The effect of interleukin-6 on L-selectin levels on polymorphonuclear leukocytes

TATSUSHI SUWA, JAMES C. HOGG, KEVIN B. QUINLAN, AND STEPHAN F. VAN EEDEN

McDonald Research Laboratory and iCAPTURE Centre, University of British Columbia, St. Paul's Hospital, Vancouver, British Columbia, Canada V6Z 1Y6

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INTERLEUKIN-6 (IL-6) is a major regulator of the acute phase response (4), and elevated blood levels have been implicated in the pathogenesis of sepsis (3), acute respiratory distress syndrome, and multiorgan failure (16). IL-6 is produced by different types of cells, including T cells, macrophages, and fibroblasts, and it mediates a wide variety of biological activities (5). IL-6 enhances differentiation and proliferation of multipotential hematopoietic progenitors in vitro (15, 19) and causes a leukocytosis characterized by a rapid neutrophilia with myeloid proliferation in the bone marrow (24). We have shown that IL-6 induces both a rapid neutrophilia by mobilization of polymorphonuclear leukocytes (PMN) from the marginated pool in the circulation and a delayed neutrophilia by releasing PMN from the bone marrow (20).

L-selectin has a crucial role in the attachment of circulating PMN to vascular endothelium during the initiation of PMN recruitment into a systemic inflammatory site (8, 11, 22). L-selectin levels on PMN are low in the mitotic pool, increases as PMN mature in the postmitotic pool of bone marrow (12, 29), and is constitutively expressed on circulating PMN (21). PMN released from the bone marrow by inflammatory stimuli such as complement fragments, pneumonia, and endotoxin express higher levels of L-selectin than their circulating counterparts (10, 25, 28) and they progressively lose this L-selectin as they age in the circulation (26). IL-6 also induces the release of PMN from the bone marrow, but in contrast to inflammatory stimuli, IL-6 treatment is associated with a decrease in L-selectin levels on circulating PMN (20). This decrease is the greatest at 6 h after IL-6 treatment (20) and is similar to that produced by glucocorticoid treatment (13). This is in contrast to a rapid decrease in PMN L-selectin levels that occurs with cell activation after stimuli such as endotoxin and C5a (7, 28).

The present study is based on the hypothesis that IL-6 decreases the L-selectin levels on circulating PMN by releasing PMN that express low levels of L-selectin from the bone marrow. To test this hypothesis, we studied the effects of IL-6 on the L-selectin levels on PMN in the bone marrow, on PMN newly released from the bone marrow by IL-6, and on PMN already in the circulation.

MATERIALS AND METHODS

Animals

This study was approved by the Animal Experimentation Committee of the University of British Columbia and was based on 39 female New Zealand White rabbits with an average weight of 2.3 ± 0.1 kg.

Experimental Design

Effect of IL-6 on L-selectin levels on all PMN in circulation. Rabbits were given 2 μg/kg of recombinant human IL-6 (catalog no. 118H0227, purity >97%, endotoxin <0.1 ng/μg IL-6) (Sigma; St. Louis, MO) in 3 ml of saline through the marginal ear vein (n = 12), and control rabbits (n = 10) received an equivalent volume of saline. This dose (2 μg/kg) of IL-6 was selected based on dose-response studies (17) in

Suwa, Tatsushi, James C. Hogg, Kevin B. Quinlan, and Stephan F. van Eeden. The effect of interleukin-6 on L-selectin levels on polymorphonuclear leukocytes. Am J Physiol Heart Circ Physiol 283: H879–H884, 2002. First published May 9, 2002; 10.1152/ajpheart.00185.2002.—In interleukin-6 (IL-6) shortens the transit time of polymorphonuclear leukocytes (PMN) through the marrow and accelerates their release into the circulation. In contrast to other inflammatory stimuli, this response is associated with a decrease in L-selectin levels on circulating PMN. The present study was designed to determine the effect of IL-6 on L-selectin levels of PMN in rabbits. Recombinant human IL-6 (2 μg/kg) caused a decrease in L-selectin levels on circulating PMN 3 to 12 h after treatment (P < 0.05). L-selectin levels decreased on PMN already in the circulation for up to 4 h (P < 0.05), on PMN released from the marrow posttreatment for up to 12 h (P < 0.01) and on PMN in the marrow for up to 6 h (P < 0.05) after IL-6 treatment. We conclude that IL-6 decreases L-selectin levels on circulating PMN by demargination of PMN with low levels of L-selectin and by releasing PMN from the marrow with low levels of L-selectin. We postulate that this prolonged downregulation of L-selectin on circulating PMN could influence their recruitment into inflammatory sites.

cytokine; bone marrow; neutrophil

Address for reprint requests and other correspondence: S. F. van Eeden, McDonald Research Laboratory, Univ. of British Columbia, St. Paul’s Hospital, 1081 Burrard St., Vancouver, BC, Canada V6Z 1Y6 (E-mail: svaneeden@mrl.ubc.ca).

