Conclusions—These results suggest that M/Ms enhance in vitro vascular calcification via 2 independent mechanisms: cell-cell interaction and production of soluble factors such as tumor necrosis factor-α. (Circulation. 2002;105:650-655.)

Key Words: muscle, smooth ▪ lipids ▪ atherosclerosis ▪ leukocytes

Vascular calcification is a major independent predictor of cardiovascular morbidity and mortality.1 Although pathologists have known for centuries that vascular calcification often includes fully formed bone tissue and even marrow within the artery wall,2 little was known of the mechanisms until this decade. Like skeletal bone, human atherosclerotic calcification contains matrix vesicles, which are key elements in organized calcium phosphate crystal formation, and the stoichiometry of crystals in atherosclerosis matches that of the bone mineral hydroxyapatite.3 In addition, a series of reports beginning in 1992 provided evidence that many osteogenic regulatory factors are expressed in atherosclerotic lesions4-6 and that the process resembles embryonic bone development.7,8

In previous studies, we9 showed that calcifying vascular cells (CVCs), a subpopulation of cells isolated from the aortic medial layer, undergo osteoblastic differentiation and mineralization in vitro. We and others10-12 have shown that vascular medial cells express bone differentiation markers, and the distinctive time sequence of expression mimics that in osteoblastic differentiation of skeletal bone cells.13,14 One of the most widely used bone differentiation markers is the bone/liver/kidney isoenzyme of alkaline phosphatase (ALP).14,15 The function of ALP in osteoblastic differentiation is believed to be hydrolysis of ester phosphates at sites of mineralization, providing ionic phosphate for incorporation into mineral. Although this enzyme is also expressed in liver and kidney cells among mesenchymal cells, induction of ALP is specific for osteoblastic differentiation.

Lipids and monocyte/macrophages (M/Ms), known atherogenic factors, colocalize with calcium deposits in plaques.3,16,17 Whereas lipids have been shown to regulate vascular calcification,18 the role of M/Ms is unknown. Factors in the subendothelial space of diseased arteries such as oxidized LDL (ox-LDL) and lipopolysaccharide (LPS) activate M/Ms to produce reactive oxygen species (ROS), transforming growth factor-β (TGF-β), osteopontin, and inflammatory cytokines including interleukins and monocyte chemotactic protein-1.19-22 Of these factors, TGF-β, oxidized lipids, tumor necrosis factor-α (TNF-α), and ROS are reported to enhance in vitro calcification of vascular cells.9,18,23,24

In the present study, we hypothesized that M/Ms regulate vascular calcification. We tested whether M/Ms promote osteoblastic differentiation and mineralization of CVCs and whether this effect is modified by ox-LDL or LPS activation of M/Ms.

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Methods

Materials
LPS (Escherichia coli 0111:B4), polymyxin B, and anti-TNF-α antibody were purchased from Sigma. The ELISA kit for TNF-α was purchased from Biosource International. The class-matched mouse anti-human antibody against pro-B-cell antigen CD19 was purchased from Dako. Ox-LDL was prepared from freshly isolated human LDL (fresh) media or overnight-conditioned media from unstimulated CVCs. CVCs were cocultured for 9 days with increasing numbers of monocytes added to the CVC culture correlated with the increasing level of ALP activity (r=0.98; Figure 1b).

Results

Effects of M/Ms on CVC Differentiation
To determine whether M/Ms have a functional role in osteoblastic differentiation of vascular cells, CVCs were cocultured with freshly isolated human peripheral blood monocytes for 2 or 5 days, and ALP activity, an established early marker of osteoblastic differentiation, was measured. Results showed that ALP activity was significantly enhanced after 5 days in cocultures compared with cultures of CVCs alone (536±9.6 versus 209±9.4 U/mg protein, P<0.0001; Figure 1a). Monocytes cultured alone produced no detectable ALP activity (Figure 1a). The increasing numbers of monocytes added to the CVC culture correlated with the increasing level of ALP activity (r=0.98; Figure 1b).

