Ligation of CD40 Activates Interleukin 1β-converting Enzyme (Caspase-1) Activity in Vascular Smooth Muscle and Endothelial Cells and Promotes Elaboration of Active Interleukin 1β*

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Uwe Schönbeck‡‡, François Mach‡‡, Jean-Yves Bonnefoy¶, Harald Loppnow**, Hans-Dieter Fland, and Peter Libby† ‡‡

From the ‡Vascular Medicine and Atherosclerosis Unit, Cardiovascular Division, Department of Medicine, Brigham and Women’s Hospital, Harvard Medical School, Boston, Massachusetts 02115, the ¶Geneva Biomedical Research Institute, 14 chemin des Aulx, 1228 Geneva, Switzerland, the ¶Department of Immunology and Cell Biology, Research Center Borstel, 23845 Borstel, Germany, and the **Martin-Luther-University, Ernst-Grube-Straße 40, 06097 Halle (Saale), Germany

Inflammation contributes to a variety of arterial diseases including atherosclerosis. Interleukin 1β (IL-1β) in its activated mature 17-kDa form may mediate aspects of vascular inflammation. As shown previously, human vascular wall cells, such as smooth muscle cells (SMC), express the IL-1β precursor upon stimulation and the IL-1β-converting enzyme (ICE) constitutively but do not produce mature IL-1β or express ICE activity. How SMC, the most numerous cell type in arteries, may release active IL-1β has therefore remained a perplexing problem. We report here that stimulation of human vascular SMC and endothelial cells (EC) through CD40 ligand, a mediator recently localized in human atheroma, induced elaboration of the IL-1β precursor as well as activation of cell-associated ICE. In addition to the constitutively expressed 45- and 30-kDa immunoreactive ICE proteins, vascular cells incubated with recombinant human CD40 ligand (rCD40L) (but not IL-1 or TNF) showed an increase of a 20-kDa immunoreactive ICE protein by Western blot analysis. Furthermore, SMC and EC stimulated through rCD40L processed recombinant human IL-1β precursor (pIL-1β), generating a cleavage product of approximately 17 kDa. Appearance of both the 20-kDa immunoreactive ICE protein and pIL-1β processing activity required at least 6 h of stimulation with 0.3 or 1.0 μg/ml rCD40L, respectively, and was inhibited by pre-incubation of the ligand with an anti-CD40L antibody. Stimulation of vascular SMC and EC through rCD40L resulted in the release of biologically active IL-1β, indicating processing of the native IL-1β precursor induced by the ligand. These findings establish a novel mechanism of IL-1β activation in human vascular and, moreover, indicate a new pathway of ICE-activation, which could participate in inflammatory aspects of atherogenesis and other disease states.

Interleukin 1 (IL-1) figures importantly in many physiological and pathological processes, notably inflammatory diseases including atherosclerosis. Two distinct genes give rise to the two IL-1 isoforms denoted IL-1α and IL-1β that bind to common receptors. Interleukin 1α, often membrane-associated, can act by contact with neighboring target cells (1). Interleukin 1β, when secreted in its mature form, can act at a distance in a paracrine manner. Acquisition of biological activity for IL-1β (but not IL-1α) requires processing into the mature, 17-kDa protein (2–5). Upon stimulation, monocytes produce a cell-associated 33-kDa precursor form of IL-1β (2, 3). Maturation of the IL-1β precursor into the active 17-kDa form results from cleavage at the Asp116-Ala117 site by a cysteine proteinase denoted IL-1-β-converting enzyme (ICE) (6–10). ICE in turn is synthesized as a precursor molecule of 45 kDa, which is thought to be autocatalytically cleaved to form an active homodimeric enzyme of 20- and 10-kDa subunits ((p20/p10)2) (11, 12). ICE was the prototype of a group of cysteine proteases, now called the caspase-family (13). In addition to ICE (caspase-1), this protease family includes pro-apoptotic enzymes, such as human ICH-1 (caspase-2) or CPP32 (caspase-3). Each of these homologous enzymes shares the active site cysteine and aspartate binding clefts. Studies of the enzymatic specificity of ICE demonstrated highly selective proteolytic activity, i.e. requiring aspartic acid in the P1-position (9, 14). Interleukin 1β and the apoptotic mediator CPP32 are among the substrates of ICE (15, 16). Although ICE can autoactivate (17), the initial mechanisms of activation and regulation of ICE-processing remain unknown.

