Five Tumor Necrosis Factor-inducible Cell Adhesion Mechanisms on the Surface of Mouse Endothelioma Cells Mediate the Binding of Leukocytes

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Abstract. We have distinguished five TNF-α-inducible cell adhesion mechanisms on microvasculature-derived endothelioma cells of the mouse which mediate the binding of different types of leukocytes. Three of these mechanisms could be identified as the mouse homologs of ICAM-1, VCAM-1, and E-selectin, of which the latter was defined by the novel mAb 21KC10. The fourth TNF-α–inducible cell adhesion mechanism was blocked by antibodies specific for mouse P-selectin. We have recently shown that TNF-α stimulates the synthesis of P-selectin in mouse endothelioma cells (A. Weller, S. Isenmann, D. Vestweber. 1992. *J. Biol. Chem.* 267:15176–15183). Here we show that this stimulation leads to maximal cell surface expression levels within 4 h after stimulation while the same endothelioma cells are also able to upregulate P-selectin at the cell surface within minutes after stimulation with PMA. Both effects are additive.

The fifth TNF-induced cell adhesion mechanism is defined by mediating the binding to the mouse monocyte/macrophage cell line J774. This adhesion mechanism is not inhibited by antibodies against any of the other four CAMs; it functions well at 7°C (in contrast to ICAM-1 and VCAM-1) and it is as active after 16 h of TNF induction as after 4 h (in contrast to E- and P-selectin). Furthermore, this new adhesion mechanism only functions on two of three endothelioma cell lines and is undetectable on the third, although ICAM-1, VCAM-1, E-selectin, and P-selectin could be demonstrated to function well on this cell line. Thus, in addition to the three known TNF-inducible CAMs, ICAM-1, VCAM-1, and E-selectin, also P-selectin and a fifth, as yet molecularly undefined cell adhesion mechanism, are TNF inducible at the cell surface of mouse endothelioma cells.

MIGRATION across the blood vessel wall is a prerequisite for leukocytes to mediate host defense mechanisms at sites of bacterial invasion or tissue damage. The central step in this migration process is the adhesion of leukocytes to activated endothelial cells lining the venules of such inflamed tissues. Cytokines and other inflammatory mediators induce cell adhesion molecules (CAMs)[1] on the surface of activated endothelial cells which allow leukocytes to recognize sites of inflammation and to bind to the blood vessel wall (Osborn, 1990). This adhesion process has been proposed to be based on a cascade of molecular interactions (Butcher, 1991; Shimizu et al., 1992) and is mediated by multiple pairs of CAMs.

The selectins (Bevilacqua et al., 1991) are at present the best candidates for CAMs which function very early in the cell binding process and may even mediate the initial cell contact. Two of them, E- and P-selectin, are expressed on endothelial cells (Bevilacqua et al., 1989; Geng et al., 1990), the third, L-selectin, is expressed on all leukocytes (Siegelman et al., 1989; Camerini et al., 1989). Antibody blocking studies have demonstrated, in vivo, that L-selectin is involved in "leukocyte-rolling" on the blood vessel wall which describes the initial, transient interactions of leukocytes with the surface of endothelial cells (von Andrian et al., 1991). This could also be the function of P-selectin which is stored in intracellular granula and is the most rapidly inducible endothelial CAM. It is transported to the cell surface within minutes upon stimulation by pre-inflammatory mediators, such as thrombin and histamine (Geng et al., 1990). For this selectin, binding to polymorphonuclear granulocytes (PMNs, neutrophils) in vitro could be demonstrated under shearing forces resembling those in flowing blood, while another endothelial CAM, ICAM-1, could only support PMN binding under static incubation conditions (Lawrence and Springer, 1991). In agreement with this, antibodies against CD18 (Mac-1) did not inhibit leukocyte rolling (von Andrian et al., 1991). Based on these in vitro and in vivo data, the selectins have been proposed to function as "rolling receptors" while ICAM-1 is probably essential for

1. *Abbreviations used in this paper*: CAM, cell adhesion molecule; HUVEC, human umbilical vein endothelial cell; PCR, polymerase chain reaction; PMN, polymorphonuclear granulocytes.

leukocyte binding at a later stage in the cell adhesion process.

In addition to mediating different molecular steps in the adhesion process, a variety of endothelial leukocyte receptors is necessary to allow the fine regulation of the extravasation of different categories of leukocytes. E- and P-selectin support the binding of PMNs and monocytes (Larsen et al., 1989; Geng et al., 1990; Bevilacqua et al., 1987) and also of certain subsets of lymphocytes (Picker et al., 1991; Shimizu et al., 1991; Damle et al., 1992). ICAM-1 binds to a larger repertoire of leukocytes including PMNs, monocytes, and lymphocytes (Kishimoto et al., 1989). VCAM-1 which is, like ICAM-1, also a member of the immunoglobulin super-gene family, mediates the binding of lymphocytes and monocytes, but not of PMNs to endothelial cells (Osborn et al., 1989).

All these endothelial CAMs are inducible at the surface of endothelial cells partly by different mechanisms and with different kinetics. On cultured human umbilical vein endothelial cells (HUVECs), the synthesis of ICAM-1, VCAM-1, and E-selectin was found to be stimulated by TNF-α and IL-1β (Osborn, 1990). In addition, γ-IFN was shown to stimulate the expression of ICAM-1 (Pober et al., 1986) and IL-4 was found to induce the expression of VCAM-1 (Thornhill et al., 1990). The kinetics of expression differs: E-selectin is rapidly induced with maximal expression levels at the cell surface 3–4 h after stimulation by TNF-α and is down regulated within the next 20 h (Bevilacqua et al., 1987). In contrast, cell surface expression of ICAM-1 and VCAM-1 reaches maximal levels 4–6 h after TNF-α induction and these levels are maintained for more than 48 h (Dustin and Springer, 1991; Osborn, 1990). For P-selectin, only the thrombin and histamine induced transport regulation, which acts within minutes, has been described on HUVECs.

In this report we have used various mouse endothelioma cell lines derived from microvasculature of different tissues (Williams et al., 1988) in order to search for TNF-inducible endothelial leukocyte adhesion molecules. We show, using cell adhesion assays, that in addition to ICAM-1, VCAM-1, and E-selectin, two more leukocyte adhesion mechanisms are TNF inducible at the surface of endothelial cells. We found recently that the synthesis of P-selectin, similar to that of E-selectin, is TNF inducible in mouse endothelioma cells (Weller et al., 1992). Here we report, that this TNF-induction leads to functionally detectable P-selectin at the cell surface and demonstrate that mouse endothelioma cells regulate the transport to the cell surface as well as the synthesis of P-selectin. The fifth TNF-inducible cell adhesion mechanism which we found on mouse endothelioma cells, mediates the binding of the mouse monocyte/macrophage cell line J774 and is not identical with any of the other four TNF-inducible CAMs.

Materials and Methods

Cells

The mouse endothelioma cell lines sEnd.1 and eEnd.2 (Williams et al., 1988) as well as bEnd.3 (W. Risau, Max-Planck-Institute for Physiological and Clinical Research, Bad Nauheim, Germany, and L. Williams, Ludwig Institute for Cancer Research, Melbourne, Australia) had been established from polyoma-induced hemangiomas in skin (sEnd.1), in the embryo (eEnd.2), and in brain (bEnd.3) and were grown in DME with 10% FCS. The mouse T cell lymphoma TK-1 and the B cell lymphoma L1-2 were both obtained from Dr. Bernhard Holzmann (Klinikum rechts der Isar, München, Germany) and cultured in RPMI, with 10% FCS and 20 μM β-mercaptoethanol.

