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Endotoxin Activates Human Vascular Smooth Muscle Cells despite Lack of Expression of CD14 mRNA or Endogenous Membrane CD14

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During infection or inflammation, cells of the blood vessel wall, such as endothelial cells (EC) and smooth muscle cells (SMC), contribute to the regulation of the immune response by production of cytokines or expression of adhesion molecules. Little is known about the mechanism(s) involved in the stimulation of vascular cells by endotoxin (lipopolysaccharide [LPS]). As reported previously, LPS antagonists reduce LPS-induced cytokine production or adhesion in vitro specifically, suggesting a specific LPS recognition mechanism. We thus investigated the role of CD14 for stimulation of vascular SMC by LPS. Complement-fixing antibodies directed against CD14 (LeuM3, RoMo I, or Mo2) lysed monocytes but failed to mediate lysis of EC or SMC, indicating the lack of endogenous membrane CD14 in vascular cells. In addition, we did not detect expression of CD14 protein on EC and SMC in cell sorting analysis or cell immunoassay experiments. These observations are in line with our finding that a CD14 probe did not hybridize with mRNA of EC or SMC in Northern (RNA) blot experiments, although it hybridized well with monocyte-derived mRNA. We obtained the same results with the much more sensitive reverse transcription-PCR. Since the vascular SMC did not express endogenous CD14, we investigated the role of human serum-derived soluble CD14 (sCD14) for activation of SMC by LPS. In medium containing human serum, anti-CD14 antibodies inhibited activation of SMC by LPS. In contrast, the same antibodies did not inhibit activation of cells cultured in medium containing fetal calf serum. SMC cultured in sCD14-depleted medium responded 1,000-fold less to LPS than cells cultured in presence of sCD14. Reconstitution of sCD14-depleted serum or supplementation of serum-free medium with recombinant CD14 restored the capacity of the cells to respond to LPS. These results show that specific activation of vascular SMC by LPS does not involve binding to endogenous membrane CD14, but that the activation of vascular SMC by LPS is mediated to a great extent by serum-derived sCD14.

Human vascular cells, such as endothelial cells (EC) and smooth muscle cells (SMC), are involved in the regulation of immune responses during infection or inflammation (32, 49) by expression of adhesion molecules and/or production of cytokines or lipid mediators. Previous reports have shown that both vascular smooth muscle and endothelial cells produce interleukin-1 (IL-1) (33, 48, 68), although SMC do not release this monokine (42). EC and SMC also produce other immunoregulators, such as IL-6 upon stimulation with IL-1 or endotoxin (lipopolysaccharide [LPS]) (25, 40, 41, 65). LPS is a potent activator of various cell types. The active moiety of LPS that induces cytokine production is the lipid A (39, 52, 55, 57). Synthetic lipid A precursor Ia (36) or nontoxic LPSs (43) specifically inhibit LPS-induced production of cytokines in monocytes. Besides cytokine production (12, 13, 29), LPS antagonists also block other endotoxin-induced activities (11, 17, 27, 45, 54, 59). These data indicated specific recognition of LPS by the target cells. One protein unequivocally identified as an LPS receptor is the CD14 molecule (18). This cell surface molecule is anchored in the membrane by phosphatidylinositol linkage (21, 64). An additional substance, the LPS-binding protein (LBP) (60, 70), enhances the response of macrophages to endotoxin (47). Other LPS receptor molecules are proposed (9, 10, 20, 26, 30, 66, 69). The CD14 molecule is also present in soluble form in the serum, as it is shedded from cells (3). Soluble CD14 (sCD14) can bind LPS and reduces endotoxin-induced activities, such as oxidative burst responses (62, 63) and tumor necrosis factor alpha (TNF-α) production by whole blood cells (22).

Recent reports have demonstrated that activation of EC by LPS is mediated by sCD14 (1, 14, 23, 51, 53). The SMC, another major cell population of the larger vessels, may also be activated by LPS during septicemia or other pathological situations following endothelial and subendothelial injury (34). An important feature of the host response during sepsis or organ failure is the loss of vascular tone. It is discussed that LPS may act directly on SMC to inhibit the vascular tone (4–6). In addition, cultured SMC or organoid explants of vessels respond to LPS by production of cytokines (41). Whether or not serum-derived CD14 mediates activation of SMC by LPS, as reported for EC, has not been published. It is also unclear whether vascular endothelial and smooth muscle cells have the capacity to express CD14 mRNA measurable in Northern (RNA) blot hybridization and PCR experiments. We thus investigated whether or not the CD14 molecule is involved in the activation of these cells. We found that vascular SMC (as well as EC) are stimulated by endotoxin through an LPS-specific mechanism; however, this response is not mediated by vascular cell-derived CD14, since vascular EC and SMC do not express CD14 mRNA. Activation of SMC by LPS is mediated mainly by soluble serum-derived forms of CD14.

