Cooperation Between Secretory Phospholipase A2 and TNF-Receptor Superfamily Signaling
Implications for the Inflammatory Response in Atherogenesis

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Abstract—Atherogenesis is the consequence of a variety of effector mechanisms rather than the result of a single functional molecule. In this connection, type IIA secretory phospholipase A2 (sPLA2) is an acute-phase reactant, which accumulates in atherosclerotic arterial walls, elicits several effects on monocytes, and has been related to the development of atherosclerosis. CD40/CD40 ligand pair is also a strong proatherogenic system. sPLA2 produced an increase of the surface expression of CD40 in THP-1 monocytes and enhanced the effect of CD40 ligation on the expression of both Fas and FasL, thus indicating the existence of a positive cooperation between sPLA2 and different elements of the TNF-receptor superfamily. Activation of the CD40/CD40L dyad with anti-CD40 antibody produced a small release of arachidonic acid and lacked any significant effect on the induction of cyclooxygenase-2, whereas the secretion of the chemokine MCP-1 and the surface display of CD11b, the α chain of the integrin Mac-1, were upregulated. Engagement of CD40 did not influence the survival of THP-1 monocytes, but coinubcation of THP-1 monocytes pretreated with anti-CD40 antibody and Jurkat cells induced a significant increase of the number of Jurkat cells showing binding of annexin-V, and nuclear condensation and fragmentation, thus indicating that this treatment might trigger a juxtacrine/paracrine mechanism of apoptotic death in sensitive cell types. This data indicates the existence of overlapping routes for the response to CD40, TNF-α, and sPLA2, thus allowing the development of distinct patterns of response in monocytic cells. (Circ Res. 2002;91:681-688.)

Key Words: apoptosis • atherosclerosis • cytokines • inflammation • lipid mediators

The development of new concepts about atherogenesis in the context of inflammation has opened new vistas for the comprehension of this disease. Recent reports have pointed to the dyad CD40/CD40 Ligand (CD40L) as a stimulus for atheroma-associated cells operating at all stages of atheroma development. CD40 is a cell-membrane spanning protein of about 50 kDa belonging to the tumor necrosis factor (TNF)-α receptor family, which also includes TNF receptors and Fas (CD95). CD40 is constitutively expressed on B cells and participates in the regulation of cell proliferation, differentiation, and apoptosis. The activator of CD40, CD40L, is a TNF-like molecule, the expression of which was originally considered as restricted to the surface of activated CD4+ T cells, thus being associated to T cell–dependent B-cell responses and isotype switching. The CD40/CD40L system is now recognized as widely distributed, and it has recently been reported that vascular cells and macrophages express functional CD40L, as well as its receptor CD40 in atherosclerotic plaques. Engagement of CD40 endows cells with functions that mediate inflammatory responses. Consequently, CD40 signaling has been associated not only with atherosclerosis, but also with the pathogenesis of other inflammatory diseases. CD40 has been related to the modulation of various stages of atherogenesis: initiation, evolution, and rupture of the plaque. In the early phase, CD40 participates through the induction of adhesion molecules and the release of chemokines. In established lesions, its function includes the production of proinflammatory cytokines. In regard to the stability of atherosclerotic plaque, CD40 ligation induces the synthesis of matrix-degrading enzymes and prothrombotic activities.

Another molecule associated with the development of atherosclerotic lesions is the secreted type IIA phospholipase A2 (sPLA2), because it has been detected in all stages of human atherosclerotic arteries, and transgenic mice hypexpressing this enzyme develop severe atherosclerosis and display lipoprotein profiles more proatherogenic than those of nontransgenic littermates. Immunohistochemical studies indicate sPLA2 is present in smooth muscle cells (SMCs) from both the media and intima layers and in macrophage-rich

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regions of atherosclerotic plaques, making SMCs the main source of the enzyme. In addition, it has been reported that proinflammatory cytokines present in atherosclerotic lesions can regulate sPLA2 transcription and secretion by arterial wall.13 Regarding the monocyte-derived macrophage contribution, in vitro studies have shown the induction of sPLA2 by mildly oxidized low-density lipoprotein (LDL).14 To date, some of the biological activities of sPLA2 have been attributed to its enzymatic capacity to hydrolyze phospholipids. However, several lines of evidence suggest that some of the physiological actions of sPLA2 are not due to its hydrolytic activity, but to specific binding to cell surface receptors. In fact, reverse transcription-polymerase chain reaction (RT-PCR) analyses have shown the presence of mRNA for the M-type sPLA2 receptor in bone marrow–derived mast cells, THP-1 cells, and human monocytes,15–17 and it has been shown that sPLA2 activates biochemical pathways such as the MAP-kinase cascade and the cytosolic phospholipase A2c (cPLA2c), thus leading to arachidonate release and to a variety of biological effects including cell migration, mitogenesis, and cytokine release.16–20 This study shows that activation of monocytic cells by sPLA2 elicits the surface display of functional CD40, thus enlarging the scope of biological responses that these cells may undergo during atherogenesis.

