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Molecular Medicine

Induction of Bone-Type Alkaline Phosphatase in Human Vascular Smooth Muscle Cells

Roles of Tumor Necrosis Factor-α and Oncostatin M Derived From Macrophages

Atsushi Shioi, Miwako Katagi, Yasuhisa Okuno, Katsuhito Mori, Shuichi Jono, Hidenori Koyama, Yoshiki Nishizawa

Abstract**—**Inflammatory cells such as macrophages and T lymphocytes play an important role in vascular calcification associated with atherosclerosis and cardiac valvular disease. In particular, macrophages activated with cytokines derived from T lymphocytes such as interferon-γ (IFN-γ) may contribute to the development of vascular calcification. Moreover, we have shown the stimulatory effect of 1α,25-dihydroxyvitamin D_3 (1,25$(OH)_2D_3$) on in vitro calcification through increasing the expression of alkaline phosphatase (ALP), an ectoenzyme indispensable for bone mineralization, in vascular smooth muscle cells. Therefore, we hypothesized that macrophages may induce calcifying phenotype, especially the expression of ALP in human vascular smooth muscle cells (HVSMCs) in the presence of IFN-γ and 1,25$(OH)_2D_3$. To test this hypothesis, we used cocultures of HVSMCs with human monocytic cell line (THP-1) or peripheral blood monocytes (PBMCs) in the presence of IFN-γ and 1,25$(OH)_2D_3$. THP-1 cells or PBMCs induced ALP activity and its gene expression in HVSMCs and the cells with high expression of ALP calcified their extracellular matrix by the addition of β-glycerophosphate. Thermostability and immunoassay showed that ALP induced in HVSMCs was bone-specific enzyme. We further identified tumor necrosis factor-α (TNF-α) and oncostatin M (OSM) as major factors inducing ALP in HVSMCs in the culture supernatants of THP-1 cells. TNF-α and OSM, only when applied together, increased ALP activities and in vitro calcification in HVSMCs in the presence of IFN-γ and 1,25$(OH)_2D_3$. These results suggest that macrophages may contribute to the development of vascular calcification through producing various inflammatory mediators, especially TNF-α and OSM. **(*Circ Res*. 2002;91:9-16.)**

Key Words: vascular calcification ■ alkaline phosphatase ■ macrophages ■ tumor necrosis factor-α ■ oncostatin M

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Dystrophic calcification is often associated with inflammatory vascular diseases such as atherosclerosis and cardiac valvular disease.[1–5] Inflammatory cells such as macrophages and T lymphocytes are frequently found in advanced atherosclerotic lesions, particularly fibrofatty or fibrous plaques in which calcification usually initiates.[6,7] Calcium deposits are usually observed at the periphery of the lipid core. It has been pointed out that macrophages are the predominant cell type associated with different stages of calcification in atherosclerotic plaques.[8] T-lymphocyte and macrophage infiltrates are also associated with calcification in human native and porcine bioprosthetic valves, and it has been suggested that production of noncollagenous proteins and cytokines by these cells may contribute to valvular calcification.[9,10] Therefore, T lymphocytes and macrophages may play an important role in the development of vascular calcification.

Vascular cells such as vascular smooth muscle cells (VSMCs), pericyte-like cells, and valvular cells play an important role in vascular calcification. VSMCs and pericyte-like cells derived from bovine aorta have calcifying capacity and express noncollagenous matrix proteins such as osteopontin (OPN), matrix gla protein (MGP), and osteocalcin (OC).[11–14] VSMCs also produce osteoprotegerin (OPG), which is a soluble decoy receptor of the tumor necrosis factor receptor superfamily and may regulate vascular calcification as well as skeletal metabolism.[15] Valvular cells derived from aortic valves can also calcify their extracellular matrix.[16] In vitro calcification by vascular cells can be modulated by calciotropic hormones, steroid hormones, transforming growth factor-β, and 25-hydroxycholesterol.[13,16–18] These results suggest that phenotypic changes of vascular cells in mesenchymal origin, especially acquisition of calcifying phenotype under various pathological conditions, may contribute to the development of vascular calcification.

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Alkaline phosphatase (ALP), one of the phenotypic markers of osteoblasts, is thought to be essential to bone mineralization in that a missense mutation in the human tissue nonspecific ALP gene was identified in hypophosphatasia, a rare heritable form of rickets.[19] ALP activity in human atherosclerotic lesions is cytochemically localized in calcified matrix vesicles and intracytoplasmic vesicles of smooth muscle cells.[20] Medial smooth muscle cells in Mönckeberg's sclerosis express ALP mRNA at higher levels than those in normal arteries.[21] Moreover, ALP activity has been implicated in calcification of aortic valves and glutaraldehyde-fixed porcine aortic valve xenografts.[22,23] By using an in vitro model, we demonstrated that expression of ALP is functionally important in bovine vascular smooth muscle cell (BVSMC) calcification.[11] These in vivo and in vitro data suggest that ALP expressed in vascular cells may play an important role in vascular calcification.