Most studies investigating expression of ICE or IL-1β activation have focused on monocytes or monocyte-derived cell lines. However, normal arteries contain few if any mononuclear phagocytes. IL-1 derived from vascular smooth muscle (SMC) and endothelial cells (EC) may initiate local immune and inflammatory responses and induce expression of adhesion molecules (18–20) and chemotaxtact cytokines, e.g. IL-8 or IL-1 itself (21–25), that can then recruit the “professional” phagocytes. In particular, inflammatory components of atherogenesis may involve IL-1 (26, 27). Although human atherosclerotic plaques contain both IL-1β and ICE (28, 29), the mechanisms that activate either the cytokine or the enzyme remain undefined.

* The abbreviations used are: IL-1, interleukin 1; EC, endothelial cells; ICE, interleukin 1β-converting enzyme; IL-1β: recombinant mature human interleukin 1β (17 kDa); rCD40L, recombinant human CD40 ligand; pIL-1β, recombinant human interleukin 1β precursor (33 kDa); SMC, smooth muscle cells; TNF, tumor necrosis factor; FCS, fetal calf serum; IT, insulin/transferrin; PAGE, polyacrylamide gel electrophoresis.

† Contributed equally to this work.
‡ To whom correspondence should be addressed: Vascular Medicine and Atherosclerosis Unit, Cardiovascular Division, Dept. of Medicine, Brigham and Women’s Hospital, Harvard Medical School, 221 Longwood Ave., LMRC 307, Boston, MA 02115. Tel.: 617-732-6628; Fax: 617-732-6691; E-mail: plibby@bustoff.bwh.harvard.edu.

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Recent work has demonstrated co-expression of CD40 and its ligand CD40L in human atherosclerotic plaques, indicating a possible role for this receptor-ligand pair in vascular pathology (30). CD40L, originally described as a 33-kDa activation-induced transiently expressed CD4+ T cell surface molecule (31–34), is also expressed on macrophages, endothelial cells, and smooth muscle cells (30). Previous studies of the interactions between CD40L and its receptor CD40 concentrated on the role of these leukocyte-surface proteins in T cell-dependent B cell differentiation and activation (35). CD40 ligation regulates a variety of activities, including B cell growth, differentiation, and death (35, 36), cytokine production by monocytes (37), and expression of leukocyte adhesion molecules on EC (38–40). Recent reports from several groups linked CD40/CD40L interaction to the mechanisms of apoptosis (41–43), a process in which ICE and other caspases play major roles, as reviewed elsewhere (44). We therefore tested the hypothesis that CD40L modulates the expression and/or activity of ICE and thus of IL-1β in cells of the vascular vessel wall, particularly smooth muscle and endothelial cells.

We demonstrate here that recombinant human CD40L (rCD40L) induces de novo synthesis of the IL-1β precursor and coordinates expression of a 20-kDa immunoreactive ICE protein with the expression of biological ICE-activity in human vascular smooth muscle and endothelial cells. Moreover, supernatants of the rCD40L-stimulated cultures, but not supernatants from cells exposed to a variety of other mediators, contained biological IL-1β activity.