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MATERIALS AND METHODS

Materials. Purified monoclonal anti-CD14 antibodies My-4, RoMo I, Mo2, LeuM3, and 63D3 (immunoglobulin G2b [IgG2b], IgG2a, IgM, IgG2b, and IgG1, respectively) were purchased from Coulter Immunology (Krefeld, Germany), Biometec (Greifswald, Germany), Coulter Immunology, Becton Dickinson (Heidelberg, Germany), and Pharmingen (Dianova, Hamburg, Germany), respectively. The LPS of *Salmonella friedenau* was phenol-water extracted and kindly provided by H. Brade (Borstel, Germany). Recombinant IL-1α and TNF-α were a kind gift of H. Galatti (Hoffmann-LaRoche, Basel, Switzerland).

Isolation and culture of vascular cells. Human vascular EC were isolated (24) from umbilical cord veins or unused portions of saphenous veins obtained following bypass surgery by collagenase treatment (0.1% collagenase H in phosphate-buffered saline [PBS]; Boehringer, Mannheim, Germany). The EC were cultured in medium 199 containing 5% fetal calf serum (FCS; BioWhittaker), endothelial cell growth factor (ECGF; 50 μg/ml; Boehringer Mannheim), heparin (25 μg/ml; Sigma, Deisenhofen, Germany), antibiotics, and L-glutamine (Biochrom, Berlin, Germany). Culture dishes were pretreated with fibronectin (1 μg/ml; Boehringer). EC were characterized by staining with anti-vWF (factor VIII) antibody (Dako Diagnostika, Hamburg, Germany) and subcultured following trypsin treatment. For experiments, EC were plated in culture dishes and grown in the endothelial culture medium until the monolayers were confluent. Prior to conditioning of the cells, medium was replaced by M199 containing 5% FCS, antibiotics, and L-glutamine.

Human vascular SMC were isolated from saphenous vein medial explants (58) after isolation of EC and removal of the intima and adventitia. The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% FCS, antibiotics, and L-glutamine and subcultured by trypsin treatment. The cells were then plated in culture dishes and incubated until confluent, and medium was replaced by fresh medium for the experiments. SMC were characterized by their typical "hill and valley" growth pattern, by staining with anti-α-SMC actin antibody (HHF35; Dako Diagnostika), and the lack of staining with the EC marker anti-factor VIII antibody (Dako Diagnostika). The culture medium contained less than 10 pg of LPS per ml, and the FCS contained less than 50 pg of LPS per ml, as measured in the *Limulus* amebocyte assay (QCL-1000; Bio Whittaker, Serva, Heidelberg, Germany).

Stimulation of vascular IL-6 production and measurement of IL-6 in bioassay. Confluent monolayers of vascular cells were incubated for 24 h with the respective stimuli. In some experiments, anti-CD14 antibodies were added prior to addition of stimuli. The supernatants were harvested and stored at −20°C. The IL-6 activity was determined in the 7TD1 assay (37, 67). Briefly, serial fourfold dilutions of samples or standard (10 ng of recombinant IL-6 per ml) were prepared in flat-bottom 96-well plates in 50 μl of DMEM containing 10% FCS, 5×10^{-5} M 2-mercaptoethanol (2-ME), L-glutamine, and antibiotics. The same volume (50^{-5} μl) of medium containing IL-6-dependent target cells (2,000 per well) was added. The cultures were then incubated for 66 h. To these cultures, MTT [2-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; 0.5 mg/ml in PBS] was added for 4 h. The cultures were incubated with dimethylformamide solution (5% sodium dodecyl sulfate [SDS] in 50% dimethylformamide) for at least 2 h, and absorption of the diazolium salt was measured at 550 nm in an enzyme-linked immunosorbent assay (ELISA) reader. The IL-6 activity in the samples was calculated by probit analysis (15) with reference to the recombinant IL-6 standard tested in parallel cultures.

