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CD36-MEDIATED SIGNAL TRANSDUCTION IN HUMAN MONOCYTES BY ANTI-CD36 ANTIBODIES BUT NOT BY ANTI-THROMBOSPONDIN ANTIBODIES RECOGNIZING CELL MEMBRANE-BOUND THROMBOSPONDIN

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Mononuclear cells (MNC) treated with anti-CD36 Fab or $F(ab')_2$ fragments and then stimulated with anti-rabbit $(F(ab')_2$ displayed an oxidative burst, suggesting that the crosslinking of CD36 promotes signal transduction in the absence of an Fc receptor involvement. Moreover, intact anti-TSP mediates a weak oxidative burst in MNC, which was strongly enhanced upon pretreatment of monocytes (but not ymphocytes) with TSP. This response, however, was mediated by Fc receptors, not by an involvement of CD36. Other means of crosslinking cell-bound TSP and exposure of MNC to surface-bound TSP failed to promote an oxidative burst. Crosscompetition tests confirmed that the interaction site(s) of TSP with monocytes are distinct from the signal-promoting sites recognized by polyclonal and 3 monoclonal anti-CD36 antibodies.

CD36 is a surface constituent of 88 kD expressed by endothelial cells, phagocytes and a variety of neoplastic cells and cell lines *(1,2)*. It is a recognition structure for Plasmodium falciparum-infected erythrocytes *(3,4)*. A structurally very similar, if not identical, glycoprotein expressed on platelets, referred to as GPIIIb *(5)* or GPIV *(2,6)*, serves as a receptor for collagen *(7)* and for the adhesion glycoprotein thrombospondin (TSP) *(2,5)*, a platelet granule constituent released from platelets upon thrombin-stimulation and involved in the second, irreversible phase of platelet aggregation *(8)*. TSP is produced by several cell types, including mononuclear phagocytes *(9)*, and is a component of the extracellular matrix *(10,11)*. CD36 on monocytes and platelet-bound TSP have been implicated in the binding of platelets to monocytes *(6,12)*. TSP also assumes other cell communication tasks: TSP is an essential growth factor for certain smooth muscle cell lines *(13)*, it mediates monocyte cytotoxicity towards TSP-receptor-expressing squamous epithelial tumor cell lines *(14)* and it may be involved in the recognition of apoptotic cells by macrophages *(15)*.

Several lines of evidence suggest that CD36 acts as a signaling molecule, capable of mediating signal transduction and induction of effector functions upon crosslinking *(16-18)*. Using the activation of an oxidative burst as a measure for signal transduction, we studied the signal transduction induced by antibodies directed against CD36 and by antibodies recognizing cell-bound TSP. Here we confirm and

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Abbreviations: TSP, thrombospondin; HBSS, Hanks' balanced salt solution; MNC, mononuclear cell(s); CL, chemiluminescence.

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extend previously reported findings *(18)* that triggering of monocytes by anti-CD36 antibodies is mediated by CD36. In contrast, the crosslinking of cell-bound TSP promotes a signal only if crosslinking antibodies bridge to an Fc receptor. We suggest that a major TSP binding site on monocytes does not directly promote an oxidative burst and is therefore distinct from the binding sites of 3 different monoclonal antibodies and a polyclonal antiserum against CD36.

MATERIALS AND METHODS

Antibodies. Polyclonal anti-TSP sera was generated either at the Cornell Medical Center as described *(19)* or at the Institute of Veterinary Virology, Berne, by repeated immunization of rabbits with FPLC-purified, native human platelet thrombospondin (see below). Anti-TSP sera also contained antibodies against fibrinogen, as revealed in immunoblots and dot blots. Monoclonal antibodies against TSP were obtained from Sigma, Chemical, St. Louis, MO (mouse IgG1, clone TSP-B7) and from Chemicon (clone P10). GPIIIb (CD36) was prepared by a variation of the method described in *(5)*. Briefly, platelet membranes were prepared from washed platelets by sonication and differential centrifugation. The membranes were solubilized in Triton X-114 and separated into aqueous and detergent phases at 37°C. The detergent phase was then further chromatographed on Q-Sepharose and fractions containing pure GPIIIb were pooled and concentrated. Denaturing detergents were strictly avoided. Polyclonal anti-CD36 was obtained by immunizing rabbits with CD36 purified from platelets. Monoclonal antibodies against CD36 were provided by Ortho, Raritan, NJ, (OKM5 and OKM8) or were purchased from Immunotech S.A., Marseille, France (clone FA6-152). Monoclonal anti-fibronectin was purchased from Milan, La Roche, Switzerland (Cat. No. 216-381). Polyclonal goat anti-fibrinogen was from Organon-Teknika-Cappel, Durham, N.C. Fab fragments and $F(ab')_2$ fragments were prepared by plasmin cleavage or pepsin cleavage, respectively, and purifying the antibodies over protein A-Sepharose columns *(20)*.