The human monocytic HL60 and mouse monocytic J774 cells were purchased from American Type Culture Collection (Rockville, MD) and cultured in DME with 10% FCS, as were the mouse myeloma cells SP2/0 and J558. PMNs were freshly isolated from the femurs of 10-wk old NMRI mice by flushing out the bone marrow cells with cold PBS, using a syringe and a 23 g needle. The cell suspension was filtered through a nylon tissue.

Antibodies

The rat mAb YN1/1 against mouse ICAM-1 (MALA-2; Takei, 1985) was kindly provided by Dr. Fumio Takei (Vancouver) and the rat mAb MK-2 against mouse VCAM-1 (Miyake et al., 1991) was a generous gift from Dr. Paul Kincade (Oklahoma Medical Research Foundation, Oklahoma City, OK) and was later produced from the American Type Culture Collection CRL 1909 hybridoma. The hybridoma RI-2 (Holzmann et al., 1989) producing a rat mAb against the integrin chain α_4 was obtained from Dr. Bernhard Holzmann. The anti-mouse CD44 mAb KM201 (Miyake et al., 1990) was produced from the ATCC rat hybridoma TIB240 and the anti-CD11a mAb M17/4.4.11.9 (Sanchez-Madrid et al., 1982) and the anti-CD11b mAb M1/70.15.11.5HL (Springer et al., 1978) were produced from the ATCC rat hybridomas TIB 217 and TIB 128, respectively. The rat mAb MECA 32, recognizing an endothelial specific antigen, was produced from a hybridoma which was kindly provided by Dr. Eugene Butcher (Stanford University School of Medicine, Palo Alto, CA).

mAbs against TNF-inducible endothelial cell surface antigens were produced by immunizing Lewis rats with bEnd.3 or sEnd.1 cells that had been treated with 200 U/ml human recombinant TNF (which was kindly provided by the Knoll AG, Ludwigshafen, Germany), 3 or 16 h before immunization. Cells were injected four times (subcutaneously, with 2×10^7 cells per injection) with 3-wk-intervals in PBS and 30% Alu-gel-s (Serva, Heidelberg, Germany) as adjuvants. 5 d after the last boost, spleen cells were fused with the mouse myeloma SP2/0 essentially as described (Vestweber and Kemler, 1985). Hybridoma supernatants were differentially screened for indirect immunoperoxidase staining on TNF activated and non-activated endothelioma cells (see cell surface ELISA, described below). Hybridomas which produced antibodies that recognized TNF-inducible cell surface antigens, were subcloned by limiting dilution. The polyclonal rabbit antiserum against a peptide containing the COOH-terminal 17 amino acids of mouse ELAM-1 has been described (Weller et al., 1992). An anti-mouse P-selectin rabbit antiserum was produced by immunizing the purified P-selectin–immunoglobulin chimeric protein (described below) at 200 μg per injection. Specific antibodies were affinity purified on the fusion protein, conjugated to CNBr-sepharose (Pharmacia, Uppsala, Sweden). Antibodies against the Fc part of human IgG were removed by a second CNBr-sepharose column with conjugated human IgG (Sigma Immunochemicals, St. Louis, MO).

Monovalent F(ab') fragments of mAbs were generated as described (Jonston and Thorpe, 1982).

Selectin–Immunoglobulin Chimeric Proteins

The 5' part of the cDNAs of mouse P-selectin and mouse E-selectin, coding for the signal sequence, the lectin domain, the EGF-repeat and the first two complement binding domains, were amplified by polymerase chain reaction (PCR) using oligonucleotides which introduced a splice donor site at the 3' end of the PCR fragment. The 3' end of the selectin cDNA fragments was defined by nucleotides A and G found in position 969 and 970 of mouse P-selectin cDNA (Weller et al., 1992) and in position 1039 and 1040 of mouse E-selectin cDNA (Weller et al., 1992). These two nucleotides represented the first half of the introduced splice donor site (AG'GT). In two separate experiments, each of the two fragments was placed into an intron of the genomic sequence of the human IgG_1 heavy chain. This intron immediately preceded the exon coding for the hinge region which was followed by the exons for the CH2 and CH3 domains. Construction was done by using the CD4-IgG expression plasmid pCD4-Hg1 CE1 (provided by Dr. Klaus Karjalainen, Basel Institute for Immunology, Basel, Switzerland; Traunecker et al., 1991) and replacing the CD4 sequences with the respective selection PCR fragment. Both PCR fragments were completely sequenced and no point mutations were found. The resulting plasmids were co-transfected together with a pSV-neo-plasmid by electroporation into the myeloma cell J558. 3×10^6 electroporated cells were plated on six 96-well plates and the supernatant of G418-resistant clones was tested in enzyme-linked

immunosorbent assays (ELISA). In these assays rabbit $F(ab)_2$ fragments directed against human IgG (dianova) were coated on to the ELISA plates, the protein binding capacity of the plastic was blocked with 10% FCS, and after one hour incubation with the myeloma supernatants, bound fusion proteins were detected with peroxidase conjugated goat $F(ab)_2$ anti-human IgG (dianova). Positive clones were subcloned twice by limiting dilution.

The selectin IgG chimeric proteins were purified from the 10× concentrated culture medium (DME, 10% FCS) with protein A-sepharose and bound proteins were eluted with 0.1 M glycin/HCl, pH 3.0; followed by immediate neutralization with 50 mM Tris, pH 8.5.

Cell Surface ELISA

Endothelioma cells were plated at a density of 2×10^4 cells per well in 96-well microtiter plates 2 d before the assay. Immediately before the test, the plates were cooled on ice for 15 min, and then the adherent cells were washed three times with ice-cold PBS/Ca^{2+} (containing 1 mM$CaCl_2$) and incubated with 100 μl of ice-cold hybridoma supernatant or other antibody solutions in DME/10% FCS on ice for 1 h. The antibody solution was removed by aspiration and the cells were washed three times with ice-cold PBS/Ca^{2+} and fixed for 30 min at 4°C in 4% paraformaldehyde in PBS/Ca^{2+}. After removal of the fixative and three washes with PBS/Ca^{2+}, the cells were incubated for 1 h at room temperature with peroxidase conjugated goat $F(ab)_2$ anti-rat IgG (or anti-rabbit IgG, both dianova) diluted 1/2,000 in DME/10% FCS. The staining reaction was done in 50 mM K_2HPO_4, 25 mM citric acid, pH 6.0, with 300 μg/ml orthophenylendiamine and 0.006% H_2O_2, and stopped by addition of HCl (final concentration 1 M). The signals were quantified by measuring light absorbance at 492 nm with an automated ELISA reader from SLT-Labinstruments (Vienna, Austria).

Cell-surface ELISA signals for the selectins were standardized by comparison with ELISA signals on the purified E-selectin-IgG_1 and P-selectin-IgG_1 fusion proteins. Highly activated 96-well ELISA plates (ICN Radiochemicals) were coated with 5 μg/ml goat $F(ab)_2$ anti-human IgG (dianova), blocked with 10% FCS in PBS, and incubated with serial 1/2 dilutions of the purified fusion proteins ranging from 200 ng/ml to 0.1 ng/ml overnight at room temperature. Antibody incubations were done with 21KC10 culture supernatant or 300 ng/ml affinity-purified rabbit antibodies against P-selectin-IgG1, followed by incubation with peroxidase-conjugated goat $F(ab)_2$ anti-rat or anti-rabbit IgG. These incubations as well as the subsequent staining reaction were done in parallel with the cell-surface ELISA under identical conditions.