Complement-mediated lysis of EC or SMC. EC or SMC were cultured in 96-well culture dishes until confluent. The cultures were washed, and complement-fixing anti-CD14 antibodies (RoMo I, Mo2, or LeuM3) or antibody (63D3) lacking the capacity to fix complement was added for 1 h. The antibodies were removed, and complement was added (1:4; Pel Freeze, Mast Diagnostika, Reinfeld, Germany) for 1 h. The EC or SMC cultures were washed again and incubated with IL-1 or medium without stimulus for 24 h. The supernatants were harvested, and IL-6 activity was measured as described above. Mononuclear cells (MNC), isolated by Ficoll gradient (7) and cultured in round-bottom 96-well plates in serum-free RPMI 1640 containing L-glutamine and antibiotics, were treated similarly with addition of centrifugation (1,200 rpm, 10 min) between the washing steps. The MNC were stimulated with LPS, since LPS is a more potent stimulus for these cells than IL-1.

Flow cytometric analysis and cell ELISA. Expression of cell surface-associated CD14 was first analyzed in a flow cytometer (19). Briefly, EC or SMC were cultured in 75-cm^2 culture flasks, trypsinized, and stored in PBS at 4°C. Cells were cultured and stored in the presence or absence of human serum or FCS and with or without LPS, respectively. Fluorescein isothiocyanate (FITC)-labeled anti-CD14 antibody (Mo2; Coulter) was added for 20 min, and the cells were washed, fixed (1.5% paraformaldehyde, 0.01% NaN_3, PBS), and analyzed in the flow cytometer (Cytofluorograf System 50H, Ortho Diagnostic Systems, Raritan, N.J.).

Second, cell ELISA experiments were performed in 96-well flat-bottom microtiter plates. Briefly, cells (10,000 cells per cm^2) were incubated for 48 h, conditioned for 24 h, washed, and fixed (1% paraformaldehyde [PFA]), and antibodies were added for 1 h on ice in PBS containing 1% bovine serum albumin (BSA). Cultures were washed, incubated with second antibody for 1 h, and washed with PBS without BSA, and substrate was added (0.4% *o*-phenylenediamine [OPD], 0.05% H_2O_2 in substrate buffer [17 mM citrate, 65 mM NaH_2PO_4, 0.01% thimerosal]). Absorption was read in an ELISA reader at 405 nm.

Isolation of total RNA and determination of CD14 mRNA by Northern hybridization or PCR. EC or SMC were cultured as described above in 75-cm^2 culture flasks. Stimuli were added to these cultures in a volume of 10 ml. After 4 h, the supernatants were removed, guanidinium thiocyanate (GITC) solution (Gibco BRL) was added, and the cell layers were lysed. Total RNA was isolated by a one-step preparation (8).

For Northern blot analysis, RNA (10 μg) was dissolved in sample buffer, heated to 56°C, and run on a denaturing 1.3% agarose gel in MOPS (morpholinepropanesulfonic acid) buffer containing formaldehyde at 35 V overnight. The RNA was blotted to Hybond-N (Amersham, Braunschweig, Germany) by capillary transfer overnight and fixed to the nylon by UV transillumination for 6 min. The Northern hybridization was performed by standard methods (46). The CD14 cDNA used in these experiments was amplified from human monocyte DNA by PCR and cloned into pUC18. For this purpose, the following primers were used: 5′ primer, G GGA TCC ATG GAG CGC GCG TCC TGC TTG T; 3′ primer, G GAA TTC GTC TTG GAT CTT AGG CAA AGC. The sequence of the CD14 cDNA was identical to the published sequence except for a base substitution at position 803 (A changed to G), also reported by others (64).

For PCR, total RNA from EC, SMC, or MNC was isolated, and RNA (1 μg) was reverse-transcribed with Superscript reverse transcriptase (RT; 200 U/μl; Gibco BRL). The cDNA products were diluted in 100 μl of bidistilled water. RT-PCR was performed with 10 μl of RT product, 10 μl of primers (20 μM), 80 μl of reaction mix (10 mM Tris [pH 8], 50 mM KCl, 2.5 mM $MgCl_2$, 0.01% gelatin, and 4 μl of the four deoxynucleoside triphosphates (200 μM each, final concentrations), and 0.5 μl of *Taq* polymerase (2.5 U; Gibco BRL). Subsequently, PCR was performed for 35 cycles at 95°C (60 s, melting), 62°C (60 s, annealing), and 72°C (90 s, extension; addition of 2 s of extension time per cycle). PCR products were run on 1.3% agarose gels, visualized by UV transilluminator, and photographed. The sequences of the CD14 primers were CCA TGG AGC GCG CGT CCT GC (sense) and GTC TTG GAT CTT AGG CAA AGC (antisense). The expected size of this PCR product was 1,140 bp. The sequences of the IL-1 primers were ATA TAA CGT TGC CAA AGT TCC AGA CAT (sense) and ATA TTC TAG ACT ACG CCT GGT TTT CCA GTA (antisense). The expected size of this product was 812 bp.