Interferon-γ (IFN-γ), one of the major cytokines produced by T lymphocytes, modulates various cellular functions of macrophages including production of proinflammatory cytokines such as tumor necrosis factor-α (TNF-α).[24] Importantly, it strongly stimulates production of 1α,25-dihydroxyvitamin D, an active metabolite of vitamin D in macrophages through inducing expression of the vitamin D–activating enzyme 25-hydroxyvitamin D 1α-hydroxylase.[25,26] Furthermore, we have demonstrated that 1α,25-dihydroxyvitamin D_3 (1,25$(OH)_2D_3$) promotes BVSMC calcification through upregulating ALP expression.[17] Therefore, we hypothesized that macrophages may induce calcifying phenotype in human vascular smooth muscle cells (HVSMCs), especially the expression of ALP in the presence of IFN-γ and 1,25$(OH)_2D_3$.

In the present study, we examined the roles of macrophages in vascular calcification by using coculture models and further explored soluble factor(s) responsible for the effects of macrophages on HVSMCs.

Materials and Methods

An expanded Materials and Methods section can be found in the online data supplement available at http://www.circresaha.org.

Results

Induction of ALP in HVSMCs by the Coculture With THP-1 Cells

To investigate the roles of macrophages in vascular calcification, we first used a mixed coculture of HVSMCs with THP-1 cells. HVSMCs were cocultured for 4 days with THP-1 cells in the presence of 100 ng/mL IFN-γ and 10^{-7} mol/L 1,25$(OH)_2D_3$. ALP expression was cytochemically detected in HVSMCs cocultured with THP-1 cells in the presence of IFN-γ and 1,25$(OH)_2D_3$ (Figure 1A), whereas ALP was only weakly positive in the cocultures without two reagents and the cultures of HVSMCs alone (Figures 1B and 1D, respectively). Moreover, ALP was not detected in THP-1 cells in the cocultures. To determine whether soluble factor(s) derived from THP-1 cells may be responsible for induction of ALP in HVSMCs, we used a transwell coculture to inhibit direct contact between THP-1 cells and HVSMCs. ALP activities in the cultures of HVSMCs separated from THP-1 cells by the membranes were increased in the presence of IFN-γ and 1,25$(OH)_2D_3$ compared with the control HVSMCs

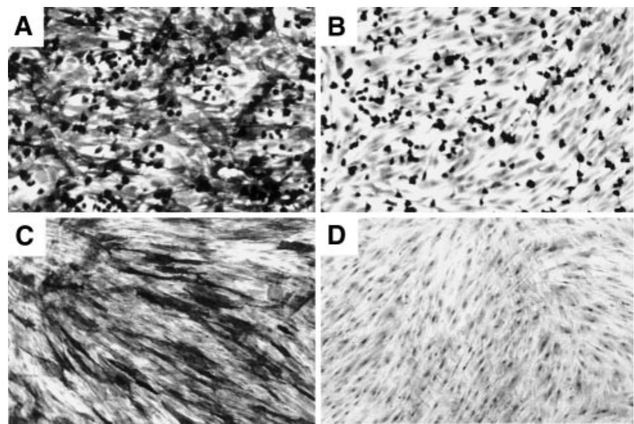

Figure 1. Cytochemical detection of ALP activities in HVSMCs by the coculture with THP-1 cells. HVSMCs were cocultured with THP-1 cells in the presence of IFN-γ and 1,25$(OH)_2D_3$ as described in Materials and Methods. The mixed cocultures in the presence (A) and absence (B) of IFN-γ and 1,25$(OH)_2D_3$. C, Transwell cocultures in the presence of IFN-γ and 1,25$(OH)_2D_3$. D, Control culture of HVSMCs.

(Figures 1C and 1D). The levels of ALP expression induced in the transwell cocultures were comparable to those in the mixed cocultures when assessed by its activities per well (transwell coculture versus mixed coculture: 29.3$\pm$2.1 versus 25.0$\pm$1.6 U/well; mean$\pm$SEM [n=3]). To further confirm production of soluble factor(s) by THP-1 cells and to clarify the mode of actions of IFN-γ and 1,25$(OH)_2D_3$, we examined the effects of conditioned media (CM) derived from the cultures of THP-1 cells for 4 days on induction of ALP in HVSMCs. The CM of THP-1 cultures treated with IFN-γ and 1,25$(OH)_2D_3$ dose-dependently increased ALP activities at the concentrations from 10% to 50% (online Figure 1a in the online data supplement available at http://www.circresaha.org) when HVSMCs were cultured in the presence of IFN-γ and 1,25$(OH)_2D_3$. On the other hand, the CM of THP-1 cells without treatment did not induce ALP activities in HVSMCs (online Figure 1a). The CM of THP-1 cells treated with either IFN-γ or 1,25$(OH)_2D_3$ were not so efficient as those with both reagents (online Figure 1b). Furthermore, the CM of THP-1 cultures treated with both reagents exerted the maximal effect on HVSMCs in the presence of IFN-γ and 1,25$(OH)_2D_3$ (online Figure 1b). These data suggest that both of IFN-γ and 1,25$(OH)_2D_3$ are necessary to stimulate ALP-inducing factor(s) production by THP-1 cells and that direct actions of these two reagents on HVSMCs are also involved in induction of ALP activities.