**Materials and Methods**

**Reagents and Cell Culture**

sPLA2 was purified from plasma of patients diagnosed of sepsis as previously described.21 This procedure provides a unique protein band, the identity of which has been ascertained by Edman’s N-terminal sequencing. Written informed consent was obtained from patients. The absence of lipopolysaccharide (LPS) in the preparation was confirmed by the Limulus amebocyte lysate assay test in the batches used for the experiments. Moreover, experiments are conducted in the absence of PBS, which ensures that the effect is observed in the absence of LPS binding protein, which is necessary for the action of low concentrations of LPS. Goat anti-cyclooxygenase-2 (COX-2, C-20) and mouse monoclonal anti-cPLA2, (4A8B-3C) were from Santa Cruz Biotechnology (Santa Cruz, Calif). THP-1 and Jurkat cells were cultured in RPMI 1640 (GIBCO) supplemented with 2 mmol/L glutamine and 5% heat-inactivated PBS. Cells were rested for 24 hours before the experiment, and then were stimulated for the indicated times with 2 μg/mL anti-CD40 mAb, 100 U/mL TNF-α, 1 μg/mL sPLA2, or combinations of agonists. The detection of monocyte chemoattractant protein-1 (MCP-1) was carried out by ELISA with reagents from R&D Systems, Inc, as described.22

**Flow Cytometry**

THP-1 cells were assayed for CD11b, CD40, CD40L, Fas, and FasL expression. For this purpose, 5×104 cells/dish were treated with the agonists for 18 hours. The cells were collected, suspended in PBS supplemented with 1% BSA and incubated with 10 μg/mL anti-human CD11b/MAC-1 IgG, mAb, 1 μg/mL anti-human CD40 5C3 IgG1, mAb, 10 μg/mL anti-human CD40L TRAP1 IgG1, mAb, 1 μg/mL anti-human Fas, NOK-1 IgG, mAb, or 500 ng/mL anti-human Fas DX2 IgG, mAb (Pharmingen) for 1 hour at 4°C. After washing with PBS, goat anti-mouse IgG-FITC conjugate (Sigma) 1:100 was added and incubated for 30 minutes at 4°C. Subsequently, cells were washed and analyzed by immunofluorescence flow cytometry in a FACSscan cytofluorometer (Becton Dickinson). Positive cells were estimated using P3×63 myeloma supernatant as a negative control or an isotype-matched control negative Ab at an equivalent concentration. To increase the levels of membrane-bound FasL by blocking FasL cleavage before the stimulation with the agonists, cells were treated with 10 μmol/L of the matrix metalloproteinase inhibitor K8301 (Pharmingen). Data were analyzed using CellQuest software (Becton Dickinson)

**Cell Cycle Analysis**

THP-1 cells were incubated for 24 hours with the agonists, washed twice with cold PBS, and fixed with 70% ethanolic cold PBS, washed twice with cold PBS, and fixed with 70% ethanol. After overnight incubation at 4°C, cells were washed and resuspended in PBS/5 mmol/L EDTA. RNA was removed by digestion with RNase A at room temperature. After 1 hour of incubation with 0.5 mL of staining solution (500 μg/mL propidium iodide in PBS/5 mmol/L EDTA), cell cycle analysis was performed by flow cytometry.

**Cell Proliferation and Apoptosis Assays**

THP-1 cells were starved overnight, and after 24 hours of stimulation, cells were pulsed with 1 μCi [3H]thymidine for 4 hours before harvesting and counting of radioactivity. For apoptosis assay, cells were starved overnight, and then washed and incubated with the different agonists. After 48 hours stimulation, apoptosis was measured with the annexin-V FITC apoptosis detection kit of Pharmingen. Apoptosis was also evaluated by DAPI staining. For this purpose, cells were fixed with 4% paraformaldehyde and then permeabilized by 0.5% Triton-X100 in PBS. After permeabilization, cells were stained for 30 minutes with DAPI (1 μg/mL) and analyzed by fluorescence microscopy to assess chromatin condensation.