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rabbits showing that it produced a high fever 30–90 min after intravenous injection and an increase in white blood cell counts 8–24 h after intravenous injection. Blood samples were obtained from the central ear artery before IL-6 or saline injection and then at 1, 3, 6, 9, 12, and 24 h after IL-6 or saline injection.

**Effect of IL-6 on L-selectin levels on PMN already in circulation.** To determine whether IL-6 has any effect on the L-selectin levels on PMN already in the circulation, the PMN of donor rabbits (n = 6) labeled with the thymidine analog 5’-bromo-2’-deoxyuridine (BrdU) (25 mg·kg⁻¹·day⁻¹; Sigma) was first infused through the marginal ear vein for 7 days and then transferred to recipient rabbits as 15 ml of whole blood. This method results in ∼80% BrdU labeling of PMN (1). The recipients were given 2 µg/kg recombinant human IL-6 (Sigma) in 3 ml of saline (n = 6) or 3 ml of saline (n = 5) intravenously 1 h after the blood transfusion (1). Blood samples were obtained from the central ear artery just before IL-6 or saline injection and at 0.5, 1, 2, 4, 6, 12, and 24 h after IL-6 or saline injection (20).

**Effect of IL-6 on L-selectin levels on PMN released from bone marrow.** The PMN in the bone marrow of 22 rabbits were pulse labeled with intravenous administration of 100 mg/kg of BrdU (Sigma), as previously described (1). Forty-eight hours later, either 2 µg/kg of IL-6 (Sigma) in 3 ml of saline (n = 12) or 3 ml of saline (n = 10) was administered intravenously. This time point was selected from previous studies (20, 23). Blood samples were obtained from the central ear artery just before IL-6 or saline injection and at 12 and 24 h after IL-6 or saline injection.

**Effect of IL-6 on L-selectin levels on PMN in bone marrow.** A 0.3-ml sample of bone marrow was aspirated from the left iliac bone 6 h after the intravenous injection of 2 µg/kg IL-6 (n = 6) or saline (n = 6) using an 18-gauge spinal needle. This time point was selected because it is associated with the maximum decrease in L-selectin levels on circulating PMN in IL-6-treated rabbits. Smears were made on 3-aminopropyltriethoxysilane-coated slides, allowed to air dry, and fixed in acetone.

**Flow Cytometry**

The blood collected in EDTA tubes was used to immunolabel circulating PMN for the presence of surface L-selectin and CD18 with the use of a whole blood method and commercially available kit (Coulter Clone, Coulter Immuno; Hialeah, FL) as previously described (25). Anti-L-selectin monoclonal antibody DREG-200 (kind gift of Dr. E. C. Butcher, Stanford University School of Medicine, Palo Alto, CA), anti-CD18 monoclonal antibody 60.3 (kind gift of Dr. J. Harlan, University of Washington, Seattle, WA) and nonimmune mouse IgG were used as negative controls. A total of 5,000 cells/specimen were evaluated, and the results are expressed as the mean fluorescence intensity.

**Immunocytochemical Detection of BrdU-Labeled PMN**

Blood collected (1 ml) in tubes containing acid-citrate-dextrose was used to obtain leukocyte-rich plasma. The cytospin specimen were made as previously described (23) and stained with the use of the alkaline phosphatase and anti-alkaline phosphatase (APAAP) method (1, 2) to determine the fraction of the BrdU-labeled PMN in each specimen (1) and calculate the transit of cells through the bone marrow, as previously described (23). All slides were coded and examined without knowledge of the group or sampling time, fields were selected in a randomized fashion, and 200 cells were evaluated per specimen.

**Number of BrdU-labeled PMN in Circulation**

The total white blood cell counts were determined with a counter (model SS80; Coulter Electronics). Differential counts of PMN were obtained by counting 100 leukocytes in randomly selected field of view on Wright’s stained blood smears. The number of BrdU-labeled PMN was then calculated with the use of these values. The number of BrdU-labeled PMN in the circulation of each recipient was expressed as a percentage of the total number of labeled PMN originally infused (14) and corrected for the calculated blood volume (18) of the recipient.

