FEBS Letters 477 (2000) 84–88 FEBS 23886

Density-dependent induction of TNF-α release from human monocytes by immobilized P-selectin

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Received 6 April 2000; received in revised form 15 June 2000

Edited by Masayuki Miyasaka

Abstract P-selectin purified from human platelets, when immobilized on a solid surface, induced monocytes to release tumor necrosis factor-α (TNF-α). The induction of TNF-α release was dependent on the concentration of P-selectin used for the immobilization, and the maximal stimulation was observed when the plate was coated with 0.3 μg/ml of P-selectin. Use of either a higher or a lower concentration of P-selectin for the plate-coating was found to elicit less TNF-α release, although the higher concentration of P-selectin caused a stronger adhesion of HL-60 leukemic cells. The expression of mRNA for TNF-α roughly paralleled the TNF-α secretion, as assessed by RT-PCR. These results indicate that monocytes are activated by immobilized P-selectin in a density-dependent manner.

Key words: P-selectin; Cell adhesion; Leukocyte activation; Cytokine production; Tumor necrosis factor-α

1. Introduction

P-selectin is a carbohydrate-binding cell adhesion molecule expressed on activated endothelial cells and platelets. A number of studies have provided evidence that members of the selectin family (E-, L- and P-selectin) control leukocyte trafficking [1–4]. P-selectin-mediated leukocyte adhesion to vascular endothelial cells and activated platelets has been shown to play a crucial role in the initial step of the recruitment of leukocytes into inflammatory and hemorrhagic sites. It has been proposed that P-selectin binds to carbohydrate chains containing sialyl Lewis X (sLeX) structure [Sialic acid α2-3Gal β1-4 (Fuc α1-3) GlcNAc β1-R] (CD15S), which is distributed on the surface of leukocytes, especially neutrophils and monocytes [5,6]. A functional ligand for P-selectin was identified and designated as PSGL-1 (P-selectin glycoprotein ligand-1) [7].

We previously showed that P-selectin-dependent adhesion of activated platelets to neutrophils and monocytes led to the activation of these leukocytes to generate the extracellular superoxide anion [8,9]. This result suggested that P-selectin sent an activation signal into the leukocytes through its counter-receptor. We have also shown that sLeX at the termini of *O*-linked carbohydrate chains of membrane glycoproteins are essential in signal transduction leading to superoxide anion production [10]. In addition, studies have shown that P-selectin is able to stimulate other leukocyte functions, such as expression of tissue factor in monocytes [11] and phagocytotic activity of neutrophils and monocytes [12,13]. It has also been demonstrated that co-stimulation with P-selectin and platelet-activating factor induces monocytes to secrete monocyte chemotactic protein-1 and tumor necrosis factor-α (TNF-α) [14]. It is likely that the P-selectin-induced production of oxygen radicals, cytokines and coagulation factors will be implicated in the initiation and progression of the inflammation and blood coagulation processes. We recently found that immobilized P-selectin but not soluble P-selectin was able to induce leukocyte activation for the superoxide anion production, and that the redistribution of the counter-receptors for P-selectin containing sLeX carbohydrate chains was an essential process for the activation [15]. Thus, the signaling for the leukocyte activation induced by P-selectin seems to be somewhat different from that induced by other soluble stimulants. In this study, we investigated the ability of P-selectin to induce TNF-α production in monocytes, and found that immobilized P-selectin was able to induce extracellular release of TNF-α in a density-dependent manner.

2. Materials and methods

2.1. Reagents

Ficoll–Paque, ConA–Sepharose 4B and heparin–Sepharose 4B were products of Pharmacia (Uppsala, Sweden). Dextran 200 (special grade for leukocyte isolation; molecular weight: 180 000–210 000) was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). 2′,7′-Bis(carboxyethyl)carboxyfluorescein tetraacetoxymethyl ester (BCECF-AM) was a product of Dojindo Laboratories (Kumamoto, Japan). RPMI 1640 medium was a product of Gibco/BRL (Gaithersburg, MD, USA).