To further delineate the cellular events that occur in HVSMCs, it is necessary to perform biochemical analyses of HVSMCs separate from THP-1 cells. Therefore, we used the transwell cocultures in the following experiments. By using this coculture model, we confirmed the roles of IFN-γ and 1,25$(OH)_2D_3$ in inducing ALP activities in HVSMCs (Figure 2A). The induction of ALP activities in HVSMCs was observed at 2 days, gradually increased until 7 days of coculture, and then slightly declined thereafter (Figure 2B). Although 1,25$(OH)_2D_3$ by itself was more potent in inducing ALP activities than IFN-γ, these two reagents synergistically increased ALP activities in the coculture at the doses of 10^{-7}

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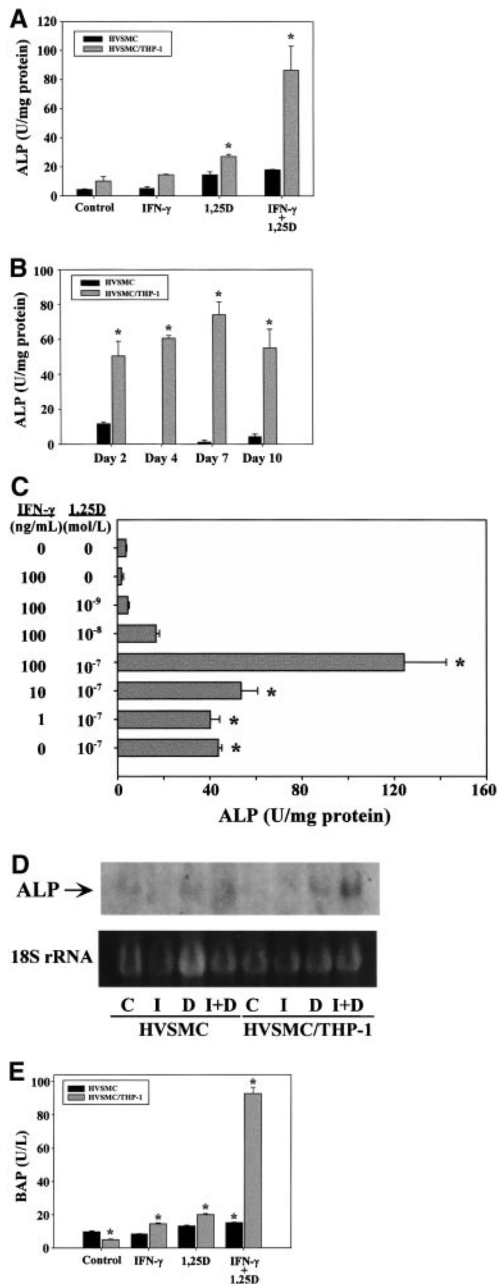

Figure 2. ALP was markedly induced in HVSMCs cocultured with THP-1 cells only in the presence of IFN-γ and $1,25(OH)_2D_3$. The data are presented as mean±SEM (n=3). HVSMC indicates HVSMC cultures; HVSMC/THP-1, transwell cocultures of HVSMCs with THP-1 cells. A, ALP activities of HVSMCs on day 7 in the cocultures with THP-1 cells. IFN-γ and $1,25(OH)_2D_3$ (1,25D) were used at the doses of 100 ng/mL and 10^{-7} mol/L, respectively. *$P<0.05$ vs control HVSMCs. B, Time courses of ALP induction in control HVSMCs and the coculture with THP-1 cells in the presence of IFN-γ (100 ng/mL) and $1,25(OH)_2D_3$ (10^{-7} mol/L). *$P<0.05$ vs HVSMCs at each time point. C, Synergistic effects of IFN-γ and $1,25(OH)_2D_3$ on ALP induction in HVSMCs. Cells were cocultured for 7 days with THP-1 cells in the presence of the indicated concentrations of IFN-γ and $1,25(OH)_2D_3$. *$P<0.05$ vs the cocultures in the absence of IFN-γ and 1,25D. D, Northern blot analysis of ALP expression in HVSMCs cultured for 7 days under the indicated conditions. 18S rRNA was used for assessing equal RNA loading. E, Expression of bone-type ALP (BAP) in HVSMCs cocultured with THP-1 cells under the indicated conditions. BAP activity in HVSMCs was measured with the enzyme immunoassay kit using a monoclonal antibody directed against human bone-specific ALP. *$P<0.05$ vs control HVSMCs.