Preparation of human CD14-depleted serum and recombinant CD14. sCD14-depleted serum was prepared by passing human serum through a column containing anti-CD14 antibody RoMo I bound to Sepharose 4B. The depleted serum contained less than 1 ng of sCD14 per ml, as determined by sCD14 capture ELISA (IBL, Hamburg, Germany).

The soluble recombinant CD14 was isolated from culture supernatants of CHO cells transfected with the human CD14 cDNA. Briefly, CD14 cDNA was amplified by PCR and cloned into the eucaryotic expression vector pPOL-DHFR. This plasmid (pPOL-DHFR/CD14) was transfected into dihydrofolate reductase (DHFR)-deficient CHO cells by electroporation. The plasmid pPOL-DHFR and the DHFR-deficient CHO cells were a kind gift of P. Kufer (Institute of Immunology, Ludwig-Maximilians-University, Munich, Germany). Amplification of the vector was selected by using methotrexate. CHO cells grown with 0.5 μM methotrexate secreted 5 to 15 μg of recombinant sCD14 per ml. These supernatants were collected and passed through an RoMo I affinity column. The recombinant CD14 was eluted from the column by using 0.1 M glycine (pH 3.0).

RESULTS

Vascular cells do not express endogenous membrane CD14 protein. EC and SMC respond specifically to LPS. Thus, we investigated the expression of CD14, an unequivocally identified LPS receptor, on the cell surface of vascular cells. First, we performed complement-mediated lysis experiments. Anti-CD14 antibody Mo2 was used for lysis in the experiments shown in Fig. 1. This and other antibodies used in separate experiments (LeuM3 and RoMo I; data not shown) are capable of fixing complement. These data show that in control experiments, coincubation of serum-free cultured monocytes (MNC) with the lytic anti-CD14 antibodies and complement killed the cells and therefore abolished the capacity of the MNC to respond to subsequent LPS stimulation (Fig. 1). A control CD14 antibody (63D3) lacking the capacity to fix complement did not reduce IL-6 production of MNC (data not shown). These results indicate the presence of endogenous membrane-bound CD14 molecules on monocytes. In order to investigate the presence of endogenous CD14 on vascular cells, we cultured EC as well as SMC in FCS-containing medium and also incubated them with the anti-CD14 antibody. The antibody used in the lysis experiment can bind to human CD14 but

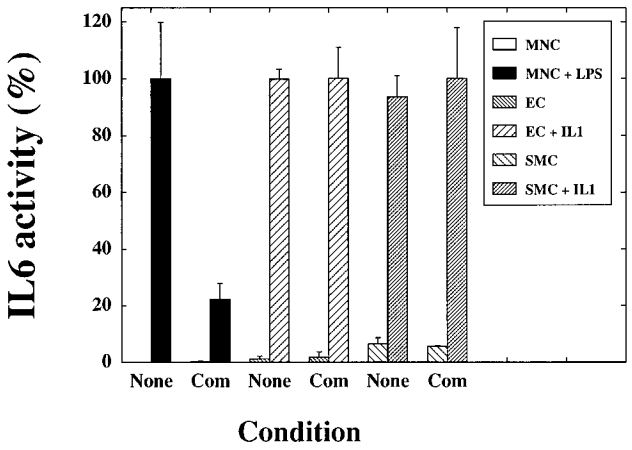

FIG. 1. Complement does not lyse SMC or EC in the presence of CD14 antibody. SMC, EC, and MNC were incubated with complement-fixing anti-CD14 antibody Mo2 (1 μg/ml) for 1 h. Cultures were washed, and complement (Com) or medium without complement (None) was added for 1 h and again washed. The cultures were then stimulated with IL-1 (10 ng/ml; EC and SMC) or LPS (1 μg/ml; MNC). After 24 h, the supernatants were harvested, and IL-6 production was determined in the 7TD1 assay. Three additional lysis experiments showed similar results. The data are presented as a percentage of the maximal IL-6 production obtained in the respective experiments, since the different cell types produced different maximal amounts of IL-6 (EC, 41,880 pg/ml; SMC, 112,191 pg/ml; and MNC, 91,363 pg/ml).