Thrombospondin isolation. TSP was isolated from supernatants of thrombin-stimulated human platelets by FPLC *(21)*. It was dialysed against Hanks' balanced salt solution (HBSS) not more than 3 days before use. On SDS-PAGE, a major band was observed. Contamination with fibronectin and fibrinogen was below or slightly over detection limit in ELISA, using monoclonal antibodies for detection. In some experiments, TSP was isolated and stored in the presence of 0.1 mM Ca^{++}.

Cell isolation. Mononuclear cell (MNC) isolation from buffy coats of whole blood donations (Swiss Red Cross Blood Transfusion Service, Berne) was performed by a modified Ficoll-Hypaque procedure aimed at minimizing platelet contamination and exposure to trace amounts of endotoxin *(20)*. In some experiments, MNC were separated into lymphocytes (> 99 % pure) and monocytes (> 98 % pure) by centrifugation-elutriation *(22)*.

Protein pretreatment of cells. MNC were preincubated for 2-60 min with TSP or various control proteins or without added proteins in a rocking 37°C water bath at 4×10^6 monocytes/ml. After termination of the incubation, cells were first chilled in an ice bath and washed three times with ice cold HBSS prior to chemiluminescence measurement.

Chemiluminescence assay for monitoring signal transduction. Aliquots of 0.25 ml cell suspension, adjusted to 4×10^5 monocytes/ml in HBSS containing luminol-saturated bovine serum albumin *(23)* (final luminol conc. 5-10 μM) were distributed to 11 x 47 mm polystyrene tubes. These were dark-adapted in the cold, brought to 37°C in a water bath (4 min, unless otherwise indicated) and stimulated with antibody or control agents. Immediately thereafter, they were placed in a luminometer (950 Biolumat, Berthold, Wildbad, Germany) for recording the light emission for 2-3 min as described *(20)*. Control stimuli were HBSS (mock stimulation), heat-aggregated (11 min at 63°C) human standard gammaglobulin (Central Laboratory, Swiss Red Cross Blood Transfusion Service; 1 mg/ml), phorbol 12-myristate 13-acetate (10^{-7} M; Sigma), or serum-opsonized zymosan (50 μg/ml, Sigma, Cat.-No. Z-4250).

In other experiments, chemiluminescence (CL) was recorded in microtiter plates, using a single photon imaging system which permits the simultaneous measurement of two 96 well plates *(24)*. Cells were added to precoated microtiter plates in HBSS containing luminol. Plates were precoated for 3 hr with either TSP or with control proteins (human IgG, human serum albumin, human fibrinogen [Kabi, Stockholm, Sweden], heparin [Hoffmann-La Roche, Basel, Switzerland]) at 1 mg/ml at room temp. and vigorously washed. In some tests, TSP was added to wells precoated with fibrinogen or with heparin; It

was confirmed in an ELISA, using monoclonal and polyclonal anti-TSP and a peroxidase-conjugated anti-IgG antibody, that similar amounts of TSP bound to uncoated, fibrinogen-coated or heparin-coated plates.

RESULTS

CD36-specific antibodies promote signal transduction in monocytes.