**Cell Fatty Acid Release**

Subconfluent cells were starved overnight and labeled for 3 hours with 0.30 μCi/mL [3H]arachidonic acid ([3H]AA) or with 0.30 μCi/mL [3H]thymidine...
Connections of the TNF Receptor/Ligand Superfamily, CD40/CD40L, and Fas/FasL to the sPLA2 Signaling Pathway in THP-1 Monocytes

Because both the CD40/CD40L dyad and sPLA2 have been detected in human atheroma lesions, we addressed the possible link between both systems by looking at the effect of sPLA2 on CD/CD40L in the monocytic cell line THP-1. These cells constitutively express CD40 on their surface, but not CD40L. Stimulation with both TNF-α and sPLA2, at concentrations similar to those detected in plasma after injection of bacteria,23 led to an upregulation of CD40 expression (Figure 1A); however, these agonists showed differences in the intensity of the induction, with sPLA2 being most effective, reaching a mean fluorescence intensity of 295±42 (mean±SD) versus 155±24 AU for TNF-α (P<0.01). Interestingly, combination of both agonists elicited a higher effect (355±19 AU; P<0.05). The effect of sPLA2 was not affected by preincubation with polymyxin B, thus ruling out a role for contamination by LPS of the sPLA2 batch. Because many effects of sPLA2 are produced by binding to the M-type receptor, which belongs to the C-type multilectin mannose receptor family, we assessed the effect of p-amino-phenyl-o-d-mannopiranoside-BSA (mannose-BSA), a noncatalytic ligand of the M-type receptor. As shown in Figure 1B, overnight treatment with different doses of mannoside-BSA led to CD40 upregulation to a similar extent to that induced by sPLA2, thus suggesting that sPLA2 receptor occupancy, rather than the catalytic activity of sPLA2, triggers CD40 expression. In contrast, all of these treatments failed to influence the expression of CD40L (data not shown).

Because the Fas/FasL dyad is influenced by sPLA2,16 and some of its functions involve cooperation with CD40,24,25 we next determined the effect of CD40 ligation on Fas/FasL induction by using anti-CD40 antibody as a cross-linking reagent for CD40. Overnight treatment of THP-1 cells with 2 μg/mL anti-CD40 resulted on the surface display of both Fas (Figure 2E) and FasL (Figure 3E). The expression of Fas was further increased when cells were coactivated with anti-CD40 together with sPLA2 or TNF-α (Figures 2B and 2D), while these stimuli on their own did not influence the surface display of Fas (Figures 2A, 2C, and 2F). Furthermore, although TNF-α did not influence the resting levels of the dyad Fas/FasL, TNF-α significantly enhanced the effect of anti-CD40 (Figures 2D and 3D), thus pointing to a cooperative effect of these elements of the TNF receptor/ligand superfamily.

Effect of CD40 Engagement on [3H]AA Mobilization

To address the functional significance of CD40 expression on arachidonate metabolism, we determined the effect of CD40

Reverse Transcription-Polymerase Chain Reaction

Total cellular mRNA was extracted by the TRIzol method (Life Technologies) according to the manufacturer’s instructions. cDNA was prepared by reverse transcription of RNA and then was amplified by PCR according to the following conditions: 1 cycle of initial denaturation at 94°C for 5 minutes, 30 cycles of denaturation at 94°C for 30 seconds, primer annealing for 30 seconds at 55°C, and extension at 72°C for 30 seconds, then 1 cycle of final extension at 72°C for 7 minutes. The PCR primers for human MCP-1 were 5'-GGATCCATGAAAGTCTCTGGCCGCCCT-3' (sense) and 5'-GAATTCTCAAGTCCTTCGGAGTTGTTG-3' (antisense). The expected PCR product, analyzed by ethidium bromide staining in 1.5% agarose gels was 300 bp for MCP-1. The expression of β-actin was used as control for the assay of a constitutive expressed gene.
ligation on cPLA₂ activation and COX-2 induction. As shown in Figure 4A, stimulation of CD40 for 15 minutes induced an almost complete phosphorylation of cPLA₂, as judged from the appearance of a shift in the electrophoretic mobility, which is due to the phosphorylation of the enzyme by MAP kinase and is associated with its activation. We next assessed whether cPLA₂ activation was accompanied by [3H]AA release. As shown in Figure 4B, sPLA₂ was the most potent agonist, and its effect was only slightly increased by both CD40 and TNF-α. To investigate whether the production of [3H]AA in response to sPLA₂ is due either to its enzymatic activity or to binding to its membrane receptor, some experiments were performed in cells labeled with [14C]OA, because sPLA₂ does not display specificity toward the fatty acid esterified at the sn-2 position of phospholipids. Under these conditions, no net release of [14C]OA was observed (data not shown), which strongly suggests that sPLA₂ is acting through a signaling mechanism involving the recruitment of a PLA₂ specific for [3H]AA and is consistent with its reported action through the activation of cPLA₂. Moreover, mannose-BSA, a ligand of sPLA₂ receptor, induced a [3H]AA build-up similar to that induced by sPLA₂ (Figure 4B). The induction of COX-2 protein was assessed after stimulation with anti-CD40 for 18 hours. Unlike sPLA₂ and TNF-α, anti-CD40 did not upregulate COX-2 expression, and combination of agonists only resulted in a mild enhancement of the response (Figure 4C). Moreover, shortest times of incubation with anti-CD40 provided the same negative results (data not shown).