**EXPERIMENTAL PROCEDURES**

**Cell Isolation and Culture**—Human vascular SMC were isolated from saphenous veins by explant outgrowth (45) and cultured in Dulbecco’s modified Eagle’s medium supplemented with 1% -glutamine, 1% penicillin/streptomycin, 10% FCS (Atlanta Biologicals, Norcross, GA) and 0.2% EDTA (EM Science, Gibbstown, NJ). Human dermal fibroblasts were isolated from saphenous veins by collagenase treatment (1 mg/ml; Worthington Biochemicals), 0.2% EDTA (EM Science, Gibbstown, NJ) (endothelial cell growth factor; Pel-Freez Biological, Rogers, AR). Both SMC or EC cultured in 75-cm² flasks were washed twice and incubated in medium lacking FCS. Vascular EC were cultured in M199 supplemented with 1% penicillin/Placidil, NY) as described elsewhere (46). Cells were maintained in medium 199 (Worthington Biochemicals), 0.2% EDTA (EM Science, Gibbstown, NJ) (endothelial cell growth factor; Pel-Freez Biological, Rogers, AR). Both cell types were sub cultured following trypsinization (0.5% trypsin (Worthington Biochemicals), 0.2% EDTA (EM Science, Gibbstown, NJ)) in 75-cm² culture flasks (Becton Dickinson, Franklin Lakes, NJ) and used throughout passages 2 to 4. Culture media and FCS contained less than 0.1% HEPES (Upstate Biotechnology Inc.) and less than 0.1% sodium deoxycholate, 1% Nonidet P-40, 0.1% SDS, 0.1% sodium deoxycholate, 1% Nonidet P-40, 150 mM NaCl, 5 mM EDTA, 20 µg/ml soybean trypsin inhibitor, 0.1% mammymethlysulfonyl fluoride, 0.2 units/ml aprotinin, 0.025% sodium azide). Cell-extracts were centrifuged (30 min, 4 °C, 10,000 × g) and supernatants were pre cleared with non-immune rabbit-serum (18 h, 4 °C; Vector, Burlingame, CA). After centrifugation (10 min, 4 °C, 10,000 × g), proteins of the supernatants were immunoprecipitated (2 h, 4 °C) with the IL-1β-specific polyclonal rabbit antibody (Upstate Biotechnology Inc.). After addition of protein A-agarose (1.5 h, 4 °C; Life Technologies, Inc.), precipitates were centrifuged (2 min, 4 °C, 300 × g), and the pellet was resuspended in 50 µl of SDS-PAGE sample buffer (0.2 µl Tris (pH 6.8), 5% glycerol, 0.1% SDS, 3% β-mercapto ethanol, 0.1 mg/ml bromphenol blue, final concentrations). After heating for 10 min at 95 °C, the samples were separated by SDS-PAGE, transferred on polyvinylidene difluoride membranes (Millipore, Bedford, MA), and exposed to autoradiography film (NEN Life Science Products).

**Western Blotting**—Cell extracts, equalized in total protein, were separated by standard SDS-PAGE under reducing conditions, and transferred to polyvinylidene difluoride membranes using a semi-dry blotting apparatus (3.0 mAc/cm², 30 min; Bio-Rad, Hercules, CA). Blots were blocked (2 h), and dilution of first and second antibody was made in 5% defatted dry milk, PBS, 0.1% Tween 20. After 1 h of incubation with the respective primary antibody (1:1,000 polyclonal rabbit anti-IL-1β (Upstate Biotechnology Inc.), 1:200 polyclonal goat anti-IL-1β-converting enzyme (M19, Santa Cruz Biotechnology, Santa Cruz, CA)) blots were washed four times (15 min in PBS, 0.1% Tween 20) and the secondary peroxidase-conjugated goat anti-rabbit antibody (1:10,000; Jackson Immunoresearch, West Grove, PA) was added for another hour. Finally, after 4 times washing (20 min, PBS, 0.1% Tween 20), immunoblots were developed using the Western blot chemiluminescence system (NEN Life Science Products) or the chromogenic system adding diaminobenzidine (50 µg/ml; Sigma) in substrate buffer (17 mAc citric acid, 65 mAc NaH2PO4, 0.1% H2O2, 0.01% (w/v) Thimerosal) to the blots. Independently produced antibodies directed against IL-1β (rabbit polyclonal anti-IL-1β (Santa Cruz) and the mouse monoclonal antibody Fiβ3 (50)) as well as ICE (rabbit polyclonal α-ICE (Upstate Biotechnology Inc.) antibody (51)) yielded similar results in the experiments performed, indicating that the reagents employed specifically recognize the intended proteins.