2.2. Antibodies

Monoclonal antibody (mAb) against human P-selectin (2T60, IgG1) [16] was a gift from Dr. K. Tanoue (the Tokyo Metropolitan Institute of Medical Science). Another mouse monoclonal anti-P-selectin antibody (2D7, IgG1) was established in our laboratory. For the purification of human P-selectin, mAb 2D7 was conjugated to CNBr-activated Sepharose 4B (Pharmacia) according to the manufacturer's instructions.

2.3. Cells

Monocytes were purified from normal human peripheral blood by a combination of dextran sedimentation and Ficoll–Paque gradient centrifugation [8,17]. Heparinized whole blood was mixed with an equal

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Abbreviations: TNF-α, tumor necrosis factor-α; sLeX, sialyl Lewis X; FBS, fetal bovine serum; PBS, phosphate-buffered saline; BSA, bovine serum albumin; RT-PCR, reverse transcription-polymerase chain reaction

PII: S0014-5793(00)01765-8

volume of 3% dextran/0.15 M NaCl and allowed to stand for 30 min. After most of the erythrocytes had settled as sediment at the bottom of the tube, the supernatant was centrifuged at 250×*g* for 10 min. The pelleted cells were then suspended in 10 mM sodium phosphate buffer (pH 7.3) containing 0.14 M NaCl (phosphate-buffered saline (PBS)) and layered on Ficoll–Paque solution. After centrifugation at 400×*g* for 20 min, cells at the interface were collected, suspended in PBS, and centrifuged at 250×*g* for 10 min. Cells were suspended at a density of 1×10^6 cells/ml in RPMI 1640 medium containing 10% FBS, and the cell suspension was incubated at 37°C for 30 min in a plastic culture dish. After non-adherent cells were removed by washing the dish twice with warm PBS, adherent cells were recovered with a cell scraper in PBS containing 0.02% EDTA. The cells were washed with RPMI 1640 medium containing 10% FBS. All procedures were performed under sterile conditions. The purity of the monocytes preparations was more than 90%, as evaluated by May–Gruenwald–Giemsa staining. The trypan blue exclusion assay showed that 99% of the cells were viable.

HL-60, a human myelocytic leukemia cell line, and L.P3, a subline of mouse L cells, were supplied by the RIKEN Cell Bank (Wako, Japan) and Health Science Research Resources Bank (Osaka, Japan), respectively. These cells were cultured in RPMI 1640 medium containing 10% FBS under a 5% CO_2 atmosphere.

2.4. Purification of P-selectin from human platelets

P-selectin was purified from human platelets as described by Johnston et al. [18] with the following modification. Human platelets (4.0 ml of packed cells) were lysed by sonication in 40 ml of PBS containing protease inhibitors (1 mM phenylmethanesulfonyl fluoride (PMSF), 0.2 mM leupeptin, 1 μM pepstatin, 1 mM *N*-ethyl maleimide, 0.1 mM aprotinin, 3.5 mM EDTA), and centrifuged at 100 000×*g* for 30 min at 4°C. The pellet was then suspended in 40 ml of PBS containing 1% NP-40 and the protease inhibitor cocktail described above, incubated at 0°C for 1 h, and centrifuged at 100 000×*g* for 30 min at 4°C. The supernatant thus obtained was applied to a column of ConA–Sepharose 4B (Pharmacia). After the column was washed with PBS containing 0.1% NP-40, bound glycoproteins were eluted with PBS containing 0.1% NP-40 and 0.5 M methyl-α-D-mannoside, and dialyzed against 10 mM sodium phosphate buffer (pH 7.3) containing 0.1 M NaCl and 0.1% NP-40 (0.1 M NaCl/0.1% NP-40/PB). The crude glycoprotein fraction was then applied to a column of heparin–Sepharose 4B (Pharmacia) which had been equilibrated with 0.1 M NaCl/0.1% NP-40/PB. The elution was sequentially conducted with 0.1 M NaCl/0.1% NP-40/PB, 0.25 M NaCl/0.1% NP-40/PB and 0.5 M NaCl/0.1% NP-40/PB. The eluate with 0.25 M NaCl/0.1% NP-40/PB from the heparin–Sepharose 4B column was then subjected to affinity chromatography on a column of anti-P-selectin antibody (2D7) conjugated to Sepharose 4B. After the column was washed with 0.1% NP-40/PBS and then with 1.5 M KSCN/0.1% NP-40, P-selectin bound to the column was eluted with 3 M KSCN/0.1% NP-40 and immediately dialyzed against PBS.