MNC triggered with polyclonal anti-CD36 displayed an oxidative burst. In contrast, Fab fragments prepared from anti-CD36 failed to induce a response at concentrations at which intact antibodies were active; however, anti-CD36 Fab-pretreated, then washed cells showed a strong CL response upon triggering with goat $F(ab')_2$ fragments directed against rabbit $F(ab')_2$ (Fig. 1). $F(ab')_2$ fragments of anti-CD36 failed to elicit a burst, but upon 2nd order crosslinking by goat $F(ab')_2$ anti-rabbit $F(ab')_2$, a response was induced (Fig. 1). Untreated and anti-CD36-pretreated cells showed a similar response upon triggering with control stimuli, e.g. aggregated IgG. Goat-anti-rabbit $F(ab')_2$ per se did not stimulate cells (not shown). It has been shown earlier that luminol-enhanced CL as a manifestation of an oxidative burst of MNC was provided by monocytes alone *(18,25)*. These experiments thus confirm and extend our recent report *(18)* that monocyte CD36 is capable of transducing a signal and activating the NAD(P)H-dependent oxidase without involvement of Fc receptors.

TSP-specific antibodies promote signal transduction in monocytes.

Since CD36 is regarded as a monocyte TSP receptor *(2,6)* TSP, which is a multivalent ligand, was tested on its capacity to induces an oxidative burst in MNC. TSP concentrations up to 200 μg/ml failed to elicit CL in MNC (not shown). However, treatment of monocytes with polyclonal or monoclonal anti-

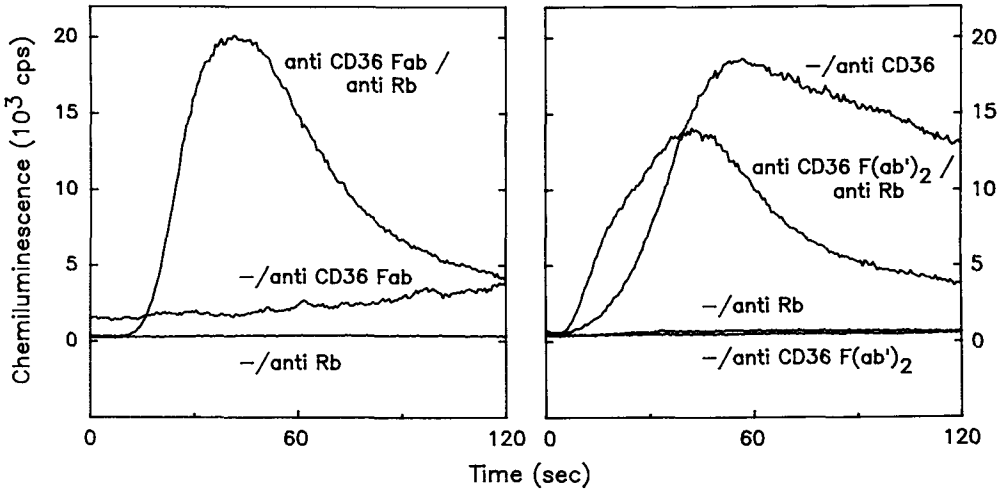

Figure 1. Signal transduction by polyclonal rabbit anti-CD36 antibodies and their fragments. Temporal traces of representative experiments obtained on two different days are shown. In some experiments, cells were preincubated with polyclonal rabbit anti-CD36 Fab (1 mg/ml at 4 x 10^6 monocytes/ml) or rabbit anti-CD36 $F(ab')_2$ (56 μg/ml for 4 x 10^6 monocytes/ml) and washed. These cells or untreated counterparts (-) were then stimulated with goat anti-rabbit $F(ab')_2$ [anti-Rb] (130 μg/ml) or with anti-CD36 in native form (170 μg/ml) or as $F(ab')_2$ fraction (112 μg/ml) or as Fab fraction (1 mg/ml), immediately prior to CL measurement.

Table 1. **The induction of an oxidative burst by triggering of TSP-pretreated MNC and untreated control cells with specific TSP antibodies**

Cell triggering prior to CL measurement	CL response *	
	Untreated MNC	TSP-pretreated MNC
Experiment 1		
Monoclonal anti-TSP (clone B7)	2,433 (108)	23,074 (96)
Monoclonal anti-TSP (P10)	3,833 (98)	52,974 (103)
Polyclonal anti-TSP	4,658 (>120)	33,366 (>120)
Preimmune rabbit serum	283 (none)	958 (61)
aggregated IgG	6,458 (46)	22,124 (36)
HBSS	219 (none)	608 (none)
Experiment 2		
monoclonal anti-TSP (B7)	1,891 (62)	13,166 (59)
monoclonal anti-TSP (P10)	3,450 (62)	15,366 (70)
polyclonal anti-TSP	3,258 (99)	14,733 (92)
monoclonal anti-fibronectin	300 (none)	1,100 (none)
aggregated IgG	4,967 (48)	14,275 (37)
HBSS	327 (none)	3,375 (none)

* Values represent peak response in counts per sec (means of 2 or 3); values in brackets designate the time (sec.) after the addition of the stimulus until peak response was reached. "none" refers to insignificant peaks.

