Hydrogen peroxide induces LFA-1-dependent neutrophil adherence to cardiac myocytes

HUIFANG LU, KEITH YOUNKER, CHRISTIE BALLANTYNE, MARK ENTMAN, AND C. WAYNE SMITH

Department of Microbiology and Immunology, Sections of Cardiovascular Science and Cardiology, The Methodist Hospital, Department of Medicine, and Speros P. Martel Laboratory of Leukocyte Biology, Department of Pediatrics, Baylor College of Medicine, Houston, Texas 77030

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
LFA-1-DEPENDENT NEUTROPHIL ADHESION TO MYOCYTES

(Indianapolis, IN). CL18/6 and CL18/1 (IgG1), anti-canine ICAM-1 MAb, were produced in our laboratory (28).

Construction of full-length LFA-1. The canine ICAM-1 cDNA sequence resides in three overlapping cDNA clones obtained from a lipopolysaccharide (LPS)-stimulated canine endothelial cell library (20). A full-length cDNA was constructed from these three clones by a series of subcloning and ligation. The first six nucleotides, AUGCUG, conserved among ICAM-1 of other known species, were subcloned into pGEM4 vector. A BglI site was introduced into the canine ICAM-1 sequence at a position homologous to that of the unique BglI site in human cDNA by PCR. The unique BglI site, at ~17 codons from the carboxyl end of domain 2 in human cDNA, was used to construct chimeric ICAM-1 having a canine amino terminus with domains D1 and D2 and a human carboxyl terminus with domains D3-D5 (Cl,2,H3–5) and vice versa (H1,2,C3–5).

L cell transfection. Canine ICAM-1 and canine-human chimeric ICAM-1-expressing L cell lines were generated using the cationic lipids N-[1-(2,3-dioleyloxy)propyl]-N,N,N-trimethyl ammonium methylsulfate (Boehringer Mannheim Biochemicals, Indianapolis, IN) transfaction by following the manufacturer’s instructions. Cells were allowed to recover in fresh medium for 72 h after the transfection and were then selected under 600 µg/ml Genticin (G418). Cells with high ICAM-1 expression were identified by flow cytometry analysis after single-colony picks with a cloning cylinder (Bellco, Vineland, N.J.). ICAM-1-transfected L cells were maintained in a selection medium that consisted of 600 µg/ml G418. Untransfected L cells were maintained in RPMI 1640 without G418. L cell transfectants were pretreated 25-mm coverslips and allowed to become visually confluent. A visual static adhesion assay was described in detail previously (29). Leukocytes were allowed to settle onto pretreated 0.1% gelatin, 0.5 mmol/l trypsinized with trypsin-EDTA. For 25-mm round coverslips samples were analyzed in a Becton Dickinson FACscan. After being incubated with antibody, cells were washed three times with DPBS and fixed in 1% paraformaldehyde. The samples were analyzed in a Becton Dickinson FACscan.

Preparation of L cell monolayers. L cell transfectants were trypsinized with trypsin-EDTA. For 25-mm round coverslips pretreated with 0.1% gelatin, 0.5 × 106 trypsinized L cells in 0.5 ml of culture medium were added and sedimented for 4 h at 37°C, 5% CO2. Two milliliters of culture medium were added, and the cells were grown to confluency within 2–3 days. Only confluent monolayers were used in the adhesion assay.

Isolation of canine polymorphonuclear neutrophils. Neutrophils were isolated from healthy mongrel dogs as described previously (30).

Canine neutrophil adherence to L cell transfectants. L cells or transfected L cells were plated onto gelatin (0.1%)-pretreated 25-mm coverslips and allowed to become visually confluent. A visual static adhesion assay was described in detail previously (29). Leukocytes were allowed to settle onto monolayers of L cells for a contact time of 500 s under static conditions. The number of neutrophils in contact with the monolayer was counted, and the chamber was inverted for an additional 500 s to allow the unattached leukocytes to fall off. The leukocytes remaining attached were counted, and this number was expressed as a percentage of the total number of cells initially contacting the monolayer. In studies designed to evaluate the involvement of β2-integrins or ICAM-1 in neutrophil adhesion, cells were preincubated as follows: the coverslip with L cell transfectants was treated with anti-ICAM-1 MAb at a concentration of 20 ng/ml in 1 ml of PBS for 30 min at room temperature and mounted in the adhesion chamber directly. Neutrophils were incubated with antibodies specific for integrin subunits at two to four times the saturating concentration at room temperature for 30 min. Chemotactic stimuli of 1% zymosan A (Sigma, St. Louis, MO)-activated serum (ZAS; prepared as previously described (12)) for canine neutrophils were added immediately before the cell mixture was injected into the adhesion chamber.

