Interaction of Monocytes With Vascular Smooth Muscle Cells Regulates Monocyte Survival and Differentiation Through Distinct Pathways

Qiangjun Cai, Linda Lanting, Rama Natarajan

**Objective**—Vascular smooth muscle cells (VSMCs) may regulate monocyte functions within atherosclerotic lesions. We investigated the impact of VSMC/monocyte interactions on monocyte apoptosis and scavenger receptor CD36 expression, key events related to monocyte survival and differentiation.

**Methods and Results**—Serum deprivation significantly increased THP-1 and human peripheral blood monocyte apoptosis. However, this was significantly reversed by physical binding to human VSMCs (HVSMCs). On binding to HVSMCs, antiapoptotic kinase Akt and its downstream targets were phosphorylated, and Bcl-2 expression was enhanced. Binding-mediated suppression of apoptosis and Akt phosphorylation were attenuated by a phosphoinositide 3-kinase inhibitor and also by an antibody to vascular cell adhesion molecule-1. CD36 expression was also significantly increased in THP-1 cells and in human peripheral blood monocytes after binding to HVSMCs, and this was mediated by both direct contact and soluble factors. Extracellular signal-regulated kinase 1/2 (ERK1/2) mitogen-activated protein kinase phosphorylation was increased in THP-1 cells after HVSMC coculture. Furthermore, an ERK1/2 inhibitor blocked monocyte CD36 upregulation. Contact-dependent CD36 induction and ERK1/2 phosphorylation in monocytes were inhibited by blocking vascular cell adhesion molecule-1 on HVSMC, whereas soluble factor–induced CD36 expression was attenuated by a monocyte chemoattractant protein-1 neutralizing antibody.

**Conclusions**—These data provide evidence of novel VSMC-dependent local regulation mechanisms for monocyte survival and differentiation in atherosclerosis. (*Arterioscler Thromb Vasc Biol. 2004;24:1-8*)

**Key Words:** atherosclerosis • monocytes • vascular smooth muscle cells • apoptosis • CD36

In response to atherogenic stimuli, monocytes in circulation adhere and migrate across the endothelium to the intimal space where they differentiate, take up lipid, and form foam cells. The role of vascular endothelial cells in the recruitment of monocytes during early steps of atherosclerosis has been well studied. However, these initial processes may be reversible and do not cause clinical consequences. It is the subsequent prolonged intimal retention of monocyte/macrophage and foam cell formation that are central features of atherogenesis. However, the mechanisms by which monocytes/macrophages are retained within the vessel wall, survive, and differentiate to foam cells are not well documented. Once they transmigrate into the subendothelial intimal space, these monocyte functions are regulated mainly by the influence of local factors that are not well characterized. Furthermore, very little is known about the role of intimal vascular smooth muscle cells (VSMCs) in regulating subendothelial monocyte functions. VSMC migration and proliferation are also well-documented hallmarks of early atherosclerotic lesions. Accumulating evidence suggests that interactions between transmigrated monocytes and VSMCs may contribute to monocyte retention and function within the vasculature. First, the potential of VSMCs to interact with monocytes is suggested by the fact that VSMCs express adhesion molecules within atherosclerotic lesions but not in the normal vascular wall. A highly significant association was found between VSMC vascular cell adhesion molecule (VCAM)-1 expression and intimal macrophage content. A strong expression of intercellular adhesion molecule (ICAM)-1 on VSMCs in atherosclerosis-prone regions was observed preceding mononuclear cell infiltration in humans. VSMCs derived from the neointima of balloon-injured rat aortas supported a greater arrest of monocytes than medial VSMCs in laminar flow assays. Furthermore, in vitro studies indicate that contact interactions between monocytes and VSMCs enhance monocyte procoagulant activity and production of metalloproteinase-1 and nitric oxide. These observations suggest that VSMCs and monocytes are not merely innocent coexistent neighbors but that VSMC/monocyte interactions are additional regulatory signals in the pathogenesis of atherosclerosis.
It is generally accepted that macrophage apoptosis within advanced atherosclerotic plaques can contribute to plaque vulnerability and thrombogenicity.\textsuperscript{11-13} In comparison, very little monocyte apoptosis is found in early atherosclerotic lesions, such as adaptive intimal thickening and fatty streaks, and this may be responsible for monocyte accumulation subsequent to transmigration into the subendothelial space.\textsuperscript{14}