TSP antibodies displayed a small but significant response whereas preimmune rabbit serum, anti-fibronectin or a number of other anti-monocyte antibodies did not (Table 1 and data not shown). MNO pretreated with TSP (up to 500 μg/ml) and washed showed a strongly enhanced oxidative burst upon treatment with anti-TSP (Table 1). CL induced with other stimuli was enhanced to a lesser degree by TSP pretreatment. CL enhancement depended on the TSP dose used for preincubation; no plateau was noted up to 1 mg/ml TSP. TSP-cell association was relatively fast; a preincubation time of 15 min was sufficient to yield a maximal antibody-induced CL response. Experiments with purified monocytes and lymphocytes proved the requirement of monocyte exposure to TSP for obtaining an enhanced burst upon antibody treatment; antibody stimulation of TSP-pretreated lymphocytes coincubated with nonexposed monocytes did not yield a burst. These experiments may be interpreted in several ways The possibility that antibodies recognize CD36-bound TSP, thereby crosslinking cellular CD36 which then mediates CL was examined more closely.

Evidence that anti-TSP-mediated signal transduction is distinct from anti-CD36-mediated signal transduction.

$F(ab')_2$ fragments prepared from anti-TSP sera failed to induce an oxidative burst in TSP-treated cells (Fig. 2). The unresponsiveness could not be attributed to an inactivation of antibody pepsin cleavage, or

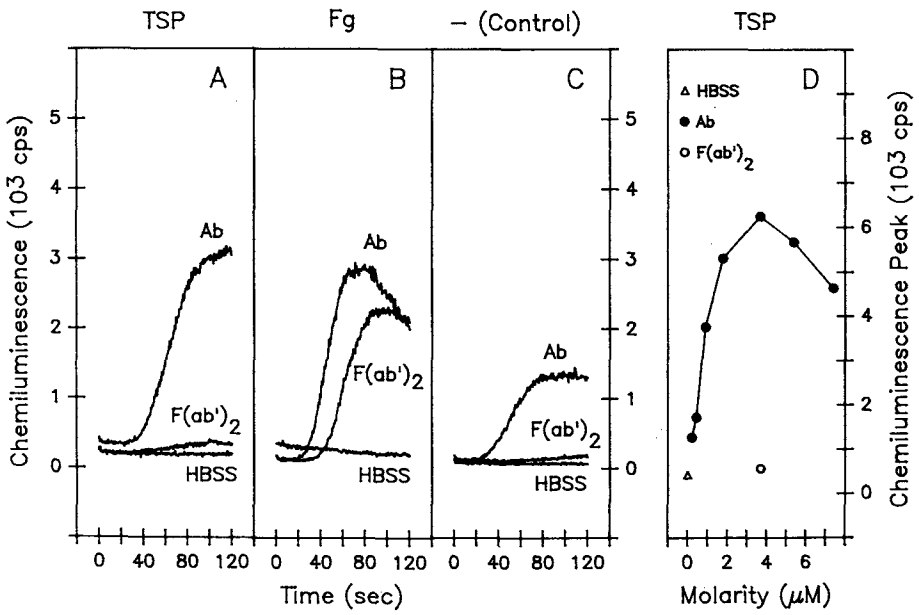

Figure 2. Failure of anti-TSP F(ab')$_2$ to elicit an oxidative burst in TSP-pretreated mononuclear cells. Cells were pretreated either with 500 μg/ml TSP or with 1 mg/ml fibrinogen, or were mock-treated, for 45 min. Then, they were washed and dark-adapted at 4°C, warmed to 37°C and stimulated with HBSS (neg. control) or with the indicated concentration of either intact anti-TSP IgG [Ab], or the F(ab')$_2$ fragments thereof [F(ab')$_2$]. A-C: Temporal traces of representative experiments all performed with the same donor cells are shown. D: Dose response relationship between antibody concentration and CL response.