Isolation of canine myocytes. Healthy mongrel dogs weighing 10–15 kg were anesthetized with pentobarbital sodium. The heart was removed through the left lateral chest under sterile conditions and immediately placed in ice-cold saline. The aorta was then cannulated using a tubing adapter set for the individual heart, and the procedure for obtaining isolated myocytes was followed as described previously (12).

Canine neutrophil adherence to canine myocytes. Isolated canine myocytes were suspended in medium A at a concentration of 50,000/ml as previously described (12). Neutrophils and myocytes were cocultured in a volume of 0.4 ml and a neutrophil-to-myocyte ratio of 10:1 for various periods of time as indicated at 37°C. The cells were resuspended, and a small aliquot was transferred to a microscope slide for examination under phase-contrast or differential interference-contrast optics. For each preparation, the number of neutrophils adherent to each of 200 myocytes was counted. Myocytes were incubated in the presence of cytokines, including human recombinant IL-1β (Genzyme, Boston, MA) and human recombinant IL-6 (rIL-6; Genzyme) for 3 h at 37°C before the 2-min exposure to H2O2 (Sigma). The H2O2 applied to myocytes was neutralized by adding catalase (Sigma) for 1 min before the addition of neutrophils. In experiments with stimulated neutrophils, ZAS was added immediately before the neutrophil suspension was mixed with the suspension of myocytes. MAb was added to the neutrophil-myocyte suspension at the beginning of incubation and were present throughout the incubation period.

RESULTS

H2O2 induces adherence of canine neutrophils to cytokine-activated cardiac myocytes. We have shown previously that adhesion of canine neutrophils to cytokine-stimulated cardiac myocytes occurs only if a chemotactic stimulus such as ZAS, PAF (12), or IL-8 (18) is added to the cell mixture. Peak adhesion occurs within 60 min. In the present experiments with rIL-6-stimulated myocytes treated for 2 min with H2O2, neutrophil adhesion occurred without added chemotactic stimulus (Fig. 1A). This effect of H2O2 was limited to the cardiac myocytes, because catalase was added after the 2-min incubation before the addition of neutrophils. In contrast to results without H2O2 exposure, peak adhesion occurred within 15–20 min, and adhesion was maximal and then began to wane (Fig. 1B). When a chemotactic stimulus was added to the cell mixture, the early kinetics of adhesion were unchanged, but the duration of peak adhesion was extended to at least 60
min (Fig. 1B). H$_2$O$_2$ exposure of neutrophils did not induce adhesion to IL-6-stimulated cardiac myocytes (Fig. 2A), and addition of a chemotactic stimulus resulted in the same slow kinetics of adhesion seen in earlier studies with no significant increase in adhesion at 15 min of contact between leukocytes and myocytes.

The effect on the rate and duration of neutrophil adhesion of exposure of cardiac myocytes to H$_2$O$_2$ required prestimulation of myocytes with cytokines (Fig. 2B). Treatment of myocytes with cytokine or H$_2$O$_2$ alone did not cause significantly increased rapid neutrophil adherence in the absence of added chemotactic factors. When myocytes were preactivated with cytokines for 3 h and then exposed to H$_2$O$_2$ for 2 min, the number of adherent neutrophils at 15 min was increased significantly. This was true for myocytes stimulated with either rIL-6 or TNF-$\alpha$ (not shown).

**ICAM-1 and LFA-1 mediate H$_2$O$_2$-induced adherence of neutrophils to myocytes.** The potential role of ICAM-1 in neutrophil adhesion induced by H$_2$O$_2$ treatment of stimulated myocytes was analyzed using the MAbs CL18/1 and CL18/6. The binding characteristics of these two antibodies were evaluated on L cells expressing full-length canine ICAM-1 (Fig. 3). CL18/1 and CL18/6 did not bind to L cells expressing human ICAM-1 (data not shown). The binding specificities of these two antibodies were further characterized by using two chimeric ICAM-1 constructs, C1,2:H3–5 and H1,2:C3–5. These chimeras were transfected into L cells, and surface expression of chimeric ICAM-1 was detected by the MAbs CA7 and R6.5 recognizing human ICAM-1 domains 5 and 2, respectively (Fig. 4). CL18/1 bound to L cells expressing H1,2:C3–5 but not C1,2:H3–5, and CL18/6 bound to C1,2:H3–5 but not H1,2:C3–5.
The blocking effect of these mAbs was also tested by static adhesion assays. Isolated canine neutrophils exhibited some baseline adhesion to the parent L cell monolayers without expressed ICAM-1 (Fig. 5). L cell monolayers expressing full-length canine ICAM-1 supported a significantly higher level of adhesion. This increase above baseline was completely inhibited by CL18/6, the MAb recognizing the C1,2:H3–5 chimera. CL18/1 was nonblocking. The suggestion that CL18/6 blocked both LFA-1 (CD11a/CD18)- and Mac-1 (CD11b/CD18)-dependent adhesion was supported by the finding that the adhesion above baseline to ICAM-1-transfected L cells was inhibited 60% (P < 0.01, n = 5) by the MAb R7.1 (anti-CD11a), 50% (P < 0.01, n = 5) by MY-904 (anti-CD11b), and completely by a combination of R7.1 and MY-904 (P < 0.01, n = 5).