The formation of lipid-laden foam cells is a hallmark of both early and late atherosclerotic lesions. This is mediated by scavenger receptors (SR) such as SR-A, CD68, and CD36 that mediate uptake of modified low-density lipoprotein (LDL).\textsuperscript{15} CD36, a member of type B SRs, plays a critical role in the pathogenesis of atherosclerosis.\textsuperscript{16-19} Markedly upregulated CD36 expression in human and murine atherosclerotic lesions has been demonstrated.\textsuperscript{20} Furthermore, apolipoprotein E–deficient mice lacking CD36 developed significantly less atherosclerosis than the control mice.\textsuperscript{21}

In this study, we examined for the first time the effects of monocyte adhesion to VSMCs on monocyte survival. Furthermore, we tested the hypothesis that this survival program is also associated with initiation of a monocyte differentiation program as exemplified by the expression of differentiation markers such as CD36 and oxidized LDL (ox-LDL) uptake. We observed that VSMC/monocyte interactions lead to anti-apoptotic effects and increased CD36 expression in monocytes. We also examined the role of adhesion molecules and soluble factors as well as key signal transduction events mediating these effects.

Materials and Methods

For details, see online Methods, available at http://atvb.ahajournals.org.

Coculture Studies

Cocultures of human VSMCs (HVSMCs) with THP-1 cells or peripheral blood monocytes (PBMCs) were performed in 3 different ways. In the first method, termed “mixed coculture,” monocytes were added to confluent HVSMC monolayers in culture dishes for indicated time periods. In this model, monocytes bound to HVSMCs are subjected to the influence of both physical contact and soluble factors, whereas cells remaining in suspension are only influenced by soluble factors. In the second method, termed “fixed coculture,” monocytes were added to confluent HVSMCs that were fixed with 3.7% paraformaldehyde (1 hour) to prevent soluble factor release. After fixation, paraformaldehyde was removed from HVSMCs by washing with PBS \textsuperscript{8}. In this second model, bound monocytes are only affected by direct contact with HVSMCs. The third method is termed as “transwell coculture.” In this model, HVSMCs were cultured in the bottom well of 6-well plates. THP-1 cells were then added to the bottom well and transwell insert simultaneously. THP-1 cells growing in the transwell insert are subjected to the influence of soluble factors released from the HVSMC/THP-1 cell cocultures in the bottom wells.

Results

Binding to HVSMCs Reverses Serum Deprivation–Induced Monocyte Apoptosis Through VCAM-1

THP-1 cells growing in serum-free medium alone (0% FBS) for 48 hours showed clear evidence of apoptosis (65.2\textpm 2.8%, \textit{P}<0.001) compared with cells in 10% FBS (22.0\textpm 0.6%) as noted by Annexin V/propidium iodide double staining (Figure 1A) and DNA fragmentation (Figure 1, available online at http://atvb.ahajournals.org). Because most apoptotic THP-1
cells were both Annexin V and propidium iodide positive (late apoptotic), only cells in late apoptosis were analyzed further. In contrast, in the mixed cocultures, THP-1 cells that bound to HVSMCs (0% FBS bound) showed only low levels of apoptosis (33.2±1.3%, P<0.001 versus cells in 0% FBS alone, Figure 1A) even though they were in serum-free medium, and this was similar to cells in 10% FBS. Interestingly, unbound THP-1 cells still in suspension in the cocultures (0% FBS unbound) showed no protection from apoptosis (59.0±4.4%, Figure 1A), indicating that only physical contact with HVSMCs, but not soluble factors, was responsible for suppressing THP-1 apoptosis. Similarly, serum depletion for 48 hours also induced early apoptosis in human PBMCs, which was prevented by binding to HVSMCs (Figure 1B).

VSMC/monocyte binding can be mediated by interactions between adhesion molecules on VSMCs (VCAM-1 and ICAM-1) and their integrin (β1 and β2) counterreceptors, respectively, on monocytes. We found that binding-mediated protection of THP-1 cells from apoptosis was partially antagonized by preincubating HVSMCs with a VCAM-1 blocking antibody (Ab) but not control nonspecific IgG (Figure 1C). In contrast, ICAM-1 Ab had no effect (not shown). These results suggest that VCAM-1–β1 integrin signaling is involved in binding-mediated protection of monocytes from apoptosis.

