itself induce β3 expression (Figure 3), perhaps to dampen downstream signaling. The absence of β3 is associated with increased TNFα, reconstitution of β3 normalizes TNFα (Figure 3), Western diet feeding to mice transplanted with β3-deficient marrow provokes inflammation manifested as pulmonary infiltration leading to death (Figure 1; supplemental Figure III) and accelerated atherosclerosis (Figure 2), and TNFα antagonism in β3-deficient animals prevents death induced by this diet (Figure 4D).

Our data show that Western diet feeding in the absence of the β3 integrin in bone marrow–derived cells affects 2 phenotypes, pulmonary infiltration and atherosclerosis.
Evolving evidence in both mice and men supports the notion that these processes are related. Although generally not appreciated, apoE-null mice, animals that develop extensive vascular disease when fed an atherogenic diet, also manifest lung infiltration with lipid-laden macrophages in response to the same diet.22 Dietary cholesterol exacerbates and statin treatment diminishes disease in a mouse model of pulmonary inflammation.23 High fat/high cholesterol feeding to apoE null and LDLR null mice transplanted with ABCG1 null marrow results in pulmonary inflammation with relative protection from atherosclerosis.24

In humans, chronic pulmonary disease is a common comorbidity that tracks with decreased survival in patients with coronary artery disease.25 Consumption of a Western diet increases the risk for chronic obstructive pulmonary disease in people.26 The use of statin drugs, which lower lipids and decrease cardiovascular event rates in people with coronary heart disease, decreases the risk of pneumonia and sepsis.27,28 Collectively, these published data suggest that pulmonary inflammation and vascular disease may share a similar inflammatory diathesis related to high fat/high cholesterol diets.

Based on the data in the current work, β3 integrin appears to be a candidate molecule for mediating clinically relevant systemic inflammation caused by Western-type diets. Both TNFα and IL-629,30 predict vascular disease, a common consequence of inflammation, and both are increased with β3 deficiency in mice (Figure 4). Chronic inhibition of β3 in vascular disease patients from populations eating Western diets increases the risk of death.31 Antagonism of TNFα decreases inflammation in patients with the metabolic syndrome, a disorder driven by Western diet feeding and obesity.31 TNFα antagonism is also associated with decreased cardiovascular disease in rheumatoid arthritis,32 a chronic inflammatory state that can also be complicated by substantial pulmonary inflammation. Because lipids increase β3 expression in macrophages (Figure 3), variations in the β3 gene impairing its response to a high fat diet could promote atherosclerosis and other chronic inflammatory diseases.

There is a conceptual framework for pursuing the signals linking β3 and inflammation. Cholesterol, shown to affect TNFα production by macrophages,33 or palmitate, which acts in part through TLR4,18 could be the specific dietary trigger for the β3 response because both are presented to macrophages as components of lipoproteins. αvβ3, a promiscuous receptor that is unlikely to exist in the unliganded state in vivo,34 is one of several cell surface receptors mediating uptake of apoptotic cells that may propagate tissue inflammation.35 αvβ3 signaling is known to stimulate phosphorylation of IRS-1,36 an adaptor protein that increases PI3 kinase/AKT signaling. Wortmannin, a PI3 kinase inhibitor, blocks late phase platelet aggregation mediated by β3,37 evidence that outside-in β3 signals involve PI3 kinase. Inhibition of the PI3 kinase/AKT pathway in mice promotes LPS-induced cytokine release,38 consistent with dampening of inflammation by β3-PI3 kinase signals.

There are apparently conflicting data in mouse tumor models39 and cell monolayer systems40 suggesting that β3 integrins actually promote the transendothelial migration of macrophages. These results, derived from experimental models strikingly different from mice fed high fat/high cholesterol diets, indicate that β3 signaling may be context-specific. The circumstance of Western diet feeding and its attendant generation of proinflammatory lipoproteins (that are presented in discrete ways to macrophages) could represent a stimulus to the β3 integrin to dampen inflammation.