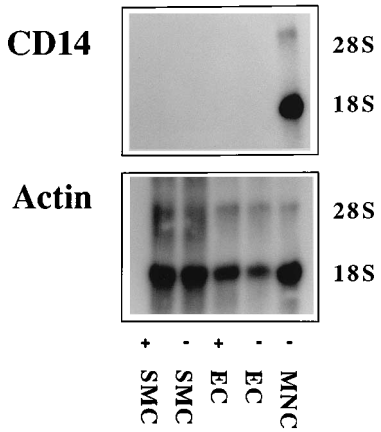

FIG. 2. EC and SMC do not express CD14 mRNA in Northern blot experiments. Total mRNA was isolated from unstimulated (−) MNC or unstimulated or LPS-stimulated (+) vascular EC and SMC and blotted to nylon membranes by capillary transfer under denaturing conditions. The blots were hybridized with CD14 or β-actin probes as described in Materials and Methods. A representative experiment of three is shown.

not to bovine CD14. Thus, under the experimental conditions used, it would only lyse cells expressing endogenous (human) CD14. However, none of the antibodies (Fig. 1 and data not shown) lysed EC or SMC, indicating that no endogenous CD14 was present on the cells. The vascular cells responded well to IL-1 stimulation, indicating viability of the cells, as also proven by lack of trypan blue staining. In separate experiments (data not shown), EC and SMC were incubated with antibodies and complement for 1 h, washed, and subsequently stimulated with LPS rather than IL-1α. These cells were also not lysed but did produce IL-6, similar to cells not treated with complement, indicating that an LPS recognition mechanism(s) other than endogenous (human membrane-bound) CD14 was present. Thus, under the conditions tested, FCS-derived bovine sCD14 might have been responsible for the activation of the cells by LPS. In addition to these investigations, we performed flow cytometry and cell ELISA experiments; however, we also did not find expression of CD14 protein by these techniques.

Vascular cells do not express CD14 mRNA. The results shown above suggest that vascular cells do not produce endogenous membrane CD14. However, it was still unclear whether the EC or SMC express CD14 mRNA. Therefore, we determined the presence of CD14 mRNA by Northern analysis and RT-PCR. The CD14 probe readily hybridized to MNC-derived RNA blotted to the same nylon as EC- or SMC-derived RNA (Fig. 2). However, it did not hybridize to the EC or SMC RNA. The presence of intact mRNA was proven by the hybridization of the same blot with a β-actin probe. These results indicate a lack of CD14 mRNA expression in the vascular cells. However, the Northern experiments might not be sensitive enough for detection of small amounts of CD14 transcripts. Thus, we performed the much more sensitive RT-PCR. In order to avoid artificial signals caused by contaminating leukocytes or genomic DNA, we performed PCR with IL-8 receptor type II primers, since neither EC nor SMC express this mRNA (59a). In contrast, granulocyte or mononuclear cell preparations do contain this mRNA. None of the vascular cell RNA preparations tested contained IL-8 receptor type II mRNA, indicating the purity of the EC and SMC isolates (data not shown). Furthermore, we excluded the presence of genomic DNA by PCR with RNA preparations not previously reverse-transcribed. For PCR experiments, we only used RNA that did not contain genomic DNA. As shown in Fig. 3, neither EC nor SMC expressed CD14 mRNA, although the primers produced the correct PCR product with MNC-derived cDNA. Six additional SMC preparations and three EC preparations also did not contain CD14 mRNA. All preparations contained intact mRNA, as indicated by RT-PCR with IL-1α primers.