Both the early neutrophil adhesion induced by H2O2 exposure of the cytokine-stimulated myocytes (Fig. 6) and the delayed adhesion induced by the addition of chemotactic stimuli (13) were inhibited by the anti-ICAM-1 MAb CL18/6. Anti-CD18 produced the same degree of inhibition of adhesion as anti-ICAM-1 (Fig. 6). Using integrin subunit-specific antibodies, we evaluated the contribution of LFA-1 and Mac-1. As shown in Fig. 7, the H2O2-induced peak of neutrophil adhesion at 15 min of incubation was completely inhibited by the MAb R7.1 (anti-CD11a) in four separate experi-

---

**Fig. 3.** Binding of monoclonal antibodies (MAbs) CL18/6 (A) and CL18/1 (B) to canine intercellular adhesion molecule (ICAM)-1. L cells transfected with canine ICAM-1 were analyzed by flow cytometry after indirect immunofluorescence staining with MAb CL18/1 (20 µg/ml) and CL18/6 (20 µg/ml). Background binding of an Ig isotype-matched control is indicated (dotted lines).

**Fig. 4.** Different binding specificities for MAbs CL18/1 and CL18/6. L cells transfected with chimeric ICAM-1 with canine domains 1 and 2 and human domains 3–5 (C1,2:H3–5; A) or human domains 1 and 2 and canine domains 3–5 (H1,2:C3–5; B) were analyzed by flow cytometry after indirect immunofluorescence staining with anti-ICAM-1 antibodies indicated. R6.5 binds to human domain 2, and CA7 binds to human domain 5. These MAbs were used to distinguish the ICAM-1 chimeras. Background binding of IgG1 is indicated (dotted lines). Data indicate that CL18/1 recognizes only H1,2:C3–5 and that CL18/6 recognizes only C1,2:H3–5.

**Fig. 5.** Effects of MAbs CL18/1 and CL18/6 on canine neutrophil adhesion to canine ICAM-1. L cells (L-mock, open bars) or L cells transfected with canine ICAM-1 (L-ICAM-1, filled bars) were grown into confluent monolayers and placed in static adhesion chambers. Isolated canine neutrophils were stimulated with ZAS (1% vol) immediately before cells were injected into adhesion chamber. When MAbs were present, they were incubated with L cells for 30 min before adhesion assay, and they were retained with cells throughout time allowed for adhesion. *P < 0.01 compared with stimulus condition without MAbs (none); n = 5 experiments.
ments. Anti-CD11b antibody MY-904 produced some reduction in this peak, but inhibition was not >30% in any experiment. R7.1 also abolished the initial rapid rise in adherence of ZAS-activated neutrophils to H2O2-treated myocytes without affecting the slow kinetics of adhesion seen in early studies of ZAS-stimulated neutrophil adherence to cytokine-activated myocytes without H2O2 treatment. This slowly developing adhesion has been shown to be Mac-1 dependent by complete inhibition in the presence of MY-904 (13). Thus LFA-1 appears to mediate the rapid adhesion of neutrophils to H2O2-treated cardiac myocytes in the presence or absence of added chemotactic stimulation.

Evidence for PAF receptor activation in H2O2-induced neutrophil adhesion. Current evidence indicates that β2-integrins on unstimulated neutrophils reside in a low-affinity (affinity) state, and to function efficiently as adhesion molecules the neutrophils must be stimulated (e.g., with chemotactic factors). Previous studies showed that H2O2 treatment of endothelial cells would cause PAF receptor stimulation (19). The PAF-receptor antagonist WEB-2086 inhibited the H2O2-induced neutrophil adhesion to cardiac myocytes in a dose-dependent manner (Fig. 8). WEB-2086 also inhibited the rapid adhesion seen when ZAS-stimulated neutrophils contacted H2O2-treated, cytokine-stimulated cardiac myocytes, but it had no effect on the slowly developing adhesion seen when ZAS-stimulated neutrophils contacted cytokine-stimulated myocytes (Fig. 9). Another PAF-receptor antagonist, SDZ-64-412 (16), also inhibited the rapid adhesion in a dose-dependent fashion (e.g., 75% inhibition at 50 µM) when the ZAS-stimulated neutrophils contacted H2O2-treated, cytokine-stimulated cardiac myocytes (data not shown).