Binding to HVSMCs Increases Activation of the Akt Pathway and Bcl-2 Expression in THP-1 Cells

We further explored the mechanisms and apoptosis-regulating factor(s) that could be activated in THP-1 cells on binding to HVSMCs. As illustrated in Figure 2A, Akt, a major antiapoptotic kinase that is downstream of phosphoinositide 3-kinase (PI3K), was time-dependently phosphorylated after binding to HVSMCs in the fixed cocultures. Furthermore, Bad, FKHR (forkhead homologue in rhabdomyosarcoma), GSK-3β (glycogen synthase kinase 3β), and IKKα (I-κB kinase-α), all downstream targets of Akt, were also phosphorylated, indicating the activation of an antiapoptotic PI3K/Akt axis in THP-1 cells. Interestingly, Akt was phosphorylated in bound but not unbound THP-1 cells in the mixed cocultures (Figure 2B), indicating that soluble factors were not involved. This agrees with our data in Figure 1A showing that unbound cells are not protected from apoptosis.

When THP-1 cells were allowed to bind to paraformaldehyde-fixed HVSMCs (to avoid release of soluble factors), Akt phosphorylation was evident in the bound cells, and this effect was attenuated by blocking HVSMC VCAM-1 (Figure 2C). This further rules out soluble factors, although supporting a role for insoluble factors, such as VCAM-1 and associated β1 integrin signaling in monocyte Akt phosphorylation.

We also observed a time-dependent increase in protein (Figure 2A) and mRNA (not shown) levels of Bcl-2, a key antiapoptotic protein, in THP-1 cells on binding to HVSMCs. In contrast, the levels of Bax, a proapoptotic protein that antagonizes Bcl-2 function, was unchanged, thereby leading to increased Bcl-2/Bax ratios.

![Figure 2. Activation of antiapoptotic signals in THP-1 cells on binding to HVSMCs.](http://atvb.ahajournals.org/)

**Figure 2.** Activation of antiapoptotic signals in THP-1 cells on binding with HVSMCs. A, Phosphorylation of PI3K/Akt pathway members and upregulation of Bcl-2 in bound THP-1 cells in the fixed cocultures. B, Akt was phosphorylated in bound but not in unbound THP-1 cells in the mixed cocultures. C, Preincubation of HVSMCs with a VCAM-1 blocking Ab (10 μg/mL) abrogated binding-mediated Akt phosphorylation (5 minutes) in bound THP-1 cells in the fixed cocultures. D, Binding-mediated protection of THP-1 cells from apoptosis was dose-dependently blocked by a PI3K inhibitor LY294002 (40 μmol/L, 48 hours; by flow cytometry). Representative data of 3 similar experiments are shown.

**Binding-Mediated Apoptosis Protection Is Through PI3K Signaling**

Specific kinase inhibitors were used to elucidate the signaling pathway(s) involved. As shown in Figure 2D, binding-mediated suppression of apoptosis in THP-1 cells was almost completely abolished (dose-dependently) by LY294002, a specific PI3K inhibitor. Binding-mediated apoptosis protection in PBMCs was also blocked by LY294002 (Figure 1B, bottom right). LY294002 also blocked the binding-induced
prevention of DNA fragmentation (not shown). In contrast, although p38 (not shown) and extracellular signal-regulated kinase 1/2 (ERK 1/2) mitogen activated protein kinases (MAPKs, seen in Figure 3) were both activated during binding to HVSMCs, a specific p38MAPK inhibitor, SB202190, and an ERK1/2 pathway inhibitor, PD98059, had no effects on apoptosis regulation (not shown). Binding-induced Akt phosphorylation as well as Bcl-2 mRNA upregulation were both inhibited by pretreatment of THP-1 cells with LY294002 (Figure II, available online at http://atvb.ahajournals.org), indicating that PI3K is the upstream activator of Akt and Bcl-2 in our model.

Induction of Monocyte CD36 Expression on Interaction With HVSMC

As illustrated in Figure 4A, in the basal state, 9.6±1.1% THP-1 cells were CD36 positive. However, after 24 hours in the mixed cocultures, 41.9±4.9% THP-1 cells bound to HVSMCs were positive for CD36 (P<0.01). Interestingly, unbound THP-1 cells also showed a significant increase in surface CD36 expression (31.7±3.5%, P<0.05). Furthermore, increased CD36 expression was associated with enhanced 1,1′-dioctadecyl-3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI)-labeled ox-LDL uptake as evidenced by flow cytometry (Figure 4B). We next examined whether similar CD36 upregulation also occurs in PBMCs obtained from normal healthy adults. As shown in Figure 4C, after coculture with HVSMCs in the mixed cocultures, human PBMCs also showed significant increase in CD36 expression.
in bound (69.5 ± 2.4%, P < 0.001) and unbound (58.5 ± 2.5%, P < 0.001) PBMCs compared with basal levels (19.2 ± 1.2%).