Cell Adhesion Assays

Endothelioma cells were plated at a density of 2×10^4 cells per well on 96-well microtiter plates 2 d before the test. For tests at 7°C, these adherent cells were washed twice in HBSS (Biochrom) at 7°C and placed at 7°C. Leukocytes were harvested, washed once in 7°C HBSS, and 5×10^5 cells were added in 100 μl per well to the microtiter plate which was rotating at 75 rpm (with 1-cm diam of orbit). After a 20-min incubation at 7°C unbound leukocytes were removed by gently flicking them off. Bound cells were fixed with 1% glutaraldehyde in PBS for 2–3 h at 7°C, usually followed by three more washes with HBSS. For assays at 37°C, cells were washed in HBSS at room temperature before the incubation which was done on a shaker at 37°C. Bound cells were quantitated in triplicate by counting five different fields per well under the microscope. In alternative assays, unbound cells were removed by centrifugation. For this, the wells were completely filled with HBSS after a 20-min cell-binding period, cautiously sealed with tape (plate sealers; Titertek Elfab a, Finland) avoiding any air bubbles and centrifuged upside down for 5 min at 500 rpm. Unbound cells were removed with the sealing tape. Quantitative analysis of these assays gave comparable results for both washing conditions with a slightly more even distribution of bound cells after washing with the centrifugation method.

Immunoprecipitation and Immunoblot

Immunoprecipitations and immunoblots were done as described (Weller et al., 1992). In immunoprecipitations with rat mAbs usually 10 ml of hybridoma supernatant was incubated for 2 h with 20 μl of protein A-sepharose (Pharmacia) to which 10 μg of affinity-purified rabbit IgG anti-rat IgG (dianova) had been previously bound. The antibody loaded beads were removed from the culture supernatant, washed three times with PBS, added to cell lysates of metabolically labeled cells, and further processed as described (Weller et al., 1992).

Results

J774 Cells Bind to TNF-stimulated sEnd.1 and bEnd.3 Cells, but Not to TNF-stimulated eEnd.2 Cells

We analyzed the ability of three mouse endothelioma cell lines eEnd.2, sEnd.1, and bEnd.3 to support leukocyte binding after stimulation with TNF-α. J774 mouse histiocytic cells were incubated with monolayers of the endothelioma cells that had been induced for 4 and 16 h with human recombinant TNF-α. The assay was carried out at 7°C for 20 min under continuous shaking (75 rpm) in microtiter plates. Under these conditions, J774 cells did not bind to either of the endothelioma cell lines in the absence of TNF-α (Fig. 1). Strong binding was observed to sEnd.1 and bEnd.3 cells after 4 h as well as after a 16-h treatment with TNF-α. However, the same treatment did not stimulate eEnd.2 cells to bind to J774 (Fig. 1 *B*). This prompted us to examine, which of the known TNF-inducible CAMs originally found on human endothelial cells were active on the three different mouse endothelioma cells.

In addition to VCAM-1, ICAM-1, and E-selectin, we also had to include P-selectin in this analysis, since we found recently that TNF-α also stimulates the synthesis of P-selectin in mouse endothelioma cells (Weller et al., 1992). To analyze the kinetics of the TNF-induced cell surface expression and the accompanying cell adhesion function, the published mAbs MK-2 against VCAM-1 and YN 1/1 against ICAM-1 could be used, while adhesion-blocking antibodies against mouse E- and P-selectin had first to be produced as described below.

Generation of Adhesion-blocking Antibodies against Mouse E- and P-selectin

A mAb against mouse E-selectin was produced by immunizing rats with intact sEnd.1 cells, which had been treated with human recombinant TNF-α, 4 h before injection. Antibodies were screened by indirect immunoperoxidase staining in "cell surface ELISA" assays for their ability to bind to antigens expressed only on the surface of TNF-treated sEnd.1 cells, but not on the surface of untreated sEnd.1 cells. A rat hybridoma was selected and subcloned, which produces the mAb 21KC10 which stains the cell surface of intact sEnd.1 cells only after TNF treatment of the cells. In immunoprecipitations of cell lysates from [^{35}S]methionine/[^{35}S]cysteine labeled, activated bEnd.3 cells, 21KC10 recognized an antigen (Fig. 2 *A*, lane *3*) of identical electrophoretic mobility as mouse E-selectin which was detected with a polyclonal antibody (Fig. 2 *A*, lane *1*) against a peptide covering the COOH-terminal 17 amino acids of mouse E-selectin (Weller et al., 1992).

The identification of the 21KC10 antigen as mouse E-selectin was further analyzed by testing the reactivity of the 21KC10 antibody with a recombinant fusion protein containing the lectin domain, the EGF repeat and the first two complement binding domains of mouse E-selectin fused to the hinge domain of human IgG_1 heavy chain. Fig. 3 demonstrates that the binding of 21KC10 to this chimeric protein in immunoblots is due to the E-selectin part in this protein, since an analogous CD4-IgG_1 chimeric protein and also human IgG were not detected. A similarly constructed P-selectin-IgG_1 chimeric protein was also not bound by 21KC10, which ex-

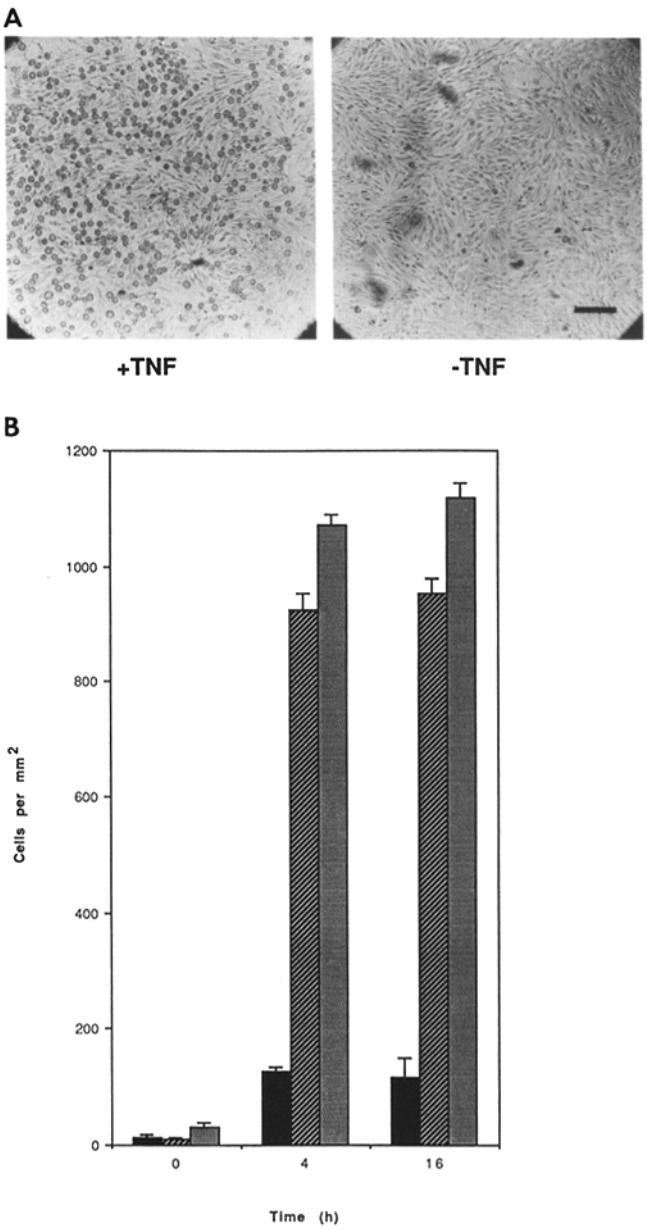

Figure 1. J774 cells bind to TNF-activated mouse endothelioma cells. J774 cells were incubated at 7°C with untreated and 16 h TNF-activated endothelioma cells in 96-well microtiter plates. (*A*) Phase contrast micrograph of two representative wells with untreated and 16 h TNF-treated bEnd.3 cells after incubation with J774 cells and three washes with HBSS. (*B*) Adhesion of J774 cells to bEnd.3, sEnd.1, and eEnd.2 cells was quantitated by counting adherent cells under the microscope at five defined areas per incubation well. Each measurement was done in three wells and one of three similar series of experiments is depicted in the graph. ■, eEnd.2; ▨, sEND.1; ▩, bEnd.3. Bar 100 μm.