Involvement of soluble forms of CD14 in the activation of vascular SMC by LPS. The results presented above show that vascular cells do not express endogenous CD14. Thus, the mechanism of activation of vascular SMC by LPS remained unclear. Recent data have shown that EC or epithelial cells are activated by LPS through a hitherto unknown pathway of CD14-mediated activation of cells. This mechanism takes advantage of the presence of shedded soluble forms of CD14 in the serum. In order to investigate whether human vascular SMC also use this pathway, we performed (i) inhibition experiments in the presence of human serum (rather than FCS), (ii) LPS stimulation experiments with human serum depleted of

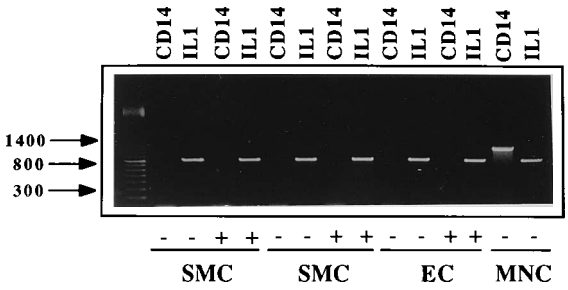

FIG. 3. PCR of reverse-transcribed EC and SMC mRNA. Total RNA from unstimulated (−) or LPS-stimulated (+) SMC, EC, or unstimulated MNC was isolated, reverse-transcribed, and amplified in PCR experiments with CD14 or IL-1α (for control of the presence of intact mRNA) primers as described in Materials and Methods. The arrows indicate the positions of the respective base pair markers (first lane). The expected sizes of the PCR products are 1,140 bp and 812 bp for CD14 and IL-1, respectively.

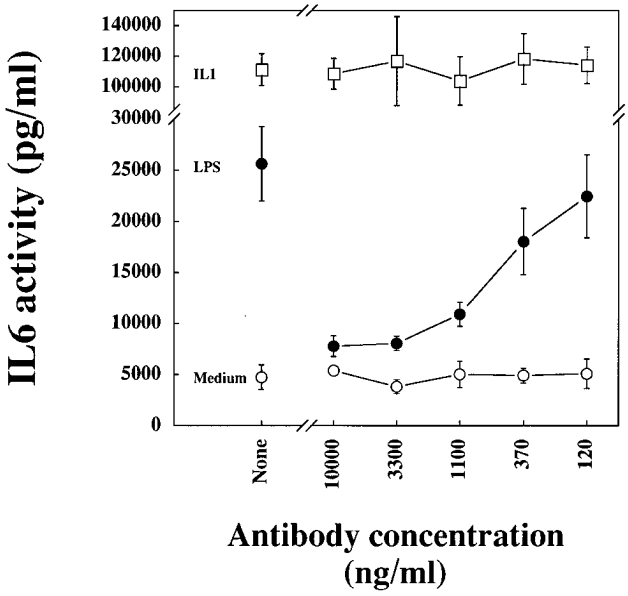

FIG. 4. Anti-CD14 antibody My4 inhibits activation of SMC by LPS in the presence of 5% human serum. SMC were cultured in 96-well culture plates until confluent and washed, and various dilutions of antibody (10 to 0.120 μg/ml) were added prior to addition of medium lacking a stimulus (open circles) or the designated stimulus: LPS, S-form LPS from *S. friedenau* (1 μg/ml; solid circles); IL-1, recombinant IL-1α (100 pg/ml; open squares). After 24 h, the supernatants were harvested, and IL-6 was measured in the 7TD1 assay.

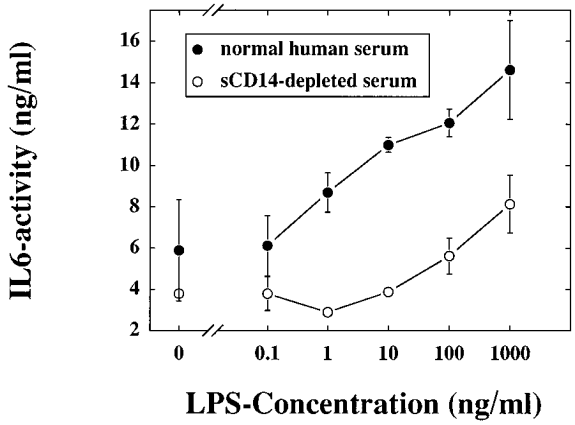

FIG. 5. SMC cultured in sCD14-depleted serum respond less potently to LPS stimulation than SMC cultured in normal human serum. SMC were incubated in 96-well culture plates until confluent. Subsequently, the cultures were washed with serum-free medium and cultured for 24 h in medium containing sCD14-depleted human serum. The indicated concentrations of LPS were then added in sCD14-depleted human serum (open circles) or normal human serum (solid circles) for an additional 24 h. Supernatants were harvested, and IL-6 was measured in the 7TD1 assay. Two additional experiments provided similar results.

sCD14, and (iii) reconstitution experiments with recombinant CD14 added under serum-free conditions.

