

Tumor Necrosis Factor α (TNF- α)-induced Cell Adhesion to Human Endothelial Cells Is under Dominant Control of One TNF Receptor Type, TNF-R55

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Summary

Tumor necrosis factor α (TNF- α) is a pleiotropic cytokine triggering cell responses through two distinct membrane receptors. Stimulation of leukocyte adhesion to the endothelium is one of the many TNF- α activities and is explained by the upregulation of adhesion molecules on the endothelial cell surface. Human umbilical vein endothelial cells (HUVEC) were isolated, cultured, and demonstrated to express both TNF receptor types, TNF-R55 and TNF-R75. Cell adhesion to HUVEC was studied using the HL60, U937, and MOLT-4 cell lines. HUVEC were activated by either TNF- α , binding to both TNF-R55 and TNF-R75, and by receptor type-specific agonists, binding exclusively to TNF-R55 or to TNF-R75. The TNF- α -induced cell adhesion to HUVEC was found to be controlled almost exclusively by TNF-R55. This finding correlated with the exclusive activity of TNF-R55 in the TNF- α -dependent regulation of the expression of the intercellular adhesion molecule type 1 (ICAM-1), E-selectin, and vascular cell adhesion molecule type 1 (VCAM-1). The CD44 adhesion molecule in HUVEC was also found to be upregulated through TNF-R55. However, both TNF-R55 and TNF-R75 upregulate α 2 integrin expression in HUVEC. The predominant role of TNF-R55 in TNF- α -induced adhesion in HUVEC may correlate with its specific control of NF- κ B activation, since κ B elements are known to be present in ICAM-1, E-selectin, and VCAM-1 gene regulatory sequences.

Tumor necrosis factor (TNF- α ; the term TNF refers to TNF- α and TNF- β jointly) was originally discovered by its antitumor activity, but is now recognized to be one of the most pleiotropic cytokines acting as a host defense factor in immunologic and inflammatory responses (1–4). Among its many different activities, TNF has effects on the vascular endothelium (5, 6) that may contribute to tissue injury in pathologies such as the disseminated intravascular coagulation seen in septic shock or in cerebral malaria, and may in part be responsible for the hemorrhagic tumor necrosis in animal models (1, 2). TNF- α triggers endothelial cells to secrete various factors, including PAI-1 (7), IL-1 (8), GM-CSF (9), and IL-6 (10). TNF- α activity also results in phenotypic changes of the endothelium characterized by an increased permeability (11) and in the induction or upregulation of various cell adhesion molecules such as E-selectin (12), intercellular adhesion molecule type 1 (ICAM-1)¹ (13), and vascular

cell adhesion molecule type 1 (VCAM-1) (14–16). The regulated expression of these adhesion molecules and of their counter-receptors on leukocytes mediates the adhesion and extravasation of white blood cells during an inflammatory reaction (17–20).

TNF- α activity is mediated through at least two distinct membrane receptors, the 75-kD receptor (also termed TNF-R α , TNF-R2, or TNF-R75) and the 55-kD receptor (TNF-R β , TNF-R1, or TNF-R55) (21). Both receptors bind TNF- α and TNF- β with similar affinities (22), but they are independently regulated (23). Their extracellular domains have similar cysteine-rich repeat motifs that are characteristic for the NGF/TNF receptor gene family (24). In contrast, their intracellular domain sequences are entirely unrelated, suggesting distinct signal transduction pathways (for reviews see references 25 and 26). Distinct functions for the two TNF receptors indeed have been found in previous studies. Specific stimulation of TNF-R55 mimics TNF- α activities such as cytotoxicity, fibroblast proliferation, resistance to chlamydiae, synthesis of prostaglandin, and induction of MnSOD mRNA (27–30). In addition, the presence of TNF-R55 alone on

¹ Abbreviations used in this paper: EMSA, electrophoretic motility shift assay; HUVEC, human umbilical vein endothelial cells; ICAM-1, intercellular adhesion molecule type 1; VCAM-1, vascular cell adhesion molecule type 1.

human keratinocytes was sufficient to mediate ICAM-1 expression (31), and TNF-R55 was reported to control NF- κ B activation in hemopoietic cell lines (23, 32, 33). Less is known about the specific function of TNF-R75. It was found to mediate mouse thymocyte proliferation (34). A recent report provides evidence that TNF-R75 induces GM-CSF secretion in PC60 cell transfectants (35). TNF-R75 was also been described to stimulate human fibroblast, mononuclear cell, and NK cell proliferation (28, 36, 37).