Both direct cell–cell contact and soluble factors released from the cocultures appeared to be responsible for CD36 induction, because THP-1 cells in a transwell coculture (24 hours) with HVSMCs also showed increased CD36 expression (Figure 4D). Furthermore, THP-1 cells bound to paraformaldehyde-fixed HVSMCs also showed increased CD36 surface expression as assessed by flow cytometry (Figure 5). Meanwhile, CD36 mRNA levels were also increased both in unbound THP-1 cells in the mixed cocultures (P < 0.05) or bound THP-1 cells in the fixed cocultures (P < 0.05) (Figure III, available online at http://atvb.ahajournals.org).

**ERK1/2 MAPK Signaling Regulates Coculture-Mediated CD36 Upregulation**

We further explored the signal transduction pathway(s) involved in THP-1 cell CD36 expression. We detected a time-dependent phosphorylation of ERK1/2 MAPK in both bound and unbound THP-1 cells in the mixed cocultures (Figure 3A, top) when compared with THP-1 cells cultured alone. Phosphorylation of ERK1/2 was also demonstrated in bound THP-1 cells in the cocultures with fixed HVSMCs as well as in THP-1 cells stimulated with HVSMC-conditioned medium (Figure 3A, middle), indicating that both direct binding as well as soluble factors released from the cocultures were responsible for monocyte ERK1/2 activation. Furthermore, THP-1 cell ERK1/2 was also phosphorylated in a transwell coculture with HVSMCs (Figure 3A, transwell), confirming the role of soluble factors.

We next examined whether ERK1/2 MAPK mediates coculture-mediated THP-1 cell CD36 expression. Figure 3B shows that induced CD36 expression (by flow cytometry) in bound and unbound THP-1 cells in the mixed cocultures were both dose-dependently blocked by the ERK1/2 inhibitor PD98059. However, although p38MAPK was activated during binding, the p38 inhibitor SB202190 had no effect (not shown). Induced surface CD36 expression on human PBMCs in the mixed cocultures and THP-1 cells in the transwell cocultures were also blocked by PD98059 (not shown). As shown in Figure 3C, increased CD36 mRNA levels in bound THP-1 cells in the fixed cocultures or in unbound THP-1 cells in the mixed cocultures were both blocked by preincubating THP-1 cells with PD98059. These results confirm that ERK1/2 MAPK can mediate coculture-induced monocyte CD36 expression.

**Role of VCAM-1 and Monocyte Chemoattractant Protein-1 in THP-1 Cell CD36 Upregulation**

Neutralizing antibodies were used to determine the roles of adhesion molecules and soluble factors involved in coculture-induced THP-1 cell CD36 expression. Upregulation of CD36 in bound THP-1 cells in the fixed cocultures was significantly blocked by preincubation of HVSMCs with an Ab to VCAM-1 (Figure 5A and 5B), but not to ICAM-1 (not shown). Furthermore, increases in both ERK1/2 phosphorylation and CD36 mRNA expression under these conditions were also inhibited by VCAM-1 Ab pretreatment of HVSMCs (Figure 5C and 5D).

Recent reports show that monocyte chemoattractant protein-1 (MCP-1) induces CD36 expression and foam cell differentiation in monocytes. Figure 6A shows a marked increase in MCP-1 mRNA levels in THP-1 cells (bound and unbound) as well as in HVSMCs on coculture. Furthermore, soluble factor–mediated upregulation of CD36 in unbound THP-1 cells in the mixed cocultures was significantly attenuated by a MCP-1 neutralizing Ab (Figure 6B and 6C). Thus CD36 expression in bound and unbound THP-1 cells may be mediated, at least in part, by MCP-1.