To determine whether M/Ms also promote mineralization, CVCs were cocultured for 9 days with increasing numbers of monocytes, and matrix calcium mineral incorporation was demonstrated by von Kossa staining. Results showed that mineralization also increased with the number of monocytes added to the CVC cultures (panels A through C in Figure 1c). Monocytes cultured alone showed no mineralization (panel D in Figure 1c).

Effects of Activated M/Ms on CVC Differentiation
Because M/Ms are exposed to activating inflammatory factors in the subendothelial space, we tested the effects of M/M activation on CVC differentiation using ox-LDL and LPS.

Effects of Ox-LDL–Activated Macrophages on CVC Differentiation
Treatment of CVC/monocyte cocultures with ox-LDL (100 μg/mL) for 3 days resulted in a 6-fold (5.9±0.2-fold) increase in ALP activity compared with untreated CVCs alone and a 2-fold (1.9±0.1-fold) increase compared with untreated coculture (Figure 2). Ox-LDL treatment of CVCs alone increased ALP activity 1.5±0.4-fold (Figure 2). Because monocyte induction of ALP in CVC plateaus at ≈6 days (data not shown), the 3-day time point, where induction is submaximal, was chosen to discern the added effects of ox-LDL.

We examined whether ox-LDL and monocytes enhanced ALP activity via secreted factors by evaluating conditioned media. CVCs were treated with conditioned media obtained from monocytes that had been activated with vehicle alone (unstimulated) or ox-LDL (100 μg/mL) for 7 hours. Before collection of the conditioned media, monocytes were washed thoroughly to remove ox-LDL and incubated in fresh medium overnight. Nonconditioned (fresh) media or overnight-conditioned media from un-
stimulated or ox-LDL–stimulated M/Ms were added to CVC culture, and ALP activity was measured after 3 days. Results showed that there was no significant difference in induction of ALP activity when conditioned media from ox-LDL–activated M/Ms were used versus conditioned media from unstimulated M/Ms (62.2±9.0 versus 57.1±5.1 U/mg protein, P=0.26; Figure 3, top). However, ALP activity was increased in both conditioned media from unstimulated and ox-LDL–stimulated M/Ms compared with nonconditioned (fresh) media (57.1±5.1 and 62.2±9.0 versus 40.0±2.3 U/mg protein, P<0.005; Figure 3, top), which suggests that conditioned media from unstimulated M/Ms secrete soluble factors that are not further enhanced by stimulation with ox-LDL.

To determine whether close contact between monocytes and CVCs is necessary for ox-LDL–induced ALP, monocytes and CVCs were cocultured in separate chambers of transwells, monocytes on the polycarbonate 0.4-μm-pore filters of the upper chamber and CVCs on the tissue culture plastic of the lower chamber. These transwells allow the exchange of soluble factors without cell-cell contact. Cultures were treated with 50 μg/mL of ox-LDL, and ALP activity was measured after 3 days of treatment. Results showed that ALP activity was not enhanced in the transwell cocultures, whereas it was enhanced in direct coculture (Figure 3, bottom), which suggests that close contact between monocytes and CVCs was required for the ox-LDL enhanced effect.

**Effects of LPS-Activated Macrophages on CVC Differentiation**

Because we have previously found that LPS strongly induces CVC differentiation (unpublished data), further induction by monocytes was not expected to be detectable. Therefore, we pretreated monocytes separately with LPS, then collected the conditioned media to treat CVCs. Freshly isolated monocytes were activated with LPS for 7 hours, and conditioned media were collected as described above. To prevent residual LPS contamination, the conditioned media were neutralized with polymyxin B before treatment of CVCs. Results showed that conditioned media from LPS-activated M/Ms significantly enhanced ALP activity compared with controls (156.8±16 versus 40.0±2.3 U/mg protein, P<0.0001), which suggests that LPS activation leads to secretion of a soluble factor that induces CVC differentiation. A quantitative chromogenic limulus amoebocyte lysate assay showed that the concentration of residual LPS in conditioned media was <50 pg/mL, which is below the threshold concentration required (>100 pg/mL) to induce CVC ALP activity (data not shown).