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mol/L and 100 ng/mL, respectively (Figure 2C). Furthermore, Northern blot analysis showed that the tissue nonspecific ALP mRNA was induced only in the coculture with THP-1 cells in the presence of IFN-γ and $1,25(OH)_2D_3$ (Figure 2D). Finally, we further examined the roles of monocyte/macrophage-lineage cells in the expression of ALP in HVSMCs by using human peripheral blood monocytes (PBMCs), instead of THP-1 cells, for the coculture. As is the case with THP-1 cells, PBMCs dramatically increased ALP activities in HVSMCs in the coculture only in the presence of IFN-γ and $1,25(OH)_2D_3$ (online Figure 2a). However, a longer period of time was necessary to induce ALP activities in the cocultures with PBMCs than those with THP-1 macrophages (7 days of coculture) (online Figure 2B), suggesting that ALP-inducing capacity may be dependent on differentiation of monocytic cells into macrophages.

ALP Induced in HVSMCs by the Coculture Is Bone-Specific Enzyme

Because several isoforms of tissue nonspecific ALP can be distinguished with respect to thermostability and immunoreactivity, we examined whether ALP induced in HVSMCs in the coculture may be bone-specific isoenzyme. Bone-type ALP is more rapidly inactivated than liver-type. The average half-inactivation time of liver-type and bone-type at 56°C has been reported to be 456 and 112 seconds, respectively.[27] By heat inactivation at 56°C for 2 minutes, ALP activities were markedly decreased compared with those in the original samples before heat inactivation (15.5±5.2%; n=3). To apply the enzyme immunoassay using a monoclonal antibody specific for human bone-type ALP to measurement of the activities in HVSMCs, the samples were prepared as described above.[28] Bone-type ALP activities as determined by this enzyme immunoassay[29] were dramatically increased only in the coculture with THP-1 cells in the presence of IFN-γ and $1,25(OH)_2D_3$ (Figure 2E). These data suggest that ALP induced in HVSMCs may be bone-specific isoenzyme.

In Vitro Calcification by HVSMCs With High Expression of ALP

Because ALP is functionally important in in vitro calcification by BVSMCs,[11] we examined whether HVSMCs with high expression of ALP can calcify their extracellular matrix. We prepared HVSMCs with high expression of ALP by the coculture for 7 days with THP-1 cells as described above. The cells were incubated for an additional 14 days with 10 mmol/L β-glycerophosphate (β-GP) in the presence or absence of IFN-γ (100 ng/mL) and $1,25(OH)_2D_3$ (10^{-7} mol/L). Interestingly, only in the presence of IFN-γ and $1,25(OH)_2D_3$ did mineral deposits develop in the cell layer at 14 days (Figure 3A). Calcium deposition by HVSMCs derived from the cocultures with THP-1 cells was cytochemically confirmed by von Kossa staining (Figure 3B). Electron microscopy revealed that electron-dense deposits were exclusively localized in the extracellular matrix, especially along with collagen fibrils (Figure 3C). Vesicular structures like matrix vesicles were also observed around calcium deposits.

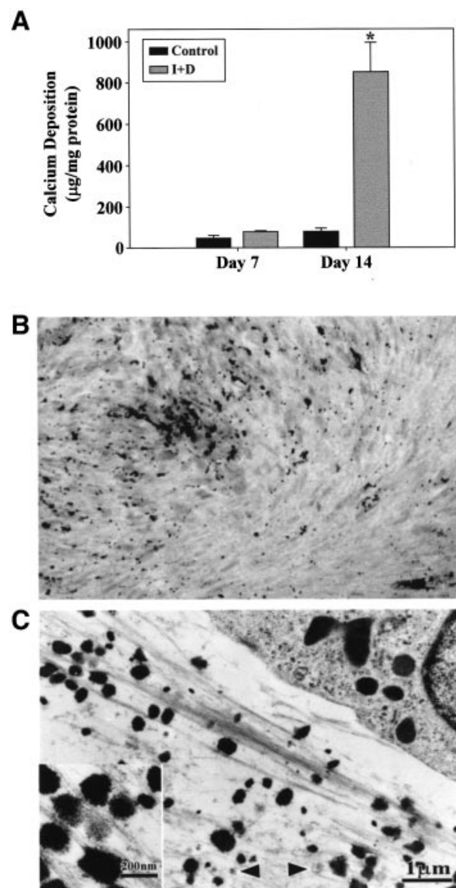

Figure 3. In vitro calcification by HVSMCs. HVSMCs were cocultured with THP-1 cells in the presence of IFN-γ and 1,25(OH)$_2$D$_3$ as described in Materials and Methods. A, Calcium deposition was quantified at the indicated time points by the *o*-cresolphthalein complexone method and normalized by cellular protein content. I+D indicates the presence of IFN-γ (100 ng/mL) and 1,25(OH)$_2$D$_3$ (10^{-7} mol/L). *$P<0.05$ vs each control. B, Calcium deposition in the cells derived from the coculture with THP-1 cells (von Kossa). C, By electron microscopy, calcification was detected as electron-dense deposits localized in extracellular matrix. Vesicle-like structures were also observed (arrowheads).