**Discussion**

Compared with circulating blood, the intimal space is less conducive for monocyte survival. Furthermore, increased
subendothelial levels of detrimental factors, such as reactive oxygen species and ox-LDL, are proapoptotic for monocytes/macrophages. However, pathological studies have found little if any monocyte apoptosis in early atherosclerosis, indicating that local subendothelial events may promote monocyte survival. Our results show for the first time that physical contact of monocytes with intimal VSMCs may represent a key mechanism to prevent migrated monocytes from apoptosis and thereby favor abnormal accumulation. This is of particular relevance for the development of early atherosclerotic lesions where abundant VSMCs are migrating from the media and proliferating in the intimal space.

We noted that serum starvation–induced monocyte apoptosis was markedly abrogated when they physically bind to VSMCs in cocultures. Furthermore, binding-mediated monocyte apoptosis protection was partially blocked by preincubating HVSMCs with a VCAM-1 Ab, suggesting that adhesive interactions between HVSMC VCAM-1 and monocyte β1 integrins were involved. To our knowledge, this is the first report of integrin signaling–mediated regulation of monocyte apoptosis, although this phenomenon has been observed in epithelial cells. The signal transduction pathway(s) involved in β1 integrin-mediated suppression of apoptosis are not fully resolved. PI3K/Akt, ERK1/2, and p38 MAPK have all been implicated and could therefore be cell-specific and contextual. In our study, although PI3K/Akt, ERK1/2, and p38 MAPK were all activated in THP-1 cells after binding to HVSMCs, only the PI3K/Akt pathway seemed to be responsible for HVSMC binding–mediated prevention of monocyte apoptosis. The PI3K/Akt pathway is of central importance in human monocyte survival. Once activated, PI3K generates inositol lipids and phosphorylates Akt, a critical regulator of PI3K-mediated cell survival. Akt stimulates the phosphorylation of the proapoptotic protein Bad, which subsequently releases and facilitates the functions of antiapoptotic mediators such as Bcl-XL or Bcl-2. Other downstream targets of Akt related to apoptosis include forkhead transcription factors of the FKHR family, IKKα, and GSK-3β, which subsequently releases and facilitates the functions of antiapoptotic mediators such as Bcl-XL or Bcl-2. Akt phosphorylation was observed only in bound but not unbound THP-1 cells in the coculture. Meanwhile, Bcl-2 expression and Bcl-2 to Bax ratios were clearly enhanced. In addition, binding-induced suppression of monocyte apoptosis, Akt phosphorylation, and Bcl-2 expression were all blocked by a specific PI3K inhibitor. These new results demonstrate that the process of binding induces a cascade of antiapoptotic signals in monocytes. Interestingly, Akt phosphorylation was blocked by treating HVSMCs with a VCAM-1 Ab, further supporting the role of VCAM-1 and associated β1 integrin signaling. The specific involvement of β1 integrin counter-receptors of VCAM-1 in this process needs to be confirmed by additional studies.

It is interesting that Bcl-2, a major antiapoptotic protein, was upregulated on binding to HVSMCs. Although the nature of adhesion molecule interactions responsible for this is not clear, VCAM-1/β1 interaction might be involved because Bcl-2 mRNA upregulation was attenuated by inhibition of PI3K/Akt, whereas Akt activation was blocked by a VCAM-1 Ab. Furthermore, Matter and Ruoslahti have found that β1 integrin–mediated Bcl-2 transcription was inhibited by LY294002 in Chinese hamster ovary cells, indicating that PI3K is a component of the β1–Bcl-2 pathway.

Because monocyte survival was enhanced on coculture with HVSMCs, we next hypothesized that they may undergo differentiation to a more atherogenic phenotype expressing SRs such as CD36. A major novel finding in our current study is that monocyte ox-LDL uptake as well as CD36 expression at both mRNA and surface protein levels were upregulated on coculture with HVSMCs. CD36 induction was mediated by both direct physical contact and soluble factors as well as by
ERK1/2 MAPK activation. We also found that contact-dependent CD36 upregulation was mediated at least in part by VCAM-1 and likely by β1 integrin interactions. Interestingly, a very recent report showed that monocyte CD36 expression and differentiation were downregulated when they bind to endothelial cells.37 Therefore, binding-mediated monocyte CD36 regulation during cell–cell interactions seems to be cell-type specific, and the subendothelial space could be more favorable than blood vessel lumen for monocyte differentiation.