DISCUSSION

The results of the experiments in the current study show for the first time that canine neutrophils can utilize the β2-integrin LFA-1 (CD11a/CD18) to adhere to canine cardiac myocytes. This conclusion is supported by the following observations. 1) ICAM-1 (CD54), a well-known ligand for LFA-1, is expressed on cardiac myocytes after exposure of the myocytes to rIL-6, IL-1β, and TNF-α (12, 33). In the current studies, neutrophil adhesion to myocytes was demonstrable only when myocytes were stimulated to express ICAM-1. 2) Canine neutrophils adhered to recombinant chimeric ICAM-1 containing canine ICAM-1 domains 1 and 2 (the region shown to support human LFA-1-dependent adhesion to human ICAM-1), and this adhesion was
inhibited by R7.1 (anti-CD11a) and CL18/6 (an MAb that maps to domains 1 and 2 of canine ICAM-1). 3) These antibodies blocked adhesion of canine neutrophils to canine cardiac myocytes. Thus canine neutrophils can use LFA-1 to adhere to ICAM-1, and they can use this mechanism to attach to cardiac myocytes.

Our results also show that stimulation of myocytes with cytokines is not sufficient to promote LFA-1-dependent neutrophil adhesion. Although the presence of ICAM-1 on the parenchymal cell surface is necessary, stimulation of the neutrophil is also required. Addition of chemotactic factors to the culture medium promotes adhesion (12, 33), and, as we have shown, exposure of myocytes to H2O2 apparently generates a stimulus sufficient to promote neutrophil attachment. The molecular nature of this stimulus is obscure, but our finding that two different PAF-receptor antagonists, WEB-2086 and SDZ-64-412 (16), inhibit neutrophil adhesion is consistent with activation through the PAF receptor. Lewis et al. (19) showed that H2O2 treatment of endothelial cells generates oxidized phospholipids apparently capable of stimulating neutrophils via the PAF receptor to transiently increase their adhesion to endothelial cells. Such a mechanism appears to function at the surface of cardiac myocytes. We recently reported that activation of canine neutrophils through the PAF receptor will stimulate LFA-1-dependent adhesion to canine pulmonary interstitial fibroblasts in vitro (4).

In earlier studies we showed that adherent canine neutrophils will release substantial amounts of reactive oxygen (27). This is true of neutrophils attached to protein-coated, plastic endothelial cells (27) and cardiac myocytes (12, 13). With regard to cardiac myocytes, this release is measurable in two distinct compartments. Initially, reactive oxygen is detected intracellularly within both neutrophils and the myocytes to which they adhere (13); later, reactive oxygen is detected in the extracellular environment (12). The latter phenomenon is quantitatively equivalent to stimulation of neutrophils with phorbol esters (12, 23, 27). Thus adherent neutrophils are potential sources of H2O2 in the microenvironment of the cardiac myocyte surface. It remains to be shown that such H2O2 could initiate the sequence of events that we generated in vitro by addition of H2O2, but the hypothesis that adherent neutrophils can recruit additional neutrophils to adhere to cardiac myocytes is of potential interest. Our results indicate that adhesion to myocytes through such a mechanism would occur maximally within 15 min, would involve LFA-1 adhesion to ICAM-1, and, when combined with exogenous chemotactic stimulation, would result in transition from LFA-1- to Mac-1-dependent adhesion.

Our recent studies in a canine model of myocardial reperfusion do not negate this hypothesis. After 1 h of ischemia, neutrophils localize in the previously ische-
LFA-1-DEPENDENT NEUTROPHIL ADHESION TO MYOCYTES

H841

This work is supported by National Institutes of Health Grants HL-42550 and ES-06091.

Address for reprint requests and other correspondence: C. W. Smith, Section of Leukocyte Biology, Dept. of Pediatrics, Baylor College of Medicine, 1100 Bates Bm. 6014, Houston, TX 77030-2600 (E-mail: csmith@bcm.tmc.edu).

Received 19 july 1999; accepted in final form 1 September 1999.

REFERENCES