**Figure 3.** FACS analysis of THP-1 cells stained for FasL (open curves) or for unspecific isotype-matched control Ab (solid gray curves). Cells were stimulated 18 hours in the presence of TNF-α, sPLA₂, anti-human CD40, or combination of agonists. FasL expression was not observed in resting cells and its curve overlaps with the unspecific control Ab. Figures on top of the open curves indicate the mean fluorescence intensity values. Representative histograms of 3 independent experiments are shown.

**Figure 4.** Effect of CD40 stimulation on the cPLA₂ system and arachidonate metabolism. THP-1 cells were incubated with 2 μg/mL anti-human CD40 mAb for the times indicated and then cell lysates were collected to assay the band-shift characteristic of cPLA₂ phosphorylation (A). Cells labeled with [3H]AA were stimulated with TNF-α, sPLA₂, 50 μmol/L mannose-BSA, anti-human CD40 mAb, or combination of agonists for 30 minutes, and then [3H]AA release was measured. Data represent mean±SD of 5 independent experiments (B). Cells treated under these conditions for 18 hours were collected for the immunodetection of COX-2. This is a representative experiment of 4 similar ones (C).

**CD40 Ligation Enhances the Proapoptotic Effect of THP-1 Cells on Coculture With Jurkat Cells and Upregulates CD11b Expression**

CD40 stimulation produces a variety of responses related to cell survival, which depend on the cell type. The effect of triggering CD40 on [3H]thymidine incorporation was assessed to address any possible mitogenic effect. However, this treatment did not induce any significant change in [3H]thymidine incorporation (30.6±2.312 dpm/μm² in control cells versus 32.2±3.976 dpm/μm² cells after anti-CD40 n=5), whereas FCS resulted in an incorporation of 55.6±5.150 dpm/μm² cells. As shown in Figure 5A, most resting THP-1 cells (80%) are found in the G1 phase of the cell cycle, and this was not significantly affected by incuba-
that belongs to the Jurkat T cells was analyzed for annexin-V binding (Figure 6A, bottom panel). The annexin-V staining for Jurkat cells and THP-1 cells reveals few apoptotic cells with no increase after CD40 ligation (Figure 6B, top panels). The same assay was performed in the cocultures using resting THP-1 or anti-CD40–treated THP-1 cells as stimulus for Jurkat cells. Although resting THP-1 cells induced a small binding of annexin-V in Jurkat cells (24.50 ± 4.37%), when THP-1 cells had been prestimulated 9 hours before the start of the coculture, the percentage of Jurkat cells expressing annexin-V increased to 49.09 ± 8.12%, (mean ± SD, n = 6, P < 0.001; Figure 6B, bottom panels). Figure 6C shows the cocultures stained with anti-CD11b (green) and DAPI (blue). In Figure 6Cb, THP-1 cells have been prestimulated and CD11b negative cells (Jurkat cells) showed the nuclear condensation and fragmentation typical of apoptosis. However, in the presence of resting THP-1 cells (Figure 6Ca) nuclear alterations were not observed.

**CD40 Ligation Upregulates the Mechanism of Monocyte Recruitment**

Monocyte recruitment to the inflammatory foci is mediated by strong interactions with the endothelium involving the β2-integrin family of receptors, as well as by the concomitant activation by chemoattractants. As mentioned before (Figure 6A), CD40 engagement upregulates the surface display of the CD11b chain of the β2-integrin Mac-1, whereas no effect was observed with both TNF-α and sPLA₂ (not shown). In keeping with an additional effect of CD40 on monocyte recruitment, THP-1 cells activated with anti-CD40 showed and enhanced expression of MCP-1 mRNA, which was accompanied by an increase of MCP-1 protein (Figures 7A and 7B). Unlike the effect on CD11b expression, this response was also enhanced by TNF-α and sPLA₂. Because κB-dependent transcription is one of the mechanisms involved in the regulation of MCP-1 expression, the possible effect of CD40 engagement on NF-κB activation was searched by looking at the degradation of the inhibitory proteins IkBα, IkBβ, and IkBε. However, treatment of THP-1 cells with anti-CD40 Ab did not produce any effect on these proteins (data not shown), which indicates that the CD40/CD40L dyad does not activate the NF-κB route in THP-1 monocytes.