In our current study, we demonstrated that MCP-1 is a candidate soluble factor mediating CD36 expression. This is consistent with recent reports that exogenous MCP-1 dose-dependently upregulates CD36 expression and monocyte differentiation in an ERK-dependent manner.23 Our data suggest that MCP-1 in the cocultures could come from both HVSMCs and monocytes, because MCP-1 mRNA levels were elevated in both cell types. In support of our data, increased MCP-1 release has also been demonstrated during monocyte transendothelial migration in vitro.38 Upregulation of MCP-1 in THP-1 cells might be caused by both direct contact and soluble factors as demonstrated by our various culture conditions. On the other hand, although the mechanism for HVSMC MCP-1 upregulation is not clear in this study, soluble factors, such as tumor necrosis factor-α, produced during coculture may be involved.38 Contact-dependent MCP-1 upregulation in HVSMCs may also be possible, because MCP-1 induction was observed in mesangial cells during interaction with extracellular matrix in β1 integrin-dependent manner.39 Because MCP-1 is a potent monocyte chemoattractant, our results suggest that MCP-1 generated in the subendothelial space during monocyte/VSMC interactions may be a key factor mediating sustained monocyte infiltration in a vicious loop. However, MCP-1 may not be the only soluble factor involved, and additional studies are needed to identify the nature of other potential mediators.

In summary, we have used coculture systems mimicking the intercellular events in the subendothelial space to investigate the functional alterations of monocytes after exposure to VSMCs. We found for the first time that tethered binding of monocytes to VSMCs initiates an orchestrated program of monocyte survival and differentiation into an atherogenic phenotype. Our data suggest that monocytes trapped in the subendothelial space may bind to VSMCs and undergo survival and differentiation through key signaling pathways and soluble and insoluble factors from VSMCs and monocytes. Overall, we have demonstrated novel new local VSMC-dependent mechanisms for monocyte dysfunction in the pathogenesis of atherosclerosis.

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References


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MATERIALS AND METHODS

Reagents and Chemicals

Inhibitors of phosphoinositide 3-kinase (PI3K, LY294002), p38 mitogen activated protein kinase (p38MAPK, SB202190), and MAPK kinase (MEK, PD98059) were from Calbiochem (San Diego, CA). Antibodies to human extracellular signal–regulated kinase (ERK), Akt, Bad, FKHR (also referred to as Forkhead Homologue in Rhabdomyosarcoma), I-κB kinases (IKKα), and glycogen synthase kinase 3β (GSK-3β) were from Cell Signaling (Beverly, MA). Human Bcl-2 and Bax antibodies were from Santa Cruz Biotechnology, Inc (Santa Cruz, CA). Human β actin antibody was from Sigma-aldrich (St. Louis, MO). Function blocking mouse anti-human ICAM-1 (clone P2A4) and VCAM-1 (clone P1B8) antibodies were from Chemicon International (Temecula, CA).

Cell Culture

Human aortic SMC (HVSMC; Clonetics, San Diego, CA) were grown in SMC basal medium (SmBM) supplemented with 10 ng/mL human epidermal growth factor, 2 ng/mL human fibroblast growth factor-2 and 5% fetal bovine serum (FBS). HVSMC were positive for both ICAM-1 and VCAM-1 as evidenced by flow cytometry. The human monocytic cell line, THP-1, was obtained from American Type Culture Collection and cultured in RPMI 1640 medium containing 10% heat-inactivated FBS and 50µmol/L β-mercaptoethanol. Human peripheral blood monocytes (PBMC) were obtained from the blood of normal healthy volunteers with informed consents according to approved City of Hope Institutional Review Board (IRB) guidelines and were separated by Ficoll as
described earlier. More than 85% of the PBMC fraction was CD14 positive as evaluated by flow cytometry.

**Preparation of Conditioned Medium (CM) from HVSMC/THP-1 cocultures**

To investigate the effect of soluble factors on THP-1 cells, CM from the cocultures were collected. Briefly, THP-1 cells were added to HVSMC monolayers from 5 minutes to 6 hours; Medium from different time points was collected and centrifuged to remove cells or cell debris. The supernatant containing the soluble factors released from the cocultures (CM) was used to treat THP-1 cells for indicated time periods from 5 minutes to 6 hours as needed.