**Determination of L-selectin Levels on BrdU-labeled PMN in Circulation**

**Evaluation of L-selectin levels on BrdU-labeled PMN.** Cells on cytopsins were stained for the presence of both cell surface L-selectin (red) and nuclear BrdU (blue) using the APAAP and previously described method (13, 28). BrdU-labeled PMN was divided into three groups according to the intensity of surface staining of L-selectin using an arbitrarily designated grading system previously described (13). In the grading system, “strong” represents cells with >75% of their surface stained deep red, “weak” represents cells with positive and/or <75% of their surface stained deep red, and “negative” represents cells with no (background) red stain. The slides were coded and examined without knowledge of the group or the sampled time. Fields were selected in a systematic randomized fashion and 100 cells were evaluated per specimen.

**Determination of L-selectin Levels on PMN in Bone Marrow**

Bone marrow smears were fixed in acetone for 10 min before immunocytochemical staining using the APAAP technique and DREG-200 to label L-selectin, as described earlier. The preparations were counterstained with Mayer’s hematoxylin for 20 s, dehydrated through graded alcohol from 70–100% xylene, mounted, and evaluated on a light microscope. One hundred cells in randomly selected fields were evaluated and graded according to the intensity of staining for L-selectin as strong, weak, or negative. Just segmented and band PMN were evaluated.

**In Vitro Exposure to IL-6**

To determine whether IL-6 causes a change in L-selectin or CD18 levels on PMN through a direct mechanism such as PMN activation (9), we exposed PMN in vitro to increasing doses of IL-6. Whole blood (1 ml) collected in EDTA tubes was incubated with IL-6 at a concentration of 0, 1, 3, 10, and 30 ng/ml for 10 min, 1 h, or 4 h and labeled for the presence of surface L-selectin and CD18 as mentioned above. Results are expressed as the mean fluorescence intensity of four experiments.

**Statistical Analysis**

All values are expressed as means ± SE. Analysis of variance for repeated measures was used for continuous data. The results of mean fluorescence intensity and immunocytochemical staining were analyzed using paired or two-sample t-tests, and Bonferroni corrections (6) were made for multiple comparisons. Statistical significance was defined as a P value of >0.05.
RESULTS

Effect of IL-6 on L-selectin Levels on All PMN in Circulation

IL-6 decreased the mean fluorescence intensity of L-selectin on circulating PMN at 3–12 h (Fig. 1A). The maximum effect was seen at 6–9 h after IL-6 treatment ($P < 0.01$) and L-selectin levels were back to pretreatment levels at 24 h (Fig. 1A). CD18 did not change with IL-6 treatment (Fig. 1B).

Effect of IL-6 on L-selectin Levels on PMN Already in Circulation

Figure 2 shows the clearance of BrdU-labeled PMN from the circulation of recipients. After transfusion of the BrdU-labeled PMN, 1 h was allowed to achieve a stable count of BrdU-labeled PMN in the circulation (1). The fractions of the transfused BrdU-labeled PMN at baseline (1 h after blood transfusion) were similar in both the IL-6-treated rabbits (39.7 ± 1.5%) and control rabbits (40.7 ± 0.9%). In control rabbits, BrdU-labeled PMN present in the circulation gradually decreased and almost disappeared by 24 h after treatment. BrdU-labeled PMN increased after IL-6 treatment ($P < 0.05$ at 2–6 h compared with control) and then decreased and almost disappeared by 24 h after treatment. Figure 3 shows examples of PMN stained for both L-selectin (red) and BrdU (blue). The cells graded as either strong or negative gave the most reproducible results and were used to compare differences. L-selectin levels on BrdU-labeled PMN at baseline were similar in IL-6-treated and control rabbits (Fig. 4A). BrdU-labeled PMN stained strongly for L-selectin were decreased by 4 h in IL-6-treated compared with control rabbits. This de-
crease in L-selectin was larger in the IL-6-treated rabbits than in the control rabbits (Fig. 4B). There were too few BrdU-labeled PMNs in circulation at 12 h to make a reproducible measurement.