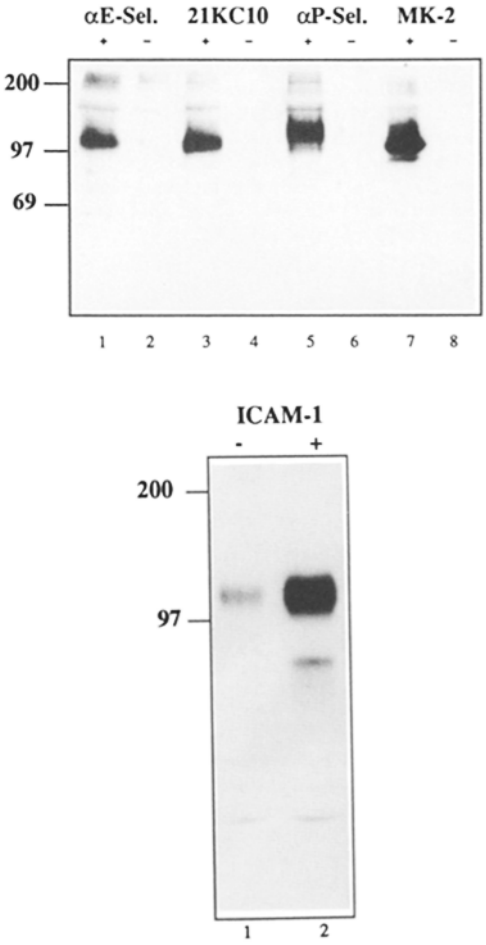

Figure 2. Immunoprecipitation of E-selectin, P-selectin, VCAM-1, and ICAM-1 from TNF-activated bEnd.3 and eEnd.2 cells. Cells were metabolically labeled with [^{35}S]methionine and [^{35}S]cysteine for 3 h in the presence and in the absence of TNF. Detergent extracts of bEnd.3 cells (*A*) and eEnd.2 cells (*B*) were immunoprecipitated with affinity-purified rabbit antibodies against a peptide consisting of the 17 COOH-terminal amino acids of mouse ELAM-1 (*A*, lanes *1* and *2*), mAb 21KC10 (*A*, lanes *3* and *4*), affinity-purified rabbit antibodies against a mouse P-selectin-IgG$_1$ chimeric protein (*A*, lanes *5* and *6*), mAb MK-2 (anti VCAM-1) (*A*, lanes *7* and *8*), mAb YN 1/1 (anti ICAM-1) (*B*, lanes *1* and *2*). Immunoprecipitated proteins were separated on 8% polyacrylamide gels under non-reducing conditions (*A*, lanes *1–8*) or reducing conditions (*B*, lanes *1–2*) and analyzed by fluorography; relative molecular masses (in kD) are marked on the left.

cludes that the antibody cross-reacts with mouse P-selectin (Fig. 3). Identical results were obtained in ELISA studies, where microtiter plates were coated with the soluble chimeric proteins which had been purified on protein A-sepharose (not shown).

Antibodies against mouse P-selectin were raised by immunizing rabbits with the soluble, purified P-selectin-IgG$_1$ chimeric protein. The antiserum obtained was affinity purified on the fusion protein which had been immobilized to CNBr-sepharose. In immunoprecipitations of metabolically labeled bEnd.3 cells, the purified antibodies specifically recognized P-selectin (Fig. 2 *A*, lane *5*).

ICAM-1, VCAM-1, E-, and P-selectin Are TNF Inducible with Distinct Kinetics on the Surface of Mouse Endothelioma Cells

We have tested the TNF inducibility of the four endothelial CAMs, ICAM-1, VCAM-1, E-, and P-selectin on the mouse endothelioma cells. The effect of TNF-α on the synthesis of these four CAMs was tested in immunoprecipitations with detergent extracts of cells, metabolically labeled for 3 h with [^{35}S]methionine and [^{35}S]cysteine in the absence or the pres-

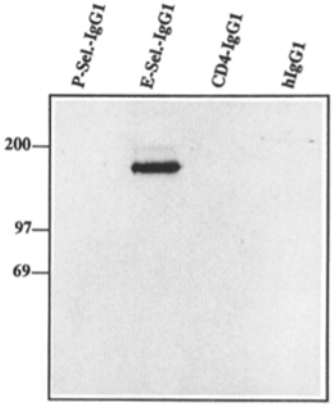

Figure 3. The mAb 21KC10 recognizes the mouse E-selectin–IgG_1 chimeric protein in immunoblots. 50 ng of each of the purified fusion proteins mouse P-selectin-IgG1, mouse E-selectin-IgG1 and CD4-IgG1, and of human IgG1 were electrophoresed on a 10% polyacrylamide gel under non-reducing conditions and analyzed in immunoblot with 21KC10 hybridoma supernatant. Bound antibodies were detected with peroxidase conjugated second antibodies; relative molecular masses (in kD) are indicated on the left.

ence of TNF-α. As shown in Fig. 2, TNF-α strongly stimulates the synthesis of E- and P-selectin and VCAM-1 in bEnd.3 cells. ICAM-1, even after stimulation with TNF-α, was only very weakly expressed on bEnd.3 cells (see below), but its synthesis was clearly stimulated on eEnd.2 cells, as shown in a separate experiment (Fig. 2). The same result was found for sEnd.1 cells (not shown).

In "cell-surface ELISA" assays, we tested the kinetics of the TNF-induced cell surface expression of all four CAMs. To ensure that only antigens on the cell surface were detected, monolayers of intact, unfixed endothelioma cells were incubated at 4°C with specific antibodies after different time periods of stimulation with TNF-α. The cells were then fixed with paraformaldehyde and the first antibodies were detected with peroxidase-conjugated second antibodies.

Cells were stimulated for 0, 2, 4, 8, 16, and 24 h with TNF-α and the cell surface expression was measured for each antigen at each time point in triple determinations. Three independent experiments were performed giving similar results. Fig. 4 shows that both selectins are only transiently induced with a maximum cell surface expression for E-selectin at 4 h and for P-selectin between 4 and 8 h after TNF stimulation. After 24 h both selectins are expressed near basal levels again. In contrasts, ICAM-1 and VCAM-1 are slightly less rapidly induced and their cell surface expression is still elevated 24 h after activation (Fig. 4). This is consistent with the time course of cell surface expression that was reported for the human homologs on HUVECs, except for P-selectin, which has not yet been described to be TNF inducible on HUVECs.

No difference between the three cell lines was found for the TNF-induced upregulation of the four CAMs, except that ICAM-1 was only very weakly expressed on stimulated bEnd.3 cells and P-selectin was only marginally induced at the surface of sEnd.1 cells. All the CAMs which were upregulated by TNF stimulation on the surface of bEnd.3 or sEnd.1 cells, were also upregulated to a similar degree and with similar kinetics on eEnd.2 cells. This argues against a direct role of these CAMs in the binding to J774 cells, since all four CAMs are present on TNF-stimulated eEnd.2 cells, yet J774 cells do not bind (Fig. 1).

The rapid down regulation of the selectins, with almost basal surface expression on bEnd.3 and sEnd.1 cells 16 h after the addition of TNF-α, is an additional argument against a role of the selectins in the binding to J774 cells, which bind equally well to both endothelial cell lines after 4 and 16 h of treatment with TNF-α (Fig. 1).