**Processing Assay**—Cultured human vascular SMC and EC as well as monocytes were harvested by scraping in processing buffer (10 mM HEPES, 1 mM dithiothreitol, 10% glycerol; final concentrations, Sigma). After 3 freeze-thaw cycles, 30 µl of cell extract (containing equal amounts of total protein) was then mixed with 10 µl of substrate buffer (37 °C with 50 ng of recombinant human IL-1β precursor (pIL-1β; Ciston, Pine Brook, NJ)). All assays were performed in a final volume of 50 µl. The processing was stopped by adding 10 µl SDS-PAGE (5 ×) sample buffer and heating the samples (10 min, 95 °C). Finally, the samples were separated by SDS-PAGE and were analyzed by immunoblotting as described above. Specificity of the processing was analyzed by pre-incubation (10 min, 37 °C) of cell extracts with 100 µg/ml ICE-inhibitor (Ac-Tyr-Val-Ala-Arg-H (aldehyde); Peptide Institute, Osaka, Japan) (8) prior to addition of the precursor.

**Measurement of IL-1β Activity**—Human vascular SMC or EC were incubated for 24 h with the respective stimuli (None, rCD40L, or IL-1β) in the absence or presence of the anti-CD40L antibody (5 µg/ml). The culture supernatants were added in the absence or presence of the neutralizing IL-1β antibody (1 µg/ml; Endogen, Cambridge, MA) to (i) the murine thymocyte cell line D10.G4.1 or (ii) subconfluent fibroblast cultures (40). The IL-1 assay was performed as described previously (48, 52). Briefly, after 72 h of stimulation, cells were pulsed for the final 24 h with tritiated thymidine ([3H]thymidine, 5 µCi/well, NEN Life Science Products) in 96-well plates and harvested, and [3H]thymidine incorporation (disintegrations per minute per culture ± S.D.) was determined. The mean of triplicate cultures was determined. Alternatively, fibroblasts were fixed with paraformaldehyde (2%), stained with crystal violet (10% in methanol), and lysed by incubation with 100 µl of SDS (1%), and finally, absorbancy was measured at 550 nm.
RESULTS AND DISCUSSION

Stimulation of Human Vascular Cells with Recombinant Human CD40L Induces de Novo Synthesis of the 33-kDa IL-1β Precursor—Human vascular SMC and EC express CD40 protein and respond to its ligand CD40L (30, 38–40). Stimulation of vascular cells with rCD40L induced concentration-dependent de novo synthesis of the 33-kDa IL-1β precursor, as shown for human vascular SMC by metabolic labeling and immunoprecipitation (Fig. 1). Induction of the protein required at least 1 μg/ml rCD40L. The precipitated IL-1β protein migrated at approximately 33 kDa as expected for the precursor form (53–55). Smaller forms of IL-1β, i.e. the biologically active mature form with a molecular mass of 17 kDa, were neither detected in cell extracts nor culture supernatants. Similar results were obtained with human vascular EC (data not shown).