Phenotypic Changes of HVSMCs by the Coculture With THP-1 Cells

To characterize the phenotype of HVSMCs cocultured with THP-1 cells, we examined the expression of phenotypic markers of differentiated VSMCs (α-smooth muscle actin and calponin), OPN, and OPG in HVSMCs. The expression of α-smooth muscle actin in HVSMCs was decreased by the cocultures in the presence of IFN-γ and 1,25(OH)$_2$D$_3$, whereas no remarkable changes in calponin expression were observed (Figure 4A). In the cultures of HVSMCs without THP-1 cells, IFN-γ depressed the expression of OPN and OPG, whereas 1,25(OH)$_2$D$_3$ did not alter the expression levels of either protein (Figure 4B). The combination of IFN-γ and 1,25(OH)$_2$D$_3$ more potently decreased the levels of OPN and OPG. In the cultures of HVSMCs with THP-1 cells, both of the reagents inhibited the expression of OPN and OPG, although the inhibitory effect of IFN-γ was more potent than that of 1,25(OH)$_2$D$_3$ (Figure 4B). Furthermore, the combination of two reagents almost completely depressed the expression of OPN and OPG.

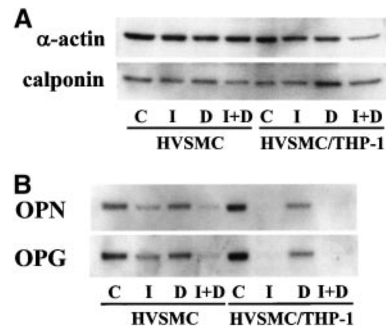

Figure 4. Analysis of phenotypic changes of HVSMCs. HVSMCs were cocultured for 7 days with THP-1 cells in the presence of IFN-γ and 1,25(OH)$_2$D$_3$. C indicates control; I, IFN-γ (100 ng/mL); D, 1,25(OH)$_2$D$_3$ (10^{-7} mol/L); I+D, IFN-γ (100 ng/mL) and 1,25(OH)$_2$D$_3$ (10^{-7} mol/L); HVSMC, HVSMC cultures; and HVSMC/THP-1, transwell cocultures of HVSMCs with THP-1. A, Immunoblot analysis of α-smooth muscle actin (α-actin) and calponin in HVSMCs cultured for 7 days under the indicated conditions. B, Immunoblot analysis of osteopontin (OPN) and osteoprotegerin (OPG) in HVSMCs cultured for 7 days under the indicated conditions.

Tumor Necrosis Factor-α (TNF-α) and Oncostatin M (OSM) Derived From THP-1 Cells Induce ALP in HVSMCs

To clarify ALP-inducing factors produced by THP-1 cells, the effects of blocking antibodies against various cytokines and adaptor molecules for their signaling pathways in this coculture system were investigated. A series of experiments revealed that antibodies against TNF-α, glycoprotein (gp) 130, leukemia inhibitory factor (LIF), and OSM partially inhibited ALP induction in the coculture (Table). Therefore, we examined whether TNF-α, LIF, and OSM can induce ALP activities in the cultures of HVSMCs without THP-1 cells in the presence of IFN-γ and 1,25(OH)$_2$D$_3$. When used alone, neither LIF nor OSM exerted ALP-inducing effects on HVSMCs (data not shown). Although TNF-α showed ≈20-fold increases of ALP activities in the cultures of HVSMCs without THP-1 cells, the levels of ALP activities induced by

Effects of Antibodies Against TNF-α, gp130, LIF, and OSM on ALP Activities in the Cocultures

Antibodies Used (10 μg/mL)	Experimental Group	ALP, U/mg Protein
TNF-α	Control IgG	286.7±16.0
	Anti–TNF-α	196.2±5.7*
gp130	Control IgG	97.0±7.3
	Anti-gp130	48.8±2.8*
LIF and OSM	Control IgG	166.1±16.9
	Anti-LIF	99.0±3.4*
	Anti-OSM	116.1±6.9*

HVSMCs were cocultured for 4 days with THP-1 cells in the presence of the indicated monoclonal antibody or control IgG, IFN-γ (100 ng/mL), and 1,25(OH)$_2$D$_3$ (10^{-7} mol/L). ALP activities were determined as described in Materials and Methods. Data are presented as mean±SEM (n=3).

*$P<0.05$ vs each control IgG.