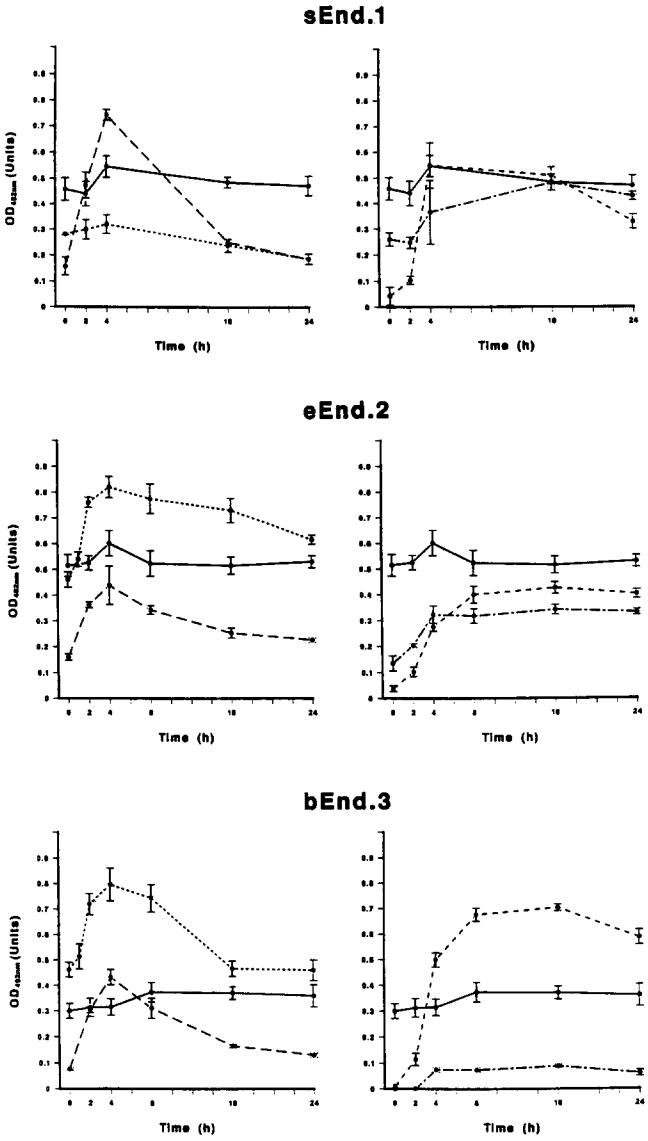

Figure 4. Kinetics of the TNF-α-induced cell surface expression of E-selectin, P-selectin, ICAM-1, and VCAM-1 on three endothelioma cell lines. 2 × 10^4 eEnd.2, sEnd.1, or bEnd.3 cells, cultured for 2 d in 96-well microtiter plates, were treated for the indicated time periods with 100 U/ml human recombinant TNF-α, and then analyzed by indirect immunoperoxidase staining with the mAbs YN 1/1 (-·-·, anti-ICAM-1), MK-2(---, anti-VCAM-1), KM 201 (———, anti-CD44), 21KC10 (– –, anti-E-selectin) and affinity-purified rabbit antibodies against mouse P-selectin (·····). The staining intensity is given in optical density units measured at 492 nm. The depicted experiment represents one out of three with equivalent results.

The Cell Surface Appearance of P-selectin on bEnd.3 Cells Is Regulated by Two Different Mechanisms

The TNF-induced synthesis and cell surface appearance of P-selectin with maximal expression levels within 4 h after stimulation is completely different from the mechanism,

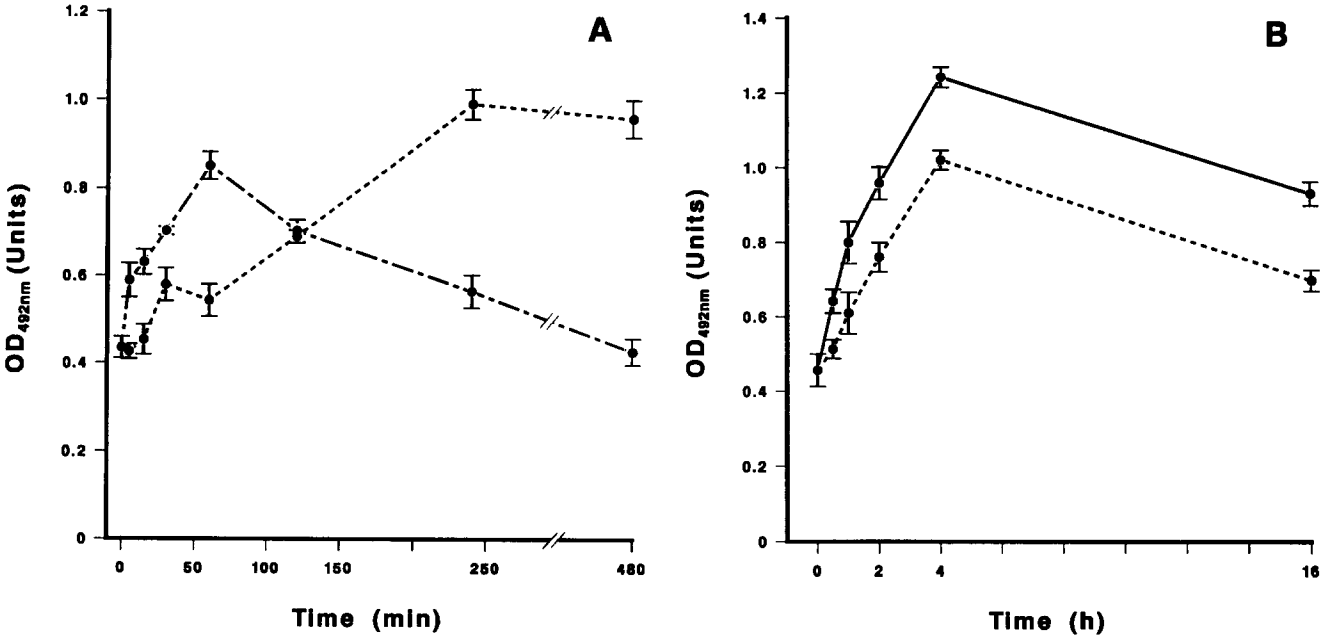

Figure 5. Time course of the cell surface appearance of P-selectin on bEnd.3 cells stimulated by PMA and TNF-α. bEnd.3 cells were cultured as described for Fig. 4 and treated for the indicated time periods with (*A*) 100 U/ml human recombinant TNF-α (----), 1 μg/ml PMA (-·-), (*B*) 100 U/ml TNF-α (-----), a mixture of TNF-α plus PMA (———) with PMA added only for the last 30 min of the indicated incubation period with TNF-α.

which has been shown to regulate the cell surface appearance of P-selectin on HUVECs. In these cells, P-selectin is stored in the so-called Weibel-Palade-Bodies and becomes redistributed to the cell surface within minutes after stimulation of HUVECs by thrombin, histamine, or phorbolesters. We wanted to test whether these two completely different regulation mechanisms would both function in the same endothelial cells.

To this end, we treated bEnd.3 cells with 1 μg/ml PMA, 2.5 U/ml thrombin, or 100 U/ml TNF-α for 5, 15, 30 min, and 1, 2, and 4 h before staining the cell surface with polyclonal affinity-purified anti-P-selectin antibodies or the anti-E-selectin antibody 21KC10 in indirect immunoperoxidase studies. Control cells were left unstimulated, but were treated with the same amount of DMSO present during the stimulation with PMA. Fig. 5 *A* shows that PMA, but not TNF-α, caused a rapid and transient increase of the P-selectin signal at the cell surface with a maximum expression level after 60 min of treatment with PMA. The same effect was seen with thrombin (not shown) while no such rapid upregulation was seen on the cell surface expression of E-selectin (not shown). In contrast to the PMA effect, TNF-α induced a less rapid increase of P-selectin as well as E-selectin at the cell surface, with a maximum after 4 h of stimulation. This effect, which is mediated by the de novo synthesis of new selectin molecules (Weller et al., 1992), was additive with the rapid effect, which was caused by PMA. Cells which were treated with TNF-α for different time periods and in addition with PMA for the last 30 min of each time period displayed a higher level of P-selectin on their surface than cells which were treated with TNF-α alone (Fig. 5 *B*). Thus, both mechanisms can function independently in mouse endothelioma cells.