2.5. Monocyte culture and measurement of TNF-α

A 96 well culture plate was coated with purified P-selectin (0.04–20 μg/ml) in 0.2 M $NaHCO_3$ at 4°C for 16 h. After the well was washed with PBS three times and then blocked with 3% bovine serum albumin (BSA)/PBS, monocytes (5×10^5 cells/ml, 0.2 ml) were placed in a well and cultured at 37°C for 0.5–6 h. TNF-α secreted into the culture supernatant was measured by the cytotoxic test using the mouse fibroblastic cell line L.P3 as described previously [19]. One unit/ml of TNF-α was defined as the concentration which gave 50% cytotoxicity against L.P3 cells. The cytotoxic activity of the monocyte culture supernatant was almost completely blocked by the addition of an anti-TNF-α antibody (a gift from Dr. Masahiro Higuchi, Institute of Molecular Medicine for the Prevention of Human Diseases, Houston, TX, USA), indicating that the cytotoxicity was mainly caused by TNF-α. In some experiments, the level of TNF-α was also determined by ELISA (R and D systems, Minneapolis, MN, USA). 1 μg of TNF-α corresponded to 5×10^4 units in the cytotoxic assay.

2.6. Cell adhesion assay

The adhesion of HL-60 cells to P-selectin was assayed essentially as described previously [20]. Briefly, HL-60 cells were labeled with a fluorescent dye, BCECF-AM (3 μM), at 37°C for 30 min. The labeled cell suspension (1×10^6 cells/ml, 0.1 ml) in RPMI 1640/1% BSA was placed in a 96 well culture plate that had been coated with P-selectin under the same conditions as used for the TNF-α assay. After the plate was incubated at 0°C for 1 h, non-adherent cells were removed, the well was gently washed three times with RPMI 1640/1% BSA, and adherent cells were lysed with 0.2 ml of 1% SDS. The fluorescence intensity was measured with a fluorescence spectrophotometer (ex = 490 nm, em = 520 nm).

2.7. Reverse transcription-polymerase chain reaction (RT-PCR) analysis of TNF-α mRNA

Total cellular RNA was isolated from monocytes incubated with P-selectin using an Ultraspec™ kit (Biotecx Laboratories Inc., Houston, TX, USA) according to the manufacturer's instructions. Reverse transcription of 2 μg of total RNA was conducted with M-MLV reverse transcriptase (4 units/μl) (Gibco/BRL) and an oligo dT_{12-18} primer (50 ng/ml) (Pharmacia). An aliquot of the cDNA was then subjected to PCR using a sense primer, 5′-ATGAGCACTGAAAGCATGATC-3′, and an antisense primer, 5′-TCACAGGGCAATGATCCCAA-AGTAGACCTGCCC-3′ [21]. The PCR protocol consisted of 30 cycles of 1.5 min at 94°C, 2 min at 45°C, and 3 min at 72°C. The PCR products were analyzed by gel electrophoresis in 1.5% agarose followed by staining with ethidium bromide.

3. Results

3.1. Purification of P-selectin from human platelets

When purified P-selectin was analyzed by SDS-polyacrylamide gel electrophoresis under a reducing condition and stained by the silver staining method, a single band with M_r of approx. 140 kDa was observed (Fig. 1). Electrophoresis under a non-reducing condition gave a band with slightly higher mobility (data not shown), which was consistent with the previous report [18].

The level of bacterial endotoxin contamination in the P-selectin preparation was lower than 0.7 pg/μg P-selectin, as determined by the Limulus test using an Endotoxin Test kit (Seikagaku Kogyo Co., Ltd., Tokyo, Japan).