In general, we cultured and stimulated vascular SMC in the presence of FCS. Since the anti-CD14 antibodies used for investigation of endogenously produced CD14 in the lysis experiments do not interact with bovine CD14, we cultured the cells for inhibition experiments in human serum. Antibodies added before LPS to cells cultured in FCS did not block the activation (data not shown). However, anti-CD14 antibody My4, as well as other anti-CD14 antibodies (data not shown), blocked LPS-mediated activation in the presence of human serum (Fig. 4). The antibody did not block the activation of SMC by IL-1α. These results indicate that serum components (i.e., sCD14) are of major importance for the activation of SMC by LPS.

In order to provide further evidence for this suggestion, we depleted human serum of sCD14 and stimulated SMC with LPS under these conditions. We show in Fig. 5 that the vascular SMC responded well to LPS in the presence of normal human serum. Depletion of sCD14 markedly reduced this response. However, high concentrations of LPS (1 μg/ml) still stimulated the cells. These results also support our conclusion that soluble forms of CD14 are involved in the activation of vascular SMC by LPS. The following experiment (Fig. 6) further verified this result. Cells cultured without serum (none) did not respond significantly to stimulation by LPS (1,000 or 1 ng/ml). Supplementation of the serum-free medium with various concentrations of recombinant CD14 restored the capacity of the cells to respond to LPS. Activation of SMC was still mediated by concentrations of sCD14 as low as 50 ng/ml. In other experiments in which 5% CD14-depleted serum was used, the cells were activated in the presence of only 1 ng of LPS per ml and 3 μg of CD14 per ml, although cells cultured in FCS-containing medium did not respond to 1 ng of LPS per ml (data not shown). The above experiments (Fig. 4 to 6) show that soluble forms of CD14 mediate the activation of SMC by LPS.

DISCUSSION

Cytokine production of vascular EC and SMC or expression of adhesion molecules on EC following endotoxin challenge or cytokine activation might contribute to the pathology of septic shock, multiorgan failure, or other disease states such as adult respiratory distress syndrome (ARDS), vasculitis, or thrombosis (31). It was therefore of interest to investigate the nature of

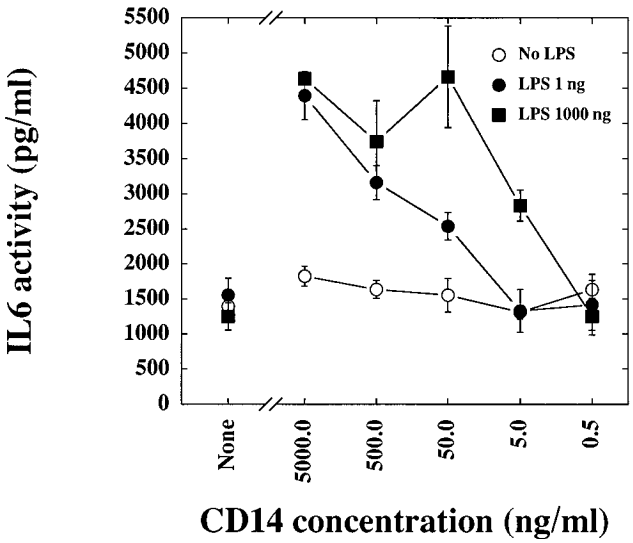

FIG. 6. Recombinant CD14 restores the capacity of serum-free-cultured SMC to respond to LPS. SMC were cultured in 96-well culture plates until confluent and incubated for 4 h in serum-free DMEM containing antibiotics and L-glutamine. The medium was replaced by serum-free medium supplemented with various concentrations of recombinant CD14 (5,000 to 0.5 ng/ml), and LPS was added (1,000 or 1 ng/ml) for 24 h. IL-6 was measured in the 7TD1 assay. One of two experiments is shown.

the LPS recognition mechanism involved in the activation of vascular cells. The application of specific LPS antagonists (13, 16, 36, 43, 50) appeared to be helpful in order to identify such a mechanism(s). Previous experiments with lipid A precursor Ia (compound 406) or a natural nontoxic LPS indicated the involvement of specific recognition molecules for LPS on vascular cells (2, 27, 35, 59). Here we provide evidence that endogenous CD14 is not expressed by vascular cells and that activation of SMC is mediated mainly by soluble serum-derived forms of CD14.