**Discussion**

The role of the CD40/CD40L dyad in atherogenesis has been highlighted by a number of significant findings: (1) the colocalization of both CD40 and CD40L in atheroma-associated cells; (2) the induction of the expression of proatherogenic mediators in vitro by engaging this system; and (3) the production of atherogenesis in vivo by acting on the system. Moreover, the connection of the CD40/CD40L system with proinflammatory cytokines has been established by several findings. Thus, CD40 induction by cytokines has been reported in macrophages, keratinocytes, vascular endothelium, and aortic smooth muscle cells, where this effect is elicited by the NF-κB route. In this study, we have shown that the CD40/CD40L system is also linked to the sPLA₂ signaling pathway, which in view of the reported proatherogenic effects of sPLA₂, is an argument in
favor of a cooperative interaction between both molecules in
atherogenesis. However, the signaling pathways triggered by
the CD40/CD40L system differ among distinct cell types, and
little information exists regarding the functional consequences of
the modulation of this system in macrophages as yet.

The present data show that ligation of CD40 on monocytes
initiates a series of proinflammatory events that includes (1)
activation of enzymes of arachidonate metabolism, (2) in-
creased expression of molecules involved in the recruitment
of mononuclear cells, and (3) upregulation of molecules that
regulate cell death. In fact, signaling through CD40 in THP-1
cells activates cPLA2, the enzyme that selectively hydrolyzes
arachidonic acid from membrane phospholipids, and allows
its conversion into compounds with functional relevance for
atherogenesis, in view of the role that eicosanoids exert on
intracellular signal transduction and leukocyte recruitment.2
In contrast, no detectable effect on the expression of COX-2 was observed,
which differs from the results reported in lung,43 orbital,44 and human fibroblasts, where the CD40/CD40L bridge upregu-
lates the expression of COX-2.

Consistent with a role for CD40 in the recruitment of
leukocytes to the plaque is the regulation of the expression of
adhesion molecules, as judged from the increased surface
display of CD11b. In fact, reduction of CD11b expression has been associated to the protective effect of a leuotriene B\textsubscript{4} antagonist in the progression of atherosclerosis.\textit{Nat Med.} 1999;5:1313–1316.


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**ALLOACTIVATION MARKERS**

MFI, mean fluorescence intensity  
A.U., arbitrary units  

**CD71 expression on Jurkat cells**

Solid curve: unspecific binding. (MFI: 1.9 A.U.)  
Empty black curve: CD71 staining on Jurkat cells. (MFI: 42.2 A.U.)  
Empty grey curve: CD71 staining on Jurkat cells from co-cultures (JurKat+THP-1). (MFI: 44.6 A.U.)

**CD69 expression on Jurkat cells**

Solid curve: unspecific binding. (MFI: 2.4 A.U.)  
Empty black curve: CD69 staining on Jurkat cells. (MFI: 13.7 A.U.)  
Empty grey curve: CD69 staining on Jurkat cells from co-cultures (JurKat+THP-1). (MFI: 10.4 A.U.)

**CD28 expression on Jurkat cells**

Solid curve: unspecific binding. (MFI: 2.04 A.U.)  
Empty black curve: CD28 staining on Jurkat cells. (MFI: 56.63 A.U.)  
Empty grey curve: CD28 staining on Jurkat cells from co-cultures (JurKat+THP-1). (MFI: 80.08 A.U.)

**CD38 expression on Jurkat cells**

Solid curve: unspecific binding. (MFI: 2.04 A.U.)  
Empty black curve: CD38 staining on Jurkat cells. (MFI: 11.32 A.U.)  
Empty grey curve: CD38 staining on Jurkat cells from co-cultures (JurKat+THP-1). (MFI: 10.07 A.U.)

**HLA-DR expression on Jurkat cells**

Solid curve: unspecific binding. (MFI: 1.9 A.U.)  
Empty black curve: HLA DR staining on Jurkat cells. (MFI: 1.7 A.U.)  
Empty grey curve: HLA DR staining on Jurkat cells from co-cultures (JurKat+THP-1). (MFI: 1.8 A.U.)

Fluorescence intensity (log$_{10}$)