to an insufficient concentration, since F(ab')$_2$ was used at concentrations which were clearly stimulatory for intact antibodies. Moreover, the same preparation which also contained anti-fibrinogen activity, could induce CL in cells pretreated with fibrinogen (Fig. 2). It has been shown earlier that crosslinking of cell-bound fibrinogen was induced by anti-fibrinogen IgG as well as by anti-fibrinogen F(ab')$_2$ *(20,26)*.

Cell-bound TSP was crosslinked in various ways other than by antibody. TSP-treated cells which then were exposed to TSP-binding ligands (heparin or fibrinogen) displayed no, or a weak burst. In other experiments, crosslinking was attempted by exposing MNC to polystyrene-bound TSP. TSP was preincubated in untreated polystyrene plates, in plates treated with heparin, or with fibrinogen. IgG-coated and fibrinogen-coated wells served as positive controls, and human serum albumin-coated wells as negative controls. Plates were vigorously washed, and MNC suspended in luminol-supplemented HBSS were distributed to these plates, which were placed in a single-photon-imaging system measuring accumulated light emission over 45 min. TSP-coated wells failed to induce CL over the background response, regardless of whether TSP was allowed to adhere at random (polystyrene coating), or to plate-bound heparin (Fig. 3). TSP adhering to plate-bound fibrinogen elicited a CL response indistinguishable from that induced by the first coat alone (Fig. 3). We thus were unable to prove that plate-bound TSP elicits a CL response by crosslinking a putative TSP receptor.

MNC were triggered with polyclonal or monoclonal anti-CD36 in the presence or absence of relatively large amounts of TSP. Anti-CD36-induced CL was enhanced in TSP-exposed cells. These cells showed

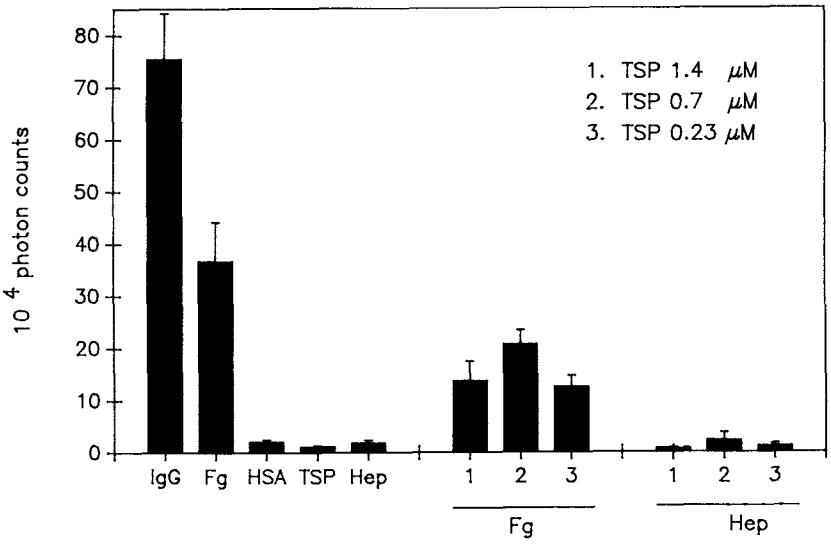

Figure 3. CL response of MNC adhering to microtiter plates precoated with TSP or control proteins (all at 1 mg/ml for 3 hr), or precoated first with heparin (Hep) or fibrinogen (Fg), and then with varying doses of TSP. Results are means of triplicates as obtained in two independent experiments.

a similar enhancement of CL triggered with aggregated IgG. Comparable results were obtained with TSP-pretreated cells. This suggests that TSP does not block the site occupied by antibodies inducing a CD36-mediated burst.