The PMA-induced increase of the P-selectin signal was about half of the maximal increase caused by TNF-α. To test whether the TNF-induced, maximal level of cell surface expression of P- and E-selectin was similar, we standardized the assay by performing ELISA assays on the purified, soluble forms of P- and E-selectin. Highly activated ELISA microtiter plates were saturated with affinity-purified anti-human IgG_1 polyclonal antibodies and incubated with serial dilutions of purified E-selectin-IgG_1 or P-selectin-IgG_1 at concentrations between 200 ng/ml and 0.1 ng/ml. Indirect immunoperoxidase staining was performed in parallel with the ELISA on endothelioma cells, using identical antibody solutions and staining parameters. We detected similar levels of cell surface expression for E- and P-selectin after maximal stimulation by TNF-α. The absolute number of selectin molecules per cell could not be determined by this assay, since 100% binding of selectin chimeras to plastic-coated anti-human IgG_1 antibodies could not be assumed. Since P-selectin was already constitutively expressed on the cell surface at much higher levels than E-selectin, TNF-α induction caused only a twofold increase of P-selectin at the cell surface while expression of E-selectin on the cell surface increased with a factor of 10–15.

Antibodies, Which Block the Adhesion Function of ICAM-1, VCAM-1, E- and P-selectin Do Not Block the Binding of J774 Cells

J774 cells do not bind to eEnd.2 cells (at 7°C) after 4 and 16 h of TNF treatment, although E- and P-selectin are clearly induced after 4 h and ICAM-1 and VCAM-1 are well expressed after 16 h of TNF stimulation. This indicates that none of the four CAMs is directly involved in the binding to J774 cells, provided it can be demonstrated that each of these CAMs functions at all on eEnd.2 cells in adhesion assays. Therefore, we compared the capability of sEnd.1, bEnd.3, and eEnd.2 cells to use each of the four adhesion proteins to bind to leukocytes.

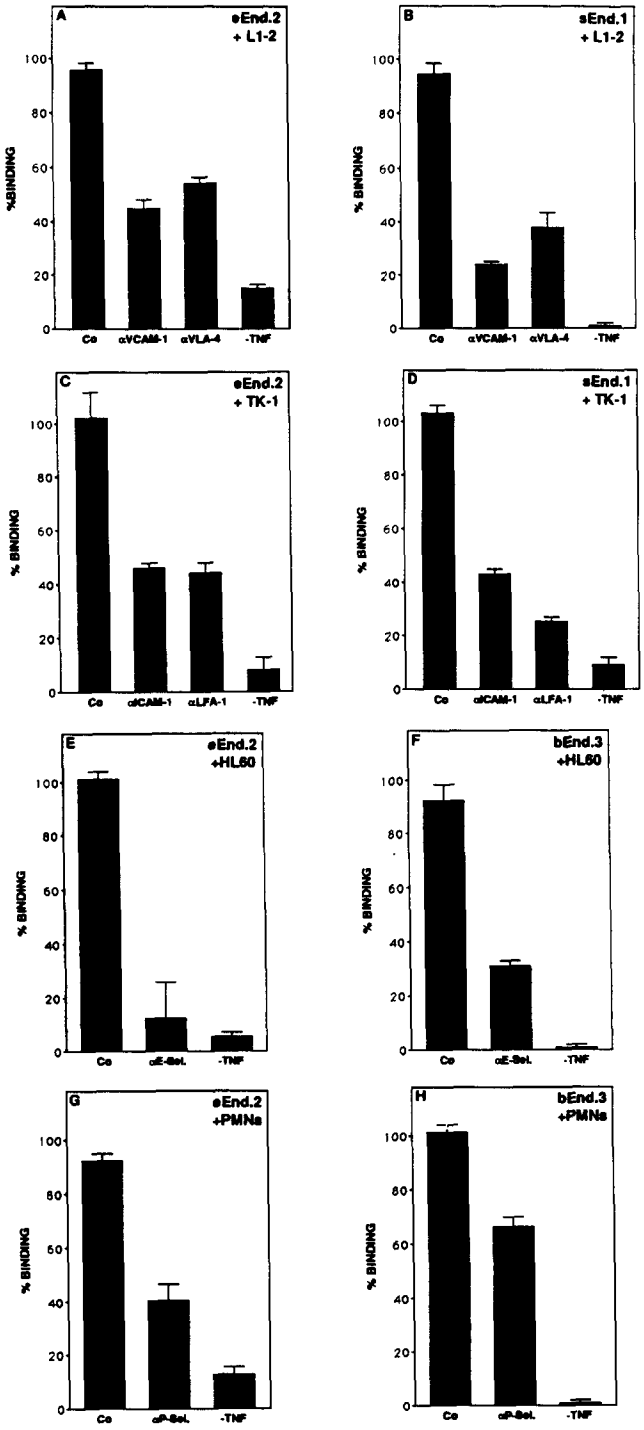

Figure 6. Comparison of the cell adhesion function of ICAM-1, VCAM-1, E- and P-selectin on TNF-activated eEnd-2, sEnd-1, and bEnd.3 endothelioma cells. Cell adhesion assays were performed as described under Materials and Methods with the mouse lymphoma L1-2 (*A* and *B*), the mouse T lymphoma TK-1 (*C* and *D*), the human monocytic cell line HL60 (*E* and *F*), and freshly isolated PMNs from mouse bone marrow (*G*, and *H*) by incubation with 16-h TNF-stimulated eEnd.2 (*A* and *C*) and sEnd.1 cells (*B* and *D*) and 4-h stimulated eEnd.2 (*E* and *G*) and bEnd.3 cells (*F* and *H*). Binding to 16-h TNF-stimulated endothelioma cells (*A–D*) and 4-h TNF-stimulated endothelioma cells (*E–H*) was defined as 100%. Inhibition of cell adhesion with antibodies was tested with 1/100 diluted ascites of mAb MK-2 (anti-VCAM-1), hybridoma supernatant of mAb R1/2 (anti-VLA-4), 10 μg/ml of purified mAb YN 1/1 (anti-ICAM-1), hybridoma supernatant of M17/4 (anti-LFA-1), fourfold-concentrated hybridoma supernatant of mAb 21KC10 (anti-E-selectin), and 10 μg/ml of affinity-purified rabbit anti-P-selectin antibodies (anti-P-selectin). Antibody dilutions were done in DME with 10% FCS. Quantitation was done by counting the cells under the microscope. In controls (*Co*), the endothelial cells were preincubated with isotype matched mAbs (*A–F*) or total IgG of a rabbit pre-immune antiserum (*G* and *H*) at the same concentration as the test antibodies. Absolute numbers of bound leukocytes to TNF-induced endothelioma cells (taken as 100% and given as cells/mm²) were: 3,511 (*A*), 2,104 (*B*), 1,060 (*C*), 932 (*D*), 1,876 (*E*), 3,756 (*F*), 2,688 (*G*), and 4,424 (*H*). The depicted experiment represents one out of five with equivalent results.

Binding of the B lymphoma line L1-2 to eEnd.2 and sEnd.1 cells was TNF-α dependent (Fig. 6, *A* and *B*) and the binding efficiency was very low at 7°C (not shown) compared to the strong binding at 37°C. This binding at 37°C was inhibited to 53% by MK-2 (anti-VCAM-1) and to 45% by R1-2 (anti-α_4) on eEnd.2 cells and to 75% by MK-2 and 63% by R1-2 on sEnd.1 cells. The human B lymphoma line Ramos behaved similarly to L1-2 cells (not shown).