Because the spread of Western habits is unlikely to abate, diseases exacerbated by high fat/high cholesterol diets will become more prevalent. Promoting antiinflammatory signaling driven by expression of the β3 integrin gene in macrophages represents a novel approach to treating these diseases.

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Disclosures
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References


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Online Supplemental Data

**Supplemental Table I.** Body weights over time in mice fed the Western diet.

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Results are presented as mean ± SEM.
Supplemental Figure I. Engraftment after bone marrow transplantation. (A) Representative H & E staining of bone marrow from β3+/+apoE-/ - mice transplanted with bone marrow from β3+/+apoE-/ - (left panel) or β3-/apoE-/ - (right panel) mice at lower (40x) and higher (100x) magnification. Minor differences in color intensity are due to the fixation process. Similar images were obtained from transplanted LDLR-/ - mice. (B) PCR genotyping of buffy coat-derived DNA or tail DNA from β3+/+apoE-/ - mice transplanted with bone marrow from β3+/+apoE-/ - (lanes 4-6) or β3-/apoE-/ - (lanes 1-3) mice. Buffy coat DNA shows the 538 bp mutant band in lanes 1-3 and the 446 bp wild type band in lanes 4-6. Tail DNA shows the wild type band in lanes 1-6. La indicates a DNA size ladder.
Supplemental Figure II. Lack of an effect on mortality in bone marrow-transplanted mice with chow (low fat) feeding. β3+/+apoE−/− mice were transplanted with bone marrow from β3+/+apoE−/− (solid line) or β3−/−apoE−/− (broken line) mice after chow diet feeding. There was no genotype effect on survival during the study period in these chow-fed mice.
Supplemental Figure III. Representative lung histology after high-fat feeding presented as H & E sections (A, B, C, D) or anti-macrophage antibody staining (E, F). For histology, tissues were formalin-fixed, paraffin-embedded, sectioned and stained with hematoxylin and eosin. For immunocytochemistry, sections were deparaffinized, rehydrated, then treated with hydrogen peroxide followed by microwave antigen retrieval at 100°C for 10 min. Slides were sequentially incubated with casein then avidin and biotin. Rat anti-mouse F4/80 monoclonal antibody CI:A3-1 (1:100, Abcam, Cambridge, MA) or nonimmune rat serum (InnoGenex, San Ramon, CA) was applied followed by biotinylated affinity-purified goat anti-rat IgG (Vector Laboratories) and detection using streptavidin-HRP/3,3'-diaminobenzidine. (A, B) Normal lung tissue from β3+/+apoE-/ or β3+/+LDLR-/ recipient mice transplanted with β3+/+apoE-/- (A) or β3+/+LDLR-/- (B) bone marrow and sacrificed after 6 weeks on Western diet. (C, D) Inflamed lung tissue from β3-/+apoE-/ or β3+/+LDLR-/ recipient mice transplanted with β3-/apoE-/ (C) or β3-/LDLR-/ (D) bone marrow and found dead with Western diet feeding. (E, F) Macrophage staining (F4/80 positivity) in lungs of β3+/+apoE-/- or β3+/+LDLR-/- mice transplanted with marrow from β3-/apoE-/ (E) or β3-/LDLR-/ (F) mice and found dead with Western diet feeding. In both hyperlipidemic models, mononuclear pulmonary inflammation was the predominant finding in necropsies of animals dying after high fat feeding. Fatty liver was also detected in some mice, but heart, kidney, intestine, and brain were normal.
Supplemental Figure IV. Serum chemistries. The left panels show results from β3+/+apoE-/- mice transplanted with bone marrow from β3+/+apoE-/- (solid bars) or β3-/-apoE-/- (open bars) mice (left panel). The right panels show results (except for cholesterol, which appears in Figure 3) from β3+/+LDLR-/- mice transplanted with bone marrow from β3+/+LDLR-/- (solid bars) or β3-/-LDLR-/- (open bars) mice. Samples were obtained at baseline on a chow diet and at subsequent time points on a Western diet. Results are presented as mean ± SEM for decreasing numbers of mice over time due to spontaneous deaths.