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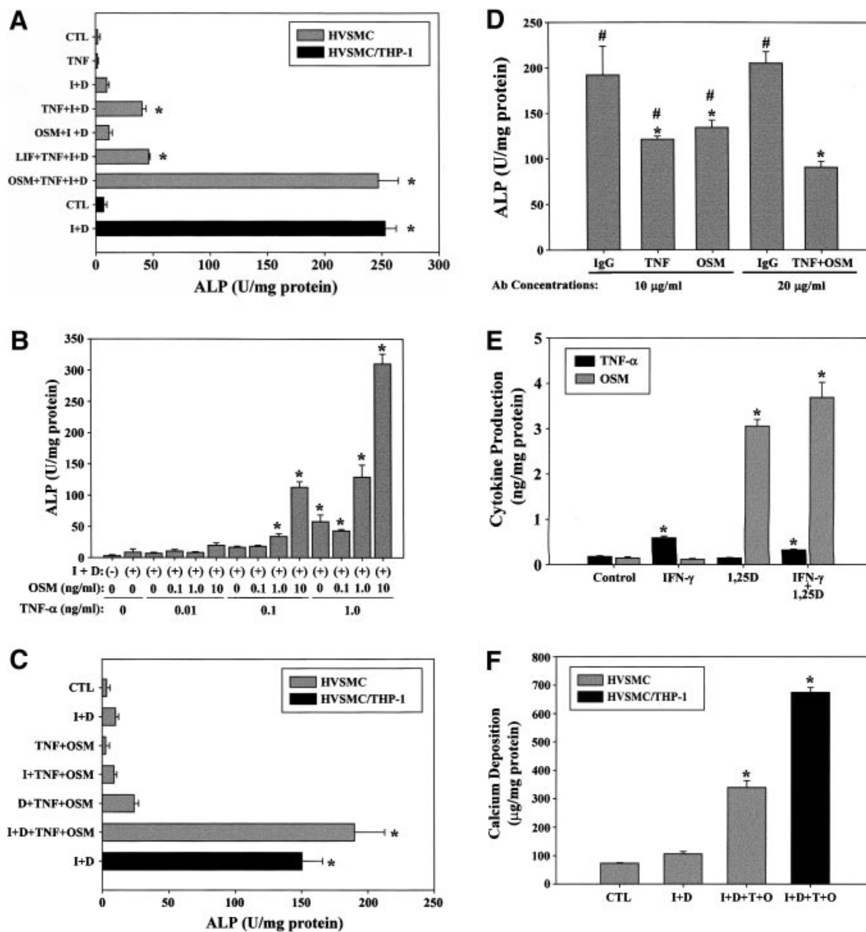

Figure 5. Involvement of TNF-α and OSM in induction of ALP and calcification in HVSMCs. The data are presented as mean±SEM (n=3). A, Effects of combinations of OSM or LIF with TNF-α on ALP activities in HVSMCs. CTL indicates control; I+D, IFN-γ (100 ng/mL) and 1,25(OH)$_2$D$_3$ (10^{-7} mol/L); TNF, TNF-α 1.0 ng/mL; LIF, LIF 10 ng/mL; OSM, OSM 10 ng/mL; HVSMC, HVSMC cultures; and HVSMC/THP-1, transwell cocultures of HVSMC with THP-1 cells. *$P<0.05$ vs CTL. B, Dose-dependent effects of TNF-α and OSM on ALP activities in HVSMCs in the presence of IFN-γ and 1,25(OH)$_2$D$_3$. *$P<0.05$ vs HVSMC culture without any treatment. C, Effects of IFN-γ and 1,25(OH)$_2$D$_3$ on ALP activities in HVSMCs in the presence of TNF-α and OSM. *$P<0.05$ vs CTL. D, Effects of antibodies against TNF-α and OSM on ALP activities in the transwell cocultures. *$P<0.05$ vs each IgG; #$P<0.05$ vs TNF+OSM. E, TNF-α and OSM production by THP-1 cells. THP-1 cells were cultured as described in the presence of the indicated reagents. After 4 days, the culture supernatants were collected and used for immunoassays for TNF-α and OSM. CTL indicates control; I+D, IFN-γ (100 ng/mL) and 1,25(OH)$_2$D$_3$ (10^{-7} mol/L). *$P<0.05$ vs each control. F, Calcium quantification by the *o*-cresolphthalein complexone method. HVSMCs were cultured for 7 days in the presence of β-GP under the indicated conditions. CTL indicates control; I+D, IFN-γ (100 ng/mL) and 1,25(OH)$_2$D$_3$ (10^{-7} mol/L); I+D+T+O, IFN-γ (100 ng/mL), 1,25(OH)$_2$D$_3$ (10^{-7} mol/L), TNF-α (1.0 ng/mL), and OSM (10 ng/mL). HVSMC indicates HVSCM culture; HVSMC/THP-1, transwell cocultures of HVSMC with THP-1 cells. *$P<0.05$ vs CTL.