The function of ICAM-1 was demonstrated using the mouse T lymphoma TK-1 which binds equally well to sEnd.1 and eEnd.2 cells after 16 h of TNF-α stimulation (Fig. 6, *C* and *D*). No, or very weak binding was observed to unstimulated cells and the TNF-α–induced binding activity was strongly temperature-sensitive, being undetectable at 4–7°C and very weak at 20°C (not shown). TK-1 binding could be inhibited by 50% with the mAbs YN 1/1 (anti-ICAM-1) and M17/4.4.11.9 (anti CD11a) on eEnd.2 cells and by 60–70% on sEnd.1 cells.

The cell adhesion function of E-selectin on bEnd.3 and eEnd.2 cells was tested with the human monocytic cell line HL60 (Fig. 6, *E* and *F*). These cells did not bind to unstimulated endothelioma cells. Binding was maximal at 4 h after TNF-α stimulation, was not temperature sensitive and could be performed at 7°C. The antibody 21KC10 blocked HL60 binding to 4 h–stimulated eEnd.2 and bEnd.3 cells by ~80 and 70%, respectively.

Binding of freshly isolated PMNs from mouse bone marrow to bEnd.3 and eEnd.2 cells (at 7°C) was also TNF dependent and was partially inhibited by 60% (on eEnd.2) and by 33% (on bEnd.3) with affinity-purified polyclonal rabbit anti-P-selectin antibodies (Fig. 6, *G* and *H*). Thus, all four TNF-inducible cell adhesion molecules were demonstrated to be functional on eEnd.2 cells similarly as on bEnd.3 or sEnd.1 cells.

Blocking of E-selectin, P-selectin, and VCAM-1 on bEnd.3 cells as well as simultaneously blocking all three of them with antibodies, did not inhibit the binding of J774 cells in adhesion assays performed at 7°C (Fig. 7). Binding of J774 cells via high-affinity Fc receptors to the antibodies bound to bEnd.3 cells was unlikely since binding was not observed to these antibodies in similar experiments performed with TNF-activated eEnd.2 cells (not shown). Since the mAb YN 1/1 against ICAM-1 strongly cross-linked J774 cells (which express ICAM-1 on their surface) to the endothelioma cells, binding of J774 cells to ICAM-1 was analyzed by testing the inhibitory effect of F(ab') fragments of mAbs against the leukocyte integrins LFA-1 (anti-CD11a)

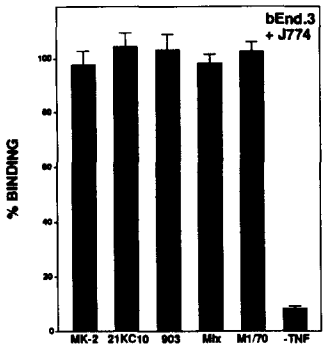

Figure 7. Antibody inhibition studies on the adhesion of J774 cells to 16 h TNF-activated bEnd.3 cells. bEnd.3 cells were cultured as described for Fig. 4, stimulated with TNF-α 16 h before the assay and incubated with J774 cells. Binding under these conditions was defined as 100%. Inhibition of cell adhesion was tried by incubating the bEnd.3 cells with 1/100 diluted ascites of mAb against VCAM-1 (*MK-2*), fourfold concentrated anti-E-selectin hybridoma supernatant (*21KC10*), 10 μg/ml of affinity-purified rabbit anti-P-selectin antibodies (*903*) and the mixture of these three antibodies at the same concentration (*Mix*) or by incubating the J774 cells with 100 μg/ml of Fab fragments of the mAb against CD11b (*M1/70*) before the cell adhesion assay. Quantitation was done by counting the cells under the microscope.

(not shown) and Mac-1 (anti-CD11b) (Fig. 7). Both antibodies did not inhibit the binding of J774 cells at 7°C.

Since all these antibodies had been found to block adhesion of other leukocyte types to the TNF-induced endothelioma cells (as described above) these results strongly indicate, that the binding of J774 cells to TNF-activated endothelioma cells defines an additional, so far unknown cytokine-induced leukocyte adhesion mechanism on endothelial cells.

Comparison of the Function of E- and P-selectin on bEnd.3 Cells

Since both endothelial selectins are known to bind PMNs we compared the effect of our antibodies against E- and P-selectin on the binding of mouse PMNs and HL60 cells to TNF-activated bEnd.3 endothelioma cells. We found that blocking of each selectin alone partially blocked the binding of PMNs by ~30% (P-selectin) and ~20% (E-selectin), while the mixture of antibodies against both selectins inhibited PMN binding by ~70% (Fig. 8 *A*). Thus, both antibodies together had a more than additive effect.

Interestingly, the binding of HL60 cells could not be blocked by antibodies against P-selectin, even the mixture of antibodies against E- and P-selectin did not inhibit cell binding more strongly than the mAb against E-selectin alone (Fig. 8 *B*). Such a lack of HL60 binding to P-selectin has also been found by others (Patel et al., 1991).

Discussion

In this study we describe five different TNF-inducible cell adhesion mechanisms on the surface of mouse endothelioma cells. Only for three of them (ICAM-1, VCAM-1, and E-selectin) has TNF induction also been demonstrated on human endothelial cells. These three CAMs are induced on the surface of the mouse endothelioma cells with kinetics similar to those reported for human primary endothelial cells.

The fourth TNF-inducible adhesion mechanism is represented by P-selectin. We have recently reported that TNF-α causes an increase of mRNA- and protein expression of P-selectin in mouse endothelioma cells (Weller et al., 1992).

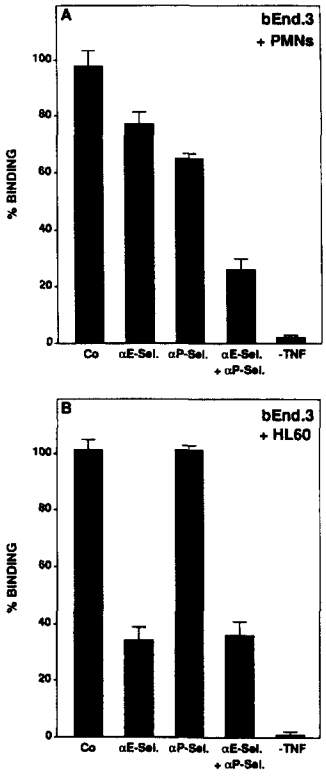

Figure 8. Comparison of the inhibitory effect of antibodies against E- and P-selectin on the binding of PMNs and HL60 cells to TNF activated bEnd.3 cells. Cell adhesion assays were performed as described under Materials and Methods with mouse PMNs (*A*) and HL60 cells (*B*) by incubation with 4 h TNF-activated bEnd.3 cells. Cell binding in the absence of antibodies was defined as 100%. Inhibition of cell adhesion with antibodies was tested with fourfold concentrated hybridoma supernatant of mAb 21KC10 (*αE-sel.*), 10 μg/ml of affinity-purified rabbit anti-P-selectin antibodies (*αP-sel.*), and the mixture of both antibodies at the same concentrations (*αE-sel. + αP-sel.*). In controls (*Co*) the endothelial cells were incubated with a mixture of a control rat IgM hybridoma supernatant (fourfold concentrated) and 10 μg/ml IgG of a rabbit pre-immune serum.

Here we show that this TNF-induced synthesis of P-selectin leads to a transient increase of P-selectin at the cell surface, with kinetics comparable to E-selectin induction. This TNF-induced up regulation of P-selectin at the cell surface is functionally significant, since the binding of PMNs to TNF-induced endothelioma cells was partially inhibited by antibodies against P-selectin. Thus, P-selectin is a true member of the TNF-regulated leukocyte adhesion molecules on endothelial cells.