3.2. Induction of TNF-α secretion by immobilized P-selectin

We first examined whether P-selectin-mediated adhesion induced the release of TNF-α from human monocytes. The level of TNF-α in the culture medium of monocytes incubated for 6 h in a P-selectin-coated plate was increased about 20-fold relative to that in the medium incubated in a BSA-coated

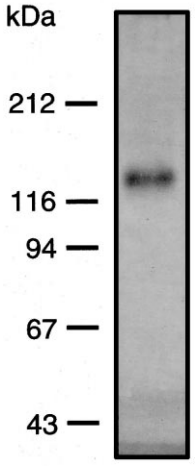

Fig. 1. Electrophoretic analysis of P-selectin purified human platelets. P-selectin purified by the procedure described in Section 2 was subjected to SDS-polyacrylamide gel electrophoresis under a reducing condition. The gel was stained by the silver-staining method. Molecular weights of marker proteins are given in kDa.

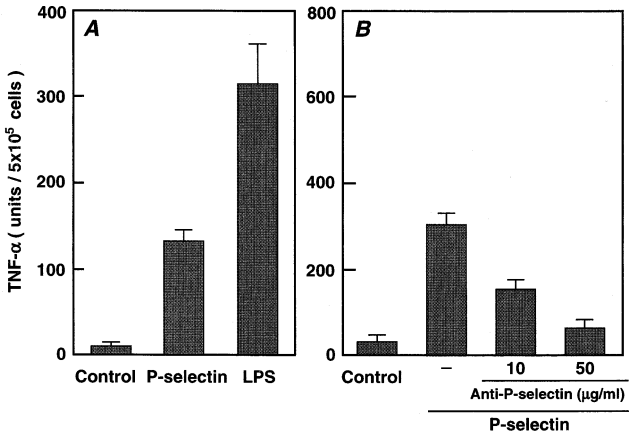

Fig. 2. Induction of TNF-α release from human monocytes by immobilized P-selectin. A: Human monocytes (5×10^5 cells/ml, 0.2 ml) were cultured at 37°C for 6 h in a 96 well plate which had been coated with P-selectin (10 μg/ml). TNF-α released in the supernatant was measured by the cytotoxic assay as described in Section 2. In a control experiment, monocytes were incubated in wells treated with the buffer (3 M KSCN/0.1% NP-40) that was used for the final step of the purification of P-selectin. Monocytes were also incubated with LPS (0.1 μg/ml) as a positive control. B: The effect of $F(ab')_2$ fragment of anti-P-selectin (2T60) was examined. The plate was coated with P-selectin (0.5 μg/ml). Triplicate assays were performed in each experiment; S.D. is indicated by an error bar.

plate (Fig. 2A). The increased TNF-α secretion was inhibited by the addition of a monoclonal antibody against human P-selectin (2T60) (Fig. 2B), indicating that the induction of TNF-α secretion was P-selectin-dependent. We next determined the time kinetics for TNF-α secretion from monocytes following their adhesion to P-selectin-coated plates. There was a significant increase in TNF-α activity in the culture supernatant 4 h following the adhesion (Fig. 3). The kinetics for P-selectin-induced TNF-α release was similar to that obtained for bacterial lipopolysaccharide (LPS).

Plates were coated with various concentrations of P-selectin, and each concentration was tested for its ability to activate monocytes. As shown in Fig. 4, the maximal TNF-α secretion was observed when monocytes were cultured in a plate coated with 0.3 μg/ml of P-selectin. The level of TNF-α in the supernatant of this culture (680 units/5×10^5 cells) was comparable to that induced by LPS. When either higher or lower concentrations of P-selectin were used to coat the plates, TNF-α release was less effectively induced. By contrast, measurement of the adhesion of HL-60 cells to plates coated with the same concentrations of P-selectin under the same condition resulted in a profile distinct from that for TNF-α secretion. The percentage of adhered cells increased with increasing concentration of P-selectin, and reached a maximal level at 5 μg/ml. This result indicated that the P-selectin-induced TNF-α secretion and adhesion were not necessarily correlated, but that there was a clear optimal density of immobilized P-selectin for the former effect.