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TNF-α were not comparable to those of the cocultures (Figure 5A). Surprisingly, OSM combined with TNF-α dramatically increased ALP activities in HVSMCs in the presence of IFN-γ and 1,25(OH)$_2$D$_3$ comparable to those induced in the coculture (Figure 5A). On the other hand, the combination of LIF and TNF-α did not show any additional effect compared with TNF-α alone (Figure 5A). The synergistic effects of TNF-α and OSM on ALP activities in HVSMCs were dose-dependent (Figure 5B) and observed only in the presence of IFN-γ and 1,25(OH)$_2$D$_3$ (Figure 5C). Moreover, antibodies against TNF-α and OSM, when used together at 10 μg/mL, significantly inhibited ALP activities in the transwell cocultures compared with each antibody used alone (Figure 5D).

We also measured the levels of these cytokines in the supernatants of THP-1 cultures treated with IFN-γ and/or 1,25(OH)$_2$D$_3$ (Figure 5E). TNF-α production was stimulated by IFN-γ, but not by 1,25(OH)$_2$D$_3$. When used together, 1,25(OH)$_2$D$_3$ attenuated the stimulatory effect of IFN-γ. On the other hand, OSM production was stimulated by 1,25(OH)$_2$D$_3$, but not by IFN-γ. Furthermore, IFN-γ strengthened the stimulatory effect of 1,25(OH)$_2$D$_3$. These data confirmed that TNF-α and OSM were produced by THP-1 cells in the presence of IFN-γ and 1,25(OH)$_2$D$_3$. Moreover, the levels of TNF-α and OSM produced by THP-1 cells were comparable to the concentrations of these cytokines used for the experiments.

Finally, we examined whether HVSMCs treated with IFN-γ, 1,25(OH)$_2$D$_3$, TNF-α, and OSM can calcify their extracellular matrix in the presence of 10 mmol/L β-GP. These four factors induced calcification in a time-dependent manner (online Figure 3a), and calcium deposition was cytochemically confirmed by von Kossa staining (online Figure 3B). On the other hand, HVSMCs cultured in the absence of TNF-α and OSM did not exhibit calcium deposition even in the presence of β-GP (Figure 5F). Additionally, calcification induced by four factors was further increased when HVSMCs were cocultured with THP-1 cells (Figure 5F).

Discussion

In this study, we examined the roles of macrophages in inducing calcifying phenotype of VSMCs using the coculture of HVSMCs with THP-1 cells. As shown in this study, ALP, which may play an important role in bone mineralization, was strongly induced in HVSMCs by the coculture and the enzyme induced in HVSMCs was bone-type isoenzyme. Furthermore, in vitro calcification was brought about in HVSMCs with high expression of ALP in the presence of β-GP. The expression of ALP has been demonstrated in various vascular-calcified lesions such as atherosclerosis, Mönckeberg-type medial calcification, and cardiac valvular disease.[20,21,30] In vitro studies revealed that vascular-

calcifying cells such as VSMCs, pericyte-like cells, and microvascular pericytes express high levels of ALP[11,14,31] and that the calcifying capacity of these cells is dependent on their activities of the enzyme.[11] Therefore, these findings suggest that induction of ALP in vascular cells by inflammatory cells may contribute to the development of calcified lesions associated with vascular inflammatory diseases.

Recently, Tintut et al[32] have reported the roles of monocytes/macrophages in vascular calcification in vitro by using the cocultures of bovine-calcifying vascular cells with human peripheral blood monocytes. They demonstrated that monocytes/macrophages enhance in vitro calcification through two independent mechanisms: cell-to-cell interaction and production of soluble factors. In this study, we examined the roles of macrophages in vascular calcification by using two coculture systems: the mixed and transwell cocultures. We confirmed that soluble factors produced by monocyte/macrophage-lineage cells are involved in the acquisition of calcifying phenotype of HVSMCs, whereas our coculture models did not clearly detect the roles of cell-to-cell interaction in induction of ALP expression in HVSMCs. This discrepancy may be due to the difference of the coculture systems used. Further studies are necessary to clarify the roles of cell-to-cell interaction in vascular calcification.

By using blocking antibodies against various cytokines and adaptor molecules for their signaling pathways, we have identified that TNF-α and OSM are the possible causative factors to induce ALP activities in HVSMCs. In contrast to OSM, LIF did not exert any direct effect on HVSMCs, although anti–LIF antibody significantly inhibited ALP induction in the coculture. These two cytokines are members of IL-6–type cytokines that share a common receptor subunit involved in signal transduction, gp130. OSM exerts its biological effects through two types of receptor complex, gp130-LIF receptor heterodimers and gp130-OSM receptor.[33] A previous report suggested that LIF receptor is absent on HVSMCs and that OSM acts specifically via the gp130-OSM receptor complex.[34] Therefore, LIF may exert its biological action mainly on THP-1 cells, but not on HVSMCs in the coculture. Moreover, antibodies against TNF-α and OSM, when used together, did not completely inhibit ALP activities in the coculture, supporting that LIF may also be involved in ALP induction in the coculture. Furthermore, TNF-α and OSM induced ALP activities in the cultures of HVSMCs without THP-1 cells only in the presence of IFN-γ and $1,25(OH)_2D_3$, suggesting that IFN-γ and $1,25(OH)_2D_3$ may directly affect HVSMCs in concert with TNF-α and OSM. However, the mechanism of action by these four factors on HVSMCs remains to be determined.