This new regulation mechanism is different from the one that was reported for P-selectin on HUVECs. There, P-selectin is stored in so-called Weibel-Palade-Bodies. Mobilization of these granules to the cell surface and their fusion with the plasma membrane can be induced by PMA, thrombin, and histamine, causing the cell surface appearance of P-selectin within minutes followed by a rapid down regulation (Hattori et al., 1989; Geng et al., 1990).

We report here that such a rapid induction mechanism also occurs in the mouse endothelioma cells, stimulated by PMA and thrombin. Although we have not analyzed whether P-selectin is indeed stored also in these cells, it is likely that this rapid induction at the cell surface is based on protein transport. This regulation mechanism seems to function independently of the regulation of the synthesis of P-selectin, since the effects of PMA and TNF-α are additive. This additivity, together with the fact that TNF-α itself cannot induce the rapid transport of stored intracellular P-selectin to the cell surface, may suggest that the TNF induced, newly synthesized P-selectin molecules directly reach the cell surface, thereby circumventing the route via storage granules. How this alternative targeting of P-selectin to storage granules or to the plasma membrane is achieved, is still unclear. One explanation would be, that the TNF-induced newly synthesized

P-selectin molecules differ in their structure from the P-selectin moieties which are intracellularly stored. However, such structural differences would have to be subtle, since the electrophoretic mobility of the TNF-induced P-selectin molecules was identical to that of the constitutively synthesized molecules. A more simple explanation for the direct targeting of TNF-induced P-selectin to the plasma membrane would be to assume, that the capacity of the sorting mechanism which targets P-selectin molecules to storage granules is limited and that the additionally synthesized molecules are sorted into a transport pathway to the cell surface by a default mechanism.

For a detailed understanding of the physiological role of P-selectin, it will be important to know to what extent both regulation mechanisms function in vivo. We could demonstrate the stimulatory effect of TNF-α on the synthesis of P-selectin in mouse tissue by immunoprecipitation analysis of metabolically labeled lung tissue of mice, which had been intravenously injected with TNF-α (Weller et al., 1992). Since the endothelium of different types of blood vessels express different levels of P-selectin (McEver et al., 1989), it is possible that endothelium with high levels of stored P-selectin preferentially regulate the cell surface expression via the transport regulation mechanism while other cells with low constitutive expression levels might preferentially regulate the synthesis of P-selectin. Alternatively, both regulation mechanisms could function in the same cells as is suggested by the parallel and independent functioning of both mechanisms in bEnd.3 cells.

The induction of P-selectin synthesis by a mechanism similar to that of E-selectin further emphasizes the importance of analyzing differences and similarities of the physiological role of both endothelial selectins. Since both adhesion molecules can bind to the tetrasaccharide sialyl-Lewis X and both mediate the binding of PMNs and monocytes, the function of the endothelial selectins appears to be redundant. The rapid transport of P-selectin to the cell surface and the more slowly acting up regulation by de novo synthesis of E-selectin had served as an explanation for this redundancy, arguing for similar functions of both selectins at different time points. The parallel expression of both selectins after induction with TNF-α, which we have described, again might argue for redundancy. However, indirect evidence has emerged recently, suggesting that the physiological ligands for both endothelial selectins on the same leukocytes might be different (Larsen et al., 1992). If this is true, the parallel expression of both selectins on endothelial cells may allow leukocytes to use different ligands to bind to both selectins at the same time. Such a multiple interaction might be necessary to guarantee sufficient strength of the binding of leukocytes to endothelial cells.

The lack of HL60-binding to P-selectin, that we found, was surprising, since HL60 cells have been shown to bind to human P-selectin (Geng et al., 1990; Larsen et al., 1989). However, also Patel et al. (1991) could not detect binding of their HL60 cells to P-selectin, although these cells bound to E-selectin. The HL60 cells we used were obtained from ATCC and had been subcloned because of initial difficulties to propagate them. The resulting subclone apparently had lost the ability to bind to P-selectin. Since binding of these cells to TNF-activated bEnd.3 cells could still be inhibited by the anti-E-selectin mAb these data suggest that HL60 cells possess different ligands for E- and P-selectin. This would be in agreement with data reported by Larsen et al. (1992).

The maximal numbers of P- and E-selectin molecules on the cell surface of bEnd.3 cells after TNF induction are similar. This quantitation was possible, since the cell surface ELISA signals could be standardized by assays with the purified E- and P-selectin–IgG fusion proteins. P-selectin was clearly detectable at the cell surface in the absence of any stimulation in contrast to E-selectin which was only very weakly expressed under such conditions. Because of this high constitutive expression level of P-selectin, TNF-α only causes a twofold increase of the amount of P-selectin at the cell surface. It is known that such differences in adhesion molecule expression of around two- to threefold are functionally significant (Dustin and Springer, 1991; Pardi et al., 1992). In agreement with this, we observed P-selectin–mediated binding of PMNs only to TNF-induced endothelioma cells. A situation similar to P-selectin was also found for ICAM-1 which is constitutively expressed on sEnd.1 and eEnd.2 cells and is also stimulated in its cell surface expression only two- to threefold by TNF-α.

The fifth TNF-inducible cell adhesion mechanism on the surface of sEnd.1 and bEnd.3 endothelioma cells is defined by the binding of the mouse monocytic/macrophage cell line J774. We can exclude that ICAM-1, VCAM-1, E-, or P-selectin mediate this binding for the following reasons: First, adhesion-blocking antibodies against all four CAMs do not interfere with the binding of J774 cells. Second, J774 cells do not bind to TNF-induced eEnd.2 cells, yet all four CAMs are TNF inducible to a similar degree on eEnd.2 as on sEnd.1 and bEnd.3 cells, and all four CAMs can be demonstrated to function in cell adhesion assays on eEnd.2 cells. Third, the induction kinetics of E- and P-selectin is different from the kinetics for the new binding mechanism: J774 cells bind equally well after 4 and 16 h of TNF stimulation while E- and P-selectin are clearly down regulated again after 16 h. Fourth, binding of J774 cells occurs at low temperature (4–7°C) where ICAM-1 and VCAM-1 hardly bind their known integrin ligands. We conclude that the binding of J774 cells to mouse endothelioma cells is mediated by a TNF-inducible adhesion mechanism which is possibly new and which functions well at low temperature.

New TNF-inducible cell adhesion mechanisms have also been described on human endothelial cells. On human dermis microvasculature–derived endothelial cells, a TNF-inducible binding mechanism for melanoma cells was defined, which could only partially be blocked by antibodies against VCAM-1, did not correlate with the expression of ICAM-1 and ELAM-1 and which was not found on HUVECs (Lee et al., 1992). Vennegoor et al. (1992) reported the binding of cultured human T cell clones to 24-h TNF-α–stimulated HUVECs which could not significantly be blocked by antibodies against human ICAM-1, VCAM-1, and E-selectin. The T cell clones which were used in the assays did not express L-selectin on their surface and the cell binding assays were carried out at 37°C. In contrast, Spertini et al. (1991) have demonstrated the binding of lymphocytes and neutrophils to 24-h TNF-α–activated HUVECs at 4°C. This binding could be inhibited with anti-L-selectin antibodies. It is unlikely that this mechanism is related to the one we have found to mediate the binding of J774 cells, since we did not

detect L-selectin on J774 cells with the MEL14 antibody in FACS analysis (not shown).

Clearly, more TNF-inducible leukocyte adhesion molecules exist on endothelial cells than have yet been characterized on the molecular level. The mouse endothelioma cells have proved to be useful in identifying P-selectin as a new member of this group of cell adhesion molecules. By using the same mAb approach which we successfully used here to obtain the mAb 21KC10 against mouse E-selectin, we should be able to identify the novel cytokine-inducible adhesion mechanism, which supports the binding of J774 cells.

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