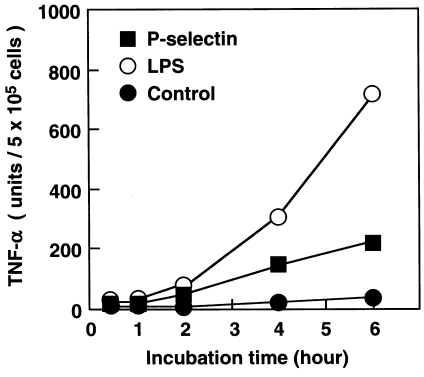

Fig. 3. Time course of TNF-α release induced by immobilized P-selectin. Human monocytes (5×10^5 cells/ml, 0.2 ml) were cultured at 37°C for 0.5–6 h in a 96 well plate which had been coated with P-selectin (10 μg/ml). TNF-α released in the supernatant was measured by the cytotoxic assay as described in Section 2. Control experiments were also conducted in the absence or presence of LPS (0.1 μg/ml) using uncoated plates.

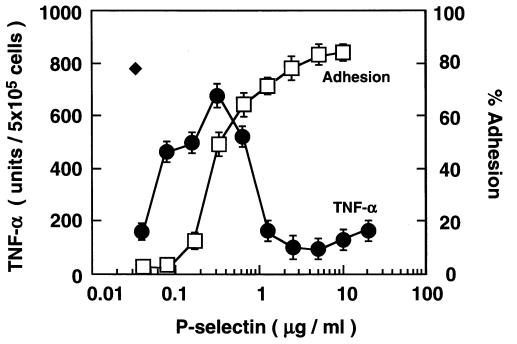

Fig. 4. P-selectin-induced TNF-α release from human monocytes and adhesion of HL-60 cells. For the TNF-α release assay, human monocytes (5×10^5 cells/ml, 0.2 ml) were cultured at 37°C for 6 h in a 96 well plate that had been coated with various concentrations (0.04–20 μg/ml) of P-selectin. TNF-α released in the supernatant was measured by the cytotoxic assay as described in Section 2 (●). TNF-α released from LPS (0.1 μg/ml)-treated monocytes was also measured (◆). For the adhesion assay, BCECF-AM-labeled HL-60 cells (1×10^6 cells/ml, 0.1 ml) were incubated at 4°C for 1 h in a 96 well plate that had been coated with P-selectin under the same conditions as for the TNF-α assay. After the non-adherent cells were washed, the fluorescence intensity of the adhered cells was determined (□).

3.3. Detection of mRNA for TNF-α by RT-PCR

Next, we attempted to detect mRNA for TNF-α in monocytes cultured in plates coated with various concentrations (0.1–10 μg/ml) of P-selectin by the RT-PCR method. The expected PCR product (702 bp) for human TNF-α was obtained from cells cultured with immobilized P-selectin (Fig. 5). Its identity was confirmed by restriction endonuclease diges-

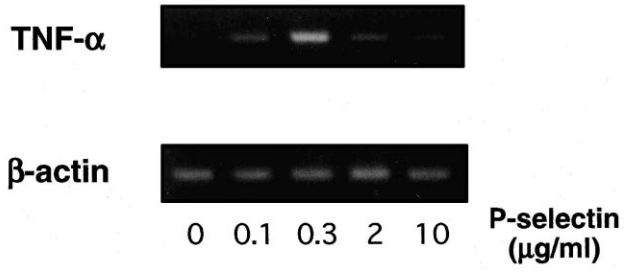

Fig. 5. Analysis of mRNA for TNF-α by the RT-PCR method. After human monocytes (10^6 cells/ml, 2.0 ml) were cultured at 37°C for 2 h in a 6 well plate that had been coated with various concentrations (0.1–10 μg/ml) of P-selectin, RNA was isolated and subjected to RT-PCR under the conditions described in Section 2. RT-PCR using primers for β-actin as an internal control was also conducted [22].

tion with *Ava* I, which yielded 452 and 250 bp fragments [21]. The relative intensities of the bands for each of the PCR products roughly paralleled the results of the TNF-α assay shown in Fig. 4. Again, this indicated that the density of P-selectin immobilized on the plate was critical for the monocyte activation. Since the control experiments showed that the levels of β-actin mRNA were similar between monocytes cultured with and those cultured without P-selectin, the efficiency of the RT-PCR was comparable among the experimental groups. The results also suggested that the induction of TNF-α secretion was transcriptionally regulated.