Effect of IL-6 on L-Selectin Levels on PMN Newly Released from Bone Marrow

Figure 5 shows the appearance of BrdU-labeled PMN from the bone marrow into the circulation. BrdU-labeled PMN counts at 48 h after labeling (baseline) were similar in both experimental and control rabbits. The number of BrdU-labeled PMN increased by 12 h and remained high until 24 h in both groups, but was greater in the IL-6-treated at 12 h compared with the control rabbits ($P < 0.01$). L-selectin levels on PMN newly released from the marrow (Fig. 6A) were higher than their counterparts already in circulation (Fig. 4A) ($P < 0.05$, L-selectin strongly expressed cells in IL-6 and control group). L-selectin levels on PMN newly released from the marrow at baseline were similar in IL-6-treated and control rabbits (Fig. 6A). L-selectin levels on BrdU-labeled PMN did not change throughout the study period in controls, but decreased by 12 h after treatment in the IL-6-treated rabbits (Fig. 6B) and recovered to baseline at 24 h (data not shown). BrdU-labeled PMN with strong staining of L-selectin decreases in the IL-6-treated rabbits at
Effect of IL-6 on L-selectin Levels on PMN in Bone Marrow

L-Selectin on PMN in the bone marrow was evaluated 6 h after IL-6 treatment. The results showed that L-selectin levels on PMN in the marrow were decreased 6 h after IL-6 treatment (Fig. 7) evident by the decrease of PMN with strong staining of L-selectin and an increase in PMN with weak staining in the IL-6-treated rabbits (P < 0.05 compared with control).

Effect of IL-6 In Vitro Exposure

L-selectin levels on PMN exposed to IL-6 (0–30 ng/ml) in vitro for 10 min are shown in Fig. 8. The results show IL-6 treatment in vitro for 10 min, 1 h, and 4 h did not change L-selectin levels on PMN (data not shown for 1 h and 4 h). CD18 levels on PMN also did not change with IL-6 treatment in vitro (data not shown).

DISCUSSION

This study shows that IL-6 decreases L-selectin levels on PMN by several different mechanisms. The early decrease (4–6 h) was associated with the IL-6-induced demargination of PMN, whereas the more prolonged reduction in PMN L-selectin levels that lasted up to 12 h was related to the release of immature PMN expressing low levels of L-selectin from the bone marrow. Furthermore, IL-6 treatment decreased L-selectin levels on PMN (segmented and band cells) in the bone marrow maturation pool. Thus this study showed that a variety of mechanisms could contribute to the IL-6-induced reduction in L-selectin levels on circulating PMN.

IL-6 causes a rapid decrease in L-selectin on circulating PMN observed as early as 3 h after treatment. Our results showed that this early decrease coincided with the demargination of PMN into the circulation induced by IL-6 (Fig. 2). We (27) have shown that demargination of PMN during exercise is associated with a decrease in L-selectin on circulating PMN and postulate that the population of PMN that reside in the marginated pool either express low levels of L-selectin or the demargination process results in shedding of L-selectin. We showed that incubation of PMN with IL-6 did not decrease their L-selectin levels (Fig. 8) or activate cells (no change in CD18 levels) that excluded the possibility that this early drop in PMN L-selectin levels is due to PMN activation by IL-6 treatment.

Pulse labeling of the dividing PMN in the bone marrow allowed us to determine L-selectin levels on labeled PMN released from the bone marrow into the circulation. Our results show that these newly released BrdU-labeled PMN express low levels of L-selectin at 12 h after IL-6 administration (Fig. 6). Our previous study (20) showed that there is a rapid release of PMN from the marrow in the 9- to 12-h period after IL-6 treatment. This association between the IL-6-induced release of PMN from the marrow and low levels of L-selectin on newly released PMN, supports the hypothesis that IL-6 promotes the release of PMN from the marrow with low levels of L-selectin. This concept was supported by the decrease in L-selectin levels on PMN in the maturation pool of the marrow (bands and segmented cells) 6 h after IL-6 treatment (Fig. 7).

Our previous study (20) showed that a single injection of IL-6 shortened the PMN transit time through the maturation pool of the bone marrow. Lund-Johansen and colleagues (12) and earlier studies from our laboratory (25) have shown that L-selectin levels increase on PMN in the maturation pool of the bone marrow. We postulate that the rapid transit of PMN through the maturation pool (20) induced by IL-6 treatment results in decreased levels of L-selectin on PMN when they are released from the bone marrow. Alternatively, IL-6 could directly reduce the transcription or translation of L-selectin on PMN in the marrow with reduced protein levels; however, further studies are needed to test this hypothesis.

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REFERENCES