TNF-α and OSM have been shown to be involved in vascular pathophysiology. TNF-α is mainly secreted by macrophages in response to various atherogenic factors such as oxidized LDL and influences many aspects of atherogenesis.[24,35] This cytokine has been shown to promote in vitro calcification of bovine vascular cells associated with their osteoblastic differentiation.[36] OSM is produced by activated macrophages and T lymphocytes and exhibits various effects on endothelial cells and VSMCs, including promoting the expression of tissue factor and adhesion molecules and stimulating angiogenesis in vitro and in vivo.[37–39] OSM has been shown to be present in macrophages infiltrating human aortic aneurysms and to be colocalized with TNF-α.[39] In this study, we have demonstrated a unique synergistic action of TNF-α and OSM on inducing ALP in HVSMCs. Therefore, it is possible that synergistic actions of these two cytokines may play an important role in inflammatory vascular diseases including vascular calcification.

In this study, we have shown for the first time that OSM production by macrophages was mainly stimulated by $1,25(OH)_2D_3$. Although OSM was originally isolated from the supernatants of U937 histiocytic leukemia cells differentiated into macrophage-like cells by treatment with phorbol myristate acetate,[40] regulation of OSM production by macrophages is largely unknown.[33] This vitamin D–dependent production of OSM by THP-1 cells may explain the requirement of $1,25(OH)_2D_3$ for the remarkable induction of ALP activities in the coculture. Moreover, the administration of vitamin D associated with a high-cholesterol diet has produced advanced atherosclerotic lesions including calcification in experimental animals.[41,42] This in vivo effect of vitamin D on progression of atherosclerotic lesions, especially atherosclerotic calcification may be mediated partly through its stimulation of OSM production by macrophages.

We have demonstrated for the first time that ALP induced in HVSMCs may be the bone-specific enzyme derived from tissue nonspecific (liver-bone-kidney type) ALP gene. Because bone-specific ALP is one of the early markers of osteoblastic differentiation, it is likely that HVSMCs may be directed toward osteoblastic differentiation by the coculture. However, expression of the phenotypic markers of differentiated VSMCs such as α-smooth muscle actin and calponin was still preserved even by the coculture. Furthermore, OPN, which is another early marker of osteoblastic differentiation and may function as an inhibitor of vascular calcification, was downregulated in the same coculture. Although recent studies suggest that osteoblastic differentiation of vascular cells such as VSMCs and pericyte-like cells may play an important role in the development of vascular calcification, it has also been reported that VSMCs in vivo and in vitro can coexpress osteoblastic and VSMC-specific genes.[43] Calcifying HVSMCs express α-smooth muscle actin, calponin, and SM22α at high levels. These cells also express high levels of MGP mRNA and very low levels of OPN mRNA. Therefore, it is possible that calcifying HVSMCs may have a unique phenotype distinct from that of osteoblasts. Moreover, OPG in HVSMCs was also downregulated by the coculture. Because OPG, a soluble decoy receptor of the TNF superfamily, may function as a local inhibitor of vascular calcification,[15] it is likely that downregulation of OPG as well as OPN in HVSMCs may serve as a prerequisite for their calcifying capacity.

We used human neonatal smooth muscle cells (SMCs) derived from umbilical artery in the present study. The neonatal SMCs cultured alone expressed low levels of ALP and did not calcify spontaneously, even in the presence of β-GP. On the other hand, bovine vascular cells including mature VSMCs express high levels of ALP and can calcify extracellular matrix spontaneously or in the presence of

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β-GP.[11,13,44] However, the neonatal SMCs acquired calcifying capacities based on high levels of ALP expression when cocultured with THP-1 cells (or incubated with TNF-α and OSM) in the presence of IFN-γ and 1,25$(OH)_2D_3$. Because ALP can degrade β-GP, releasing inorganic phosphate, ALP-dependent calcification in the presence of β-GP may be mediated through increased concentrations of phosphorus in culture media.[11,44] Recently, it has been reported that inorganic phosphate itself induces in vitro calcification of human fetal and mature SMCs.[45] Therefore, the findings of this study with neonatal SMCs expressing high levels of ALP are comparable to those with mature SMCs. Moreover, it has been well recognized that neointimal and neonatal SMCs share similarities in morphology and expression of genes for matrix proteins and some differentiation factors.[46,47] Because neointima is formed in various vascular inflammatory diseases, the coculture presented in this study may be a useful tool to analyze the mechanism of atherosclerotic calcification.

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