**Flow Cytometry Analysis of CD36**

After 24 hours of coculture with HVSMC, unbound monocytes were collected by gentle aspiration, and bound monocytes were collected by gently shaking the plates and gentle pipetting. The latter process is gentle enough that the HVSMC monolayer remained undisturbed as noted under a microscope. Monocyte purity was confirmed with CD14 staining by flow cytometry. Monocyte surface CD36 expression was measured by flow cytometry with a R-phycoerythrin (R-PE)-conjugated antibody to human CD36 (BD Pharmingen, San Diego, CA) according to supplier’s protocols. Samples were analyzed on a MoFlo MLS (Dako-Cytomation, Fort Collins, CO) flow cytometer. The results were expressed as percent cells positive for CD36.

**Flow Cytometric Analysis of Cellular Uptake of Ox-LDL**

To examine cellular uptake of Ox-LDL, THP-1 cells were incubated with 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (DiI)-labeled Ox-LDL (5µg/mL) in DMEM/10% FBS for 4 hours and washed 3 times with PBS/5%FBS. Flow
Cytometry was performed to measure amounts of DiI-Ox-LDL internalized in cells on a MoFlo MLS flow cytometer with excitation and emission wavelengths set at 520 and 580 nm respectively.

**Western Blotting**

Cells were lysed and used for immunoblotting as described earlier.² Protein bands on western blots were visualized using Supersignal chemiluminescence reagent (Pierce). Immunoblots were scanned using GS-800 densitometer and protein bands quantified with Quantitation One software (Bio-Rad Laboratories, Hercules, CA).

**Reverse Transcriptase–Polymerase Chain Reaction (RT-PCR)**

Total RNA isolation and relative RT-PCR reactions were performed as described earlier.¹ The 5' and 3' primers were 5'-GTGACTCATCAGTTCTTTCC-3' and 5'-GAA TACCTCCAAACACAGCC-3' (CD36); 5'-ATTATAAGCTGTGGACAGGGGGA-3' and 5'-TCATCCACAGGGCGATGTTGCA-3' (Bcl-2); and 5'-TAGCAGGCACCTT CATTCCC-3' and 5'-CCTGAACCACCTTGCTGG-3' (human MCP-1). PCR consisted of 35 cycles of 30 seconds at 94°C, 30 seconds at 56°C, and 30 seconds at 72°C. 18S RNA was used as internal standard for quantitation in these relative RT-PCRs.

**Detection of Apoptosis by Annexin V Staining**

Five million monocytes were added to confluent monolayers of HVSMC and cocultured in RPMI1640 medium containing 0.2% bovine serum albumin (BSA) for 48 hours. Bound and unbound THP-1 cells were collected separately and double-stained with Annexin V-FITC and propidium iodide (PI) using an Annexin V-FITC apoptosis detection kit (BD Pharmingen). Monocyte apoptosis was induced by culturing in serum-free medium alone for 48 hours. Samples were analyzed on a MoFlo MLS flow
cytometer. Cell debris was excluded from the analysis by conventional gating of forward scatter versus side scatter dot plots. Four cell populations were defined as follow: *upper left panel*, necrotic cells; *upper right panel*, late stage apoptosis; *lower right panel*, early stage apoptosis; *lower left*, viable cells.

**Analysis of DNA Fragmentation**

This was performed by using an Apoptotic DNA Ladder Kit (Roche Applied Science, Indianapolis, IN) according to company instructions. DNA electrophoresis was carried out in a 1.5% agarose gel and DNA fragments were examined and photographed under UV light.

**Statistical Analysis**

Data are expressed as mean ± SE of multiple experiments. Student’s *t*-tests were used to compare two groups, or Analysis of Variance (ANOVA) with Tukey’s post tests for multiple groups using PRISM software (Graph Pad, San Diego, CA). Values of *P*<0.05 were considered statistically significant.

**REFERENCES**


Figure Legends

**Figure I.** Binding to HVSMC reverses serum depletion-induced THP-1 cell DNA fragmentation. Serum depletion-induced THP-1 cell DNA fragmentation was reversed by binding with HVSMC in the mixed cocultures. M indicates 100bp DNA markers.

**Figure II.** Binding-mediated Akt phosphorylation (5 minutes) (A) and Bcl-2 mRNA upregulation (30 minutes) (B) in THP-1 cells were blocked by LY294002 (40 µmol/L) in the fixed cocultures.

**Figure III.** Upregulation of THP-1 cell CD36 mRNA upon coculture with HVSMC. CD36 mRNA expression was upregulated in unbound THP-1 cells in the mixed cocultures (peak at 6 hours) (unbound), or in bound THP-1 cells in the fixed cocultures for (peak at 1 hour) (fixation).
Figure I
Figure II

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