We have shown previously that lipid A is the active portion of LPS responsible for induction of immunostimulatory cytokines, such as IL-1, IL-6, and TNF-α, in human MNC (38, 56). The synthetic lipid A precursor Ia, an incompletely acylated lipid A partial structure, or inactive LPS of *Rhodobacter capsulatus* did not induce cytokine production in MNC. However, they potently inhibited LPS-induced cytokine production in human MNC (36, 44). This concept was proven to be applicable in various test systems (11, 17, 27, 45, 54). In contrast, only some data showed that LPS antagonists block endotoxin-induced responses of vascular cells cultured under serum-containing conditions. Pohlmann and colleagues reported that an enzymatically deacylated LPS inhibited stimulation of EC by LPS in the presence of 5% FCS (50). The same antagonist also inhibited LPS-induced prostacyclin production of EC (54). In IL-6 induction and adhesion experiments with vascular SMC or EC in the presence of FCS, the antagonists inhibited only lipid A- or endotoxin-stimulated responses, but not cytokine-induced activation (44, 59; unpublished results). This reduction of LPS-induced responses in vascular cells is possibly caused by a competition at the receptor level. Furthermore, it has also been shown that deacylated LPS bound to THP-1 cells as potently as lipid A or LPS but did not activate the cells, and an inhibitory regulation pathway was proposed (28).

Despite numerous efforts to define LPS-binding structures, only a few LPS receptors besides CD14 are well characterized. Some additional binding or receptor molecules have been isolated by biochemical methods, but have not been purified to homogeneity or cloned (69). Furthermore, an LPS-binding protein (LBP) that interacts with LPS was isolated and cloned (60, 61, 70). The LPS-LBP complex has been shown to bind to the CD14 surface molecule (66) present on monocytes. The LPS may also bind directly to CD14, since stimulation of MNC with LPS under serum-free conditions can be blocked by monoclonal anti-CD14 antibodies (35a).

The CD14 molecule is a potential candidate for an LPS receptor on vascular cells. It was therefore of interest to show whether this molecule might be involved in the activation of vascular cells by LPS. However, incubation of vascular cells with monoclonal anti-CD14 antibody Mo2 did not block LPS-mediated stimulation if the cells were cultured in FCS-containing medium, although it inhibited the response of monocytes to LPS (data not shown). These results, obtained in functional inhibition assays in biological systems, showed that vascular cells probably do not express endogenous CD14 on their surface. We provided further evidence for this hypothesis by using a more sensitive assay system, the complement-mediated lysis of cells. Under these conditions, we also did not detect CD14. In addition, we did not observe staining of either vascular EC or SMC in flow cytometry or cell ELISA experiments. In order to exclude the possibility that CD14 proteins of vascular and monocytic origin exhibit different antigenic properties, we also investigated the presence of CD14 mRNA. In Northern blot analysis, we did not find accumulation of CD14 mRNA in human vascular EC and SMC, indicating the lack of endogenous CD14 mRNA. This assumption was further verified by RT-PCR. In the experiments with RT-PCR, which is much more sensitive than the Northern analysis, we also did not detect CD14 mRNA, although sufficient intact mRNA was present in the preparations, as indicated by the positive RT-PCR of the same samples for IL-1α mRNA.

The results discussed above emphasize the presence of recognition mechanisms other than endogenous CD14 on vascular cells. The character of these molecules was unknown. It has been proposed that sCD14 molecules exist, which interfere with the activation of cells lacking phosphatidylinositol-anchored CD14. Recent publications have shown that these forms of CD14 contribute to the activation of endothelial or epithelial cells (1, 14, 23, 51, 53). Our own observations regarding endothelial cells support this view. Using recombinant CD14 under serum-free conditions or sCD14-depleted as well as sCD14-containing human serum, we could demonstrate that the soluble form of CD14 is also involved in the activation of SMC. However, the sCD14 mechanism may not represent the only mechanism for the activation of SMC, since high concentrations of LPS stimulate SMC, even in the absence of sCD14.

The data in this report indicate that vascular EC as well as SMC do not express CD14 mRNA or protein but that soluble serum-derived forms of CD14 are the main factors involved in activation of these cells by LPS. However, there is still the possibility that additional receptor molecules present on vascular SMC may contribute to LPS-mediated activation of these cells. Future work has to be performed to unravel the structure of these recognition sites and the mechanisms of LPS-induced activation of EC and SMC.

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