4. Discussion

It has been well demonstrated that P-selectin on activated endothelium and platelets plays a crucial role in leukocyte trafficking through its interaction with the counter-receptors containing carbohydrate chains on the leukocyte surface. In addition to its function in mediating intercellular adhesion, P-selectin has also been demonstrated to serve as a signal transmitting molecule. Several researchers, including the present authors, have shown that leukocyte functions such as tissue factor expression on monocytes [11], phagocytotic activity of neutrophils and monocytes [12,13], inflammatory cytokine secretion [14] and superoxide anion production in neutrophils [15] are potentiated by P-selectin alone or in combination with other agonists. On the other hand, Wong and co-workers reported that P-selectin had a suppressive effect on the production of oxygen radicals in neutrophils [23]. Thus, the function of P-selectin in cellular signaling remains controversial.

In the present study, we demonstrated that P-selectin purified from human platelets was capable of activating monocytes to release TNF-α (Fig. 2). Furthermore, the induction of TNF-α release was dependent on the density of P-selectin immobilized on the culture plates, and an optimal density of P-selectin for the efficient production of the cytokine was determined (Fig. 4). In the RT-PCR analysis, mRNA for TNF-α was found to be upregulated and the quantities of the amplified products were roughly correlated with the TNF-α release (Fig. 5). These results indicated that the high density of P-selectin was not necessarily required for the monocyte activation, although such density did support the firm cell adhesion. This observation might partly explain the disagreement in the reported effects of P-selectin on leukocyte activation. Although the reason for the insufficient activation of monocytes by higher density of P-selectin remains uncertain, we assume that the biphasic effect of immobilized P-selectin on TNF-α production was due to the difference in the extent of the cross-linking of the counter-receptor for P-selectin on the cell surface. We recently reported that the redistribution of the counter-receptor containing sLeX carbohydrate chains was essential for the leukocyte activation leading to superoxide anion production through P-selectin [15]. It is likely that moderate cross-linking of the counter-receptors is crucial for transmission of a signal into cells. However, the correlation between the distribution of sLeX epitopes on the cell membranes and TNF-α production, when various concentrations of P-selectin were used for the plate coating, has not been clearly demonstrated, and further studies will be needed on this subject.

It cannot be ruled out that the secretion of TNF-α may be negatively regulated by a feedback mechanism mediated by other cytokines [24] or cellular regulatory proteins [25]. Considering that no such phenomenon was observed when monocytes were stimulated by LPS and that TNF-α secretion occurred over relatively short periods, it is likely that the density-dependent and biphasic induction of TNF-α release by P-selectin involves a specific mechanism. Another possibility is that certain inhibitory factors contaminated the P-selectin preparation and reduced the efficiency of the TNF-α production. This seems unlikely, however, since a partial absorption experiment in which a high concentration (10 μg/ml) of P-selectin was adsorbed with a small amount of the anti-P-selectin antibody–protein G-agarose conjugate prior to the plate coating resulted in potentiation of TNF-α production (data not shown).

TNF-α is known to play a key role in orchestrating inflammation, immunity and thrombosis [26–28]. P-selectin has also been shown to play a major role in thrombogenic and inflammatory processes in the vascular system via its function in leukocyte recruitment. The expression of various cell adhesion molecules including E-selectin, another member of the family of endothelial selectins, is also induced by TNF-α. The combination of the stimulation by inflammatory cytokines and by adhesion molecules may exacerbate the pathogenesis of various inflammatory disorders. Wakefield and coworkers recently reported that the combined administration of anti-TNF-α and anti-P-selectin antibodies effectively attenuated the inflammation induced by venous thrombosis in rats [29]. The interaction of P-selectin and leukocytes may thus be one of the key steps in the etiology of various inflammatory diseases.

Acknowledgements: This study was supported by the Ministry of Education, Science, Sports and Culture of Japan, and by the Japan Health Science Foundation. We are grateful to Dr. K. Tanoue (Tokyo Metropolitan Institute of Medical Science) for his generous donation of monoclonal antibody 2T60. We also thank Dr. E. Miyashita (University of Tokyo Hospital) for her initial guidance in the isolation of human platelets.

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