Kornbluth et al21 showed that LPS induces TNF-α in M/Ms, and we22 previously found that TNF-α is a potent inducer of CVC differentiation and mineralization. Therefore, we examined whether TNF-α is responsible for the osteoinductive effect of the conditioned medium from LPS-activated monocytes. CVCs were incubated with
conditioned media from LPS-activated M/Ms that had been pretreated for 1 hour with either control or TNF-α neutralizing antibody, and ALP activity was measured after 2 days. Results showed that TNF-α neutralizing antibody attenuated the induction of ALP activity (Figure 4, top). Class-matched irrelevant antibody (mouse anti-human CD19) had a small but not significant effect on ALP activity (data not shown). ELISA of the conditioned media from LPS-activated M/Ms showed immunoreactivity for TNF-α (Figure 4, bottom) at a level previously shown to induce CVC differentiation.23 Conditioned media from irrelevant cell types (CVCs or smooth muscle cells) showed no significant level of TNF-α (Figure 4, bottom).

**Discussion**

The present results indicate that monocytes enhance osteoblastic differentiation of CVCs, as evidenced by increased ALP activity and increased matrix mineralization. Activation of M/Ms further enhanced CVC differentiation. Interestingly, mechanisms by which activated monocytes induced CVC differentiation varied depending on the activating agent; the effect of ox-LDL activation required close contact with the CVCs, whereas the effect of LPS activation was mediated at least in part through secretion of TNF-α.
Macrophages play a critical role in pathogenesis of chronic inflammatory conditions, including atherosclerosis.26 The present results suggest that both unstimulated monocytes and activated macrophages contribute to in vitro vascular calcification through effects on osteoblastic differentiation. This finding is supported by the report of Jeziorska and colleagues16 that macrophages are associated with various phases of calcification in atherosclerotic lesions. Interestingly, histological analyses identify macrophages and giant cells near the calcified regions in other chronic pathological conditions, including aortic valvular sclerosis8–27 and calcifying tendinitis.28

Chronic infection has been implicated in atherogenesis. For example, Chlamydia pneumoniae infection is associated with coronary artery disease,20 and its antigen has been detected in atherosclerotic lesions.29 Ericson et al30 reported that the degree of immunoreactivity of C. pneumoniae correlates with the severity of atherosclerosis. This may be due to inflammatory changes in macrophages that contribute to lesion progress in concert with hyperlipidemia. Our data suggest that bacterial products such as endotoxin (LPS) may contribute to vascular cell calcification indirectly through production of cytokines by activated M/Ms. These results are in agreement with reports of the possible role of C. pneumoniae or other infectious agents in valvular calcification.31,32

Our data also show that activation of M/Ms by ox-LDL enhances monocyte-induced calcification. Our findings suggest that cell-cell contact between CVCs and M/Ms is necessary for ox-LDL–enhanced osteogenic effects. A similar cell-cell contact requirement has been reported for M/M stimulation of prostanoid synthesis in vascular smooth muscle cells.33 Cell-cell interaction between monocytes and CVCs could be operating through engagement of cell-surface integrins with extracellular matrix proteins. Integrins such as α2β1 regulate osteoblastic differentiation and mineralization in vitro through binding to the extracellular matrix protein osteopontin.34 Osteopontin is abundant in several pathological conditions, including atherosclerosis and aortic stenosis, especially at sites of mineralization, and is synthesized by macrophages, smooth muscle cells, and CVCs.6,12,28 Other possible mechanisms include production of TGF-β or ROS by macrophages. These factors contribute to vascular calcification,9,24 and cell-cell contact may be required because TGF-β is likely to bind to extracellular matrix and because other cellular processes mediated by macrophage-derived oxygen radicals also require cell-cell contact, as reported by Mix and colleagues.35

Vascular calcification is a complex phenomenon whose regulation is not well understood. Our findings indicate a role for M/Ms in this process. Moreover, oxidized lipids deposited in the artery wall and LPS released by bacteria such as C. pneumoniae may further enhance vascular calcification by activating M/Ms to induce osteoblastic differentiation of CVCs. Additional in vivo and human studies are required to extend the relevance of these data to the human condition.

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References


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