DISCUSSION

CD36 expressed by monocytes is regarded as a TSP receptor *(2,5)*, mediating in part the adherence of thrombin-stimulated platelets to monocytes *(6,12)*, and the phagocytosis of apoptotic cells *(15)*. We have reported that monocyte CD36 is a signaling molecule, capable of mediating an oxidative burst *(18)*. Anti-CD36-mediated signal transduction was also reported for platelets, although a participation of antibody Fc portion has not been ruled out *(16,17)*. More recently, divalent $F(ab')_2$ fragments of antibodies were shown to promote platelet aggregation (Clemetson et al., unpublished observation). These fragments as well as monovalent (Fab) fragments did not stimulate an oxidative burst in monocytes, but did transmit a signal upon 2nd-order crosslinking by anti-Fab fragments (Fig. 1). Taken together, this suggests that CD36 is a signaling molecule, and that monocyte CD36 requires crosslinking by antibodies, or polymeric natural ligands. Why polyclonal antisera required 2nd-order crosslinking to induce CL in monocytes, whereas monoclonal antibodies or their $F(ab')_2$ fragments were directly stimulatory, is presently not known.

Thrombospondin has recently been implied as a mediator of monocyte-mediated cytotoxicity towards a squamous epithelial tumor cell line *(14)* and serves as a mediator of cellular function(s) in other systems as well *(13)*. This notion prompted us to test whether TSP may promote signal transduction in monocytes. The induction of CL by crosslinking cell-bound TSP appeared to be an appropriate system for several reasons. Firstly, previous studies showed that antibodies against another monocyte ligand,

fibrinogen, elicit a CL signal *(20)*. Secondly, antibody crosslinking of many surface determinants of monocytes was followed by a CL signal, and the close temporal association points to a causal relationship *(18,20)*. Thirdly, crosslinking of anti-CD36, a putative TSP receptor, ensued one of the strongest CL responses induced by antibodies *(18)*. Finally, CL recording in monocytes is more sensitive that the measurement of enzymatic activities. Our findings suggested that signaling through monocyte-bound TSP, if it occurs, must be distinct from CD36-mediated signaling in important respects, since no burst could be elicited by treating MNC with TSP, or by crosslinking cell-bound by multivalent TSP ligands (heparin, fibrinogen) or by exposure of cells to surface-bound TSP. The only way cell-bound TSP could be induced to mediate a burst was by treating MNC with intact monoclonal or polyclonal anti-TSP antibodies. However, this reaction was not mediated by a putative TSP receptor, but by antibody bridging to an Fc receptor, as shown by the use of anti-TSP $F(ab')_2$ fragments. The antibody-crosslinking of TSP, therefore, does not mimic the antibody crosslinking of CD36, which may transmit a signal without the involvement of Fc receptors. This is true for both constitutively expressed TSP giving rise to a small antibody-induced burst, and for TSP-pretreated MNC showing a much stronger burst upon antibody treatment.

The failure to detect an oxidative burst upon crosslinking of cell-bound TSP does not rule out that signals not involving the burst-generating system may be perceived upon TSP crosslinking, but they demonstrate that signaling by cell-bound TSP is distinct from that mediated by CD36.

The ability of intact anti-TSP antibodies to elicit enhanced CL in TSP-pretreated MNC suggested that some cells firmly bind TSP. Experiments with elutriation centrifugation-separated lymphocytes and monocytes proved that TSP has to be bound by monocytes in order to mediate CL. The exact manner how TSP interacts with monocytes in our system remains to be determined. However, regardless of whether this TSP binding site or monocytes is, or is not, on CD36, it must be distinct from the site on monocyte CD36 to which burst-inducing antibodies bind. Further evidence for a dissociation of the TSP binding site from the anti-CD36 interaction sites stems from cross competition experiments which showed that anti-CD36 readily stimulates monocytes regardless of co-incubation or pretreatment with high concentrations of TSP. Monocyte determinants other than CD36, e.g. integrins and heparin sulfate proteoglycans have also been implicated in TSP binding *(27)*.

In conclusion we show here that certain properties attributed to anti-CD36 antibodies, namely their capacity to elicit an oxidative burst by antigen crosslinking, are not mimicked by antibodies directed towards cell-bound TSP. It remains to be seen whether TSP in this setting is bound by a silent, non-signaling site on CD36, or by another monocyte surface-determinant. Our studies call for further analysis of TSP-independent functions of CD36 in host defense.

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