Recombinant Human CD40L Increases Expression of a 20-kDa Immunoreactive ICE Protein—Human vascular SMC and EC produce the IL-1β precursor (1, 21, 56) but do not release mature forms of IL-1β upon stimulation with IL-1α, IL-1β, TNF, endotoxin etc. (51). We therefore further analyzed the effect of rCD40L on the expression and/or activation of the ICE, the enzyme responsible for production of biologically active, mature IL-1β (6–10). We first investigated whether or not stimulation of vascular cells affected the expression of ICE proteins. In monocytes or monocyctic cell lines, this enzyme exists as a 45-kDazymogen, an intermediate form of 30 kDa, and active subunits of 20 (p20) and 10 kDa (p10) (8, 17). An antibody raised against the p20 subunit detects a 45-, 30-, and 20-kDa band in vascular SMC and EC (51). Neither regulation of these immunoreactive ICE proteins nor biologically active ICE forms have been previously found in vascular cells. However, stimulation of vascular cells through rCD40L increased the expression of the 20-kDa immunoreactive ICE protein, as illustrated here for SMC (Fig. 2). This increase did not occur in cells cultured in the presence of serum, an unphysiologic condition for SMC (47). Thus, the following experiments were performed using vascular cells cultured in the absence of serum. Howard et al. (57) showed that activation of ICE requires co-expression with IL-1β. We therefore further explored the influence of recombinant human mature IL-1β (rIL-1β) or rIL-1β/rCD40L co-stimulation on ICE-expression. However, rIL-1β either alone or in combination with rCD40L did not alter the expression of the 20-kDa immunoreactive ICE protein (Fig. 2). Appearance of the 20-kDa immunoreactive ICE protein depended on the rCD40L concentration (Fig. 3A). Furthermore, the increase of the 20-kDa immunoreactive ICE protein depended on the time of stimulation with rCD40L, first detected after 2 h (Fig. 3B). The early detection of the ICE protein could be due to processing rather than de novo synthesis of the constitutively expressed ICE precursor. The increasing strength of the 20-kDa band, compared with the weakzymogen band, may be due to additional de novo synthesis and subsequent processing of the ICE precursor, which is highly autocatalytic. In addition to the p20 subunit, active ICE contains the p10 subunit (12). We did not detect the p10 subunit in these analyses because the anti-p20 antibody used does not recognize p10.

Induction of IL-1β Converting Enzyme Activity in Vascular Cells by Recombinant Human CD40L—To analyze whether the increase of the 20-kDa immunoreactive ICE protein in
Western blotting with an IL-1
Aliquots (30 μg/ml) of these cell extracts were incubated with recombinant human CD40L-stimulated vascular cells correlates with the induction of biologically active ICE, as we performed processing assays in which cell extracts were incubated with recombinant human IL-1β precursor (pIL-1β). We monitored processing of exogenous pIL-1β as described above. Specificity of the processing was analyzed by pre-incubating cell extracts from cultures stimulated for 24 h with 10 μg/ml rCD40L with an ICE-inhibitor, before addition of the recombinant IL-1β precursor. As a standard, recombinant mature human IL-1β (rIL-1β, 20 ng/ml; 17 kDa) was applied on the right lanes.

cCD40L-stimulated vascular cells correlates with the induction of biologically active ICE, we performed processing assays in which cell extracts were incubated with recombinant human IL-1β precursor (pIL-1β). We monitored processing of exogenous pIL-1β into smaller fragments of the cytokine by Western blot analysis. Extracts of vascular cells stimulated with rCD40L (Fig. 4A) or with 5 μg rCD40L/ml for the indicated times (C). Cell extracts were analyzed for processing of pIL-1β as described above. Specificity of the processing was analyzed by pre-incubating cell extracts from cultures stimulated for 24 h with 10 μg/ml rCD40L with an ICE-inhibitor, before addition of the recombinant IL-1β precursor. As a standard, recombinant mature human IL-1β (rIL-1β, 20 ng/ml; 17 kDa) was applied on the right lanes.

FIG. 4. Induction of ICE-activity by recombinant human CD40L. A, human vascular SMC and EC were cultured for 24 h in serum-free medium and thereafter incubated with fresh medium containing 10 ng/ml rIL-1β, 5 μg/ml rCD40L, or a combination of both. Aliquots (30 μl) of these cell extracts (20,000 cells/μl) were incubated for 10 min at 37 °C with pIL-1β (50 ng/μl; 33 kDa) and analyzed by Western blotting with an IL-1β specific antibody. As a control, monocyte extracts (MØ) were also applied. Time- and concentration-dependence of the induction of ICE-activity was investigated using cell extracts from SMC cultures stimulated for 24 h with the indicated concentrations of rCD40L (B) or with 5 μg rCD40L/ml for the indicated times (C). Cell extracts were analyzed for processing of pIL-1β as described above. Specificity of the processing was analyzed by pre-incubating cell extracts from cultures stimulated for 24 h with 10 μg/ml rCD40L with an ICE-inhibitor, before addition of the recombinant IL-1β precursor. As a standard, recombinant mature human IL-1β (rIL-1β, 20 ng/ml; 17 kDa) was applied on the right lanes.

FIG. 5. Human vascular smooth muscle cells stimulated through rCD40L process the IL-1β precursor in a time- and concentration-dependent fashion. A, extracts from SMC cultures stimulated for 24 h (serum-free) with 5 μg/ml rCD40L were incubated with recombinant IL-1β precursor (50 ng/μl; 33 kDa) for the indicated time, and processing of pIL-1β was analyzed by Western blotting. B, the cell concentration required for processing of the precursor was determined by incubating cell extracts (24 h, 5 μg/ml rCD40L) of the indicated concentrations with the IL-1β precursor (50 ng/ml). Processing of pIL-1β was analyzed by immunoblotting as described under “Experimental Procedures.”
Inhibitors of ICE activity in Vascular Cells by CD40L

Fig. 6. Recombinant CD40L induces expression of IL-1β activity in the supernatant of human vascular smooth muscle cells. A, Human vascular SMC were incubated 24 h in IT medium prior to replacement with fresh medium alone (None), medium containing rCD40L (5 μg/ml), or rIL-1β (10 ng/ml rIL-1β) in the presence or absence of an α-CD40L antibody (α-CD40L, 5 μg/ml). After 24 h, supernatants were harvested and assayed for IL-1β activity in the absence or presence of an anti-IL-1β antibody (α-IL-1β, 1 μg/ml) to the IL-1-dependent cell line D10.G4.1. B, Human vascular SMC (IT medium, 24 h) were stimulated (24 h) with the indicated concentrations of rCD40L (μg/ml), and the resulting supernatants were assayed for IL-1β activity using a bioassay. C, Supernatant of rCD40L-stimulated SMC (Fig. 6A) or EC (data not shown) demonstrated the rCD40L-dependent release of bioactive IL-1β. The α-CD40L antibody did not affect the proliferation induced by supernatants of rIL-1β-stimulated vascular cells. Induction of IL-1β activity in rCD40L-stimulated vascular cells depended on the concentration and required ≥ 1 μg/ml rCD40L (Fig. 6B). Thus, the concentration dependence of the induction of biological IL-1β activity in the supernatant correlated with the concentration required for both, the induction of ICE activity and the expression of the IL-1β precursor. Both of the conventional IL-1 bioassays employed (proliferation of D10.G4.1 cells or human dermal fibroblasts) yielded similar results (data not shown).

The presented data indicate that CD40/CD40L interaction regulates IL-1β activity in the vessel wall by both induction of IL-1β precursor expression and activation of the IL-1β-converting enzyme. This pathway may have particular relevance for atherosclerosis, as cells in atheroma express CD40, its ligand CD40L, as well as IL-1β, a cytokine implicated in regulation of many aspects of vascular pathology. As other stimuli tested previously (e.g. IL-1α, IL-1β, TNFα, IL-8, and endotoxin) do not cause release of active IL-1β from vascular wall cells, CD40 ligation may prove important in initiating and sustaining inflammatory and host defense processes involving blood vessels. Moreover, inhibition of CD40L/CD40 signaling may represent a novel therapeutic target in arterial inflammation and atherosclerotic diseases.

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