Here we describe the activities of the two TNF receptor types in the induction of cell adhesion to human endothelial cells. We report a specific role of TNF-R55 in the induction of cellular adhesion to endothelium. ICAM-1, E-selectin, VCAM-1, and CD44 are induced through TNF-R55, but not through TNF-R75. In contrast, the expression of α 2 integrin was found to be mediated through TNF-R75 and TNF-R55.

Materials and Methods

Cell Lines. HL60, U937, and MOLT-4 cell lines were obtained from the American Type Culture Collection (Rockville, MD). Media and usual culture reagents were purchased from Gibco Laboratories (Grand Island, NY). HL60 and U937 cells were cultured in RPMI 1640, 10% FCS, 2 mM glutamine, 100 U/ml penicillin, and 100 μ g/ml streptomycin. MOLT-4 cells were cultured in Iscove medium, 10% FCS, 2 mM glutamine, 100 IU/ml penicillin, 100 μ g/ml streptomycin, and 50 mM 2-ME (Fluka Chemie AG, Buchs, Switzerland).

Human Umbilical Vein Endothelial Cells (HUVEC). HUVEC were purified from human umbilical cords as detailed by Jaffe et al. (38). HUVEC were cultured in medium M199 (Sigma Chemical Co., St. Louis, MO) supplemented with 20% FCS, 100 U/ml penicillin, 100 μ g/ml streptomycin, 2 mM glutamine, 2.5 μ g/ml fungizone (Gibco Laboratories), 20 μ g/ml gentamycin (Gibco Laboratories), 100 μ g/ml endothelial cell growth factor (ECGF; Collaborative Research, Inotech AG, Dottikon, Switzerland), and 100 μ g/ml heparin sodium salt (Sigma Chemical Co.). Culture flasks were precoated for 1 h at ambient temperature with 1% BSA (Sigma Chemical Co.) in PBS, then washed once with HBSS (Gibco Laboratories). Cells isolated from different cords were not pooled. HUVEC were used after at least three passages. Cellular homogeneity in the culture was controlled by staining with mouse anti-human Von Willebrand factor:FVIII:c/v WF complex antibody (Immunotech SA, Marseille, France).

Reagents. Mouse mAbs to TNF-R55 (htr-9, htr-5, htr-1, htr-20) and to TNF-R75 (utr-1) have been described previously (26, 39). IgG antibodies were purified from hybridoma supernatants on a protein G-Sepharose column (Pharmacia LKB Biotechnology, Uppsala, Sweden). IgM antibodies were purified by affinity chromatography using goat anti-mouse Ig (μ chain specific) covalently attached to cyanogen bromide-activated agarose beads (Sigma Chemical Co.). Mouse anti-human ICAM-1, anti-human VCAM-1, and anti-human E-selectin were purchased from British Biotechnology Products Ltd. (Oxon, UK), mouse anti-human MHC class I from Immunotech SA, mouse anti-human CD44 (F10-44-2) from Serotec (Inotech AG), and mouse anti-human integrin molecule antibodies from Telios Pharmaceuticals Inc. (San Diego, CA). Polyclonal anti-TNF receptor antibodies were raised by immunizing rabbits with recombinant extracellular regions of human TNF-R75 or TNF-R55 expressed in the Sf9/baculovirus system or CHO cells

as previously described (22). Specific antibodies against TNF-R75 or TNF-R55 were purified from rabbit antisera by affinity chromatography using CNBr-activated Sepharose 4B (Pharmacia LKB Biotechnology) coupled with recombinant soluble TNF-R75 or TNF-R55, respectively. The resulting affinity-purified rabbit antibodies were termed RaTNF-R75 (anti-TNF-R75) and RaTNF-R55 (anti-TNF-R55). Goat anti-mouse Ig antibodies labeled with PE were purchased from Southern Biotechnology Associates Inc. (Birmingham, AL). Recombinant human TNF- α and TNF- α with point mutations at residues 32 and 86, R32W/S86T-TNF- α (termed Trp32 Thr86 TNF), purified from *Escherichia coli* (Loetscher, H., D. Stueber, D. Banner, F. Mackay, and W. Lesslauer, manuscript in preparation).

Western Blotting Analysis. HL60 cells and HUVEC were grown to 90% confluency ($\sim 10^8$ cells total) and incubated for 2 h in medium at 4°C with 15 μ g/ml of htr or utr mAbs. HL60 cells were then harvested and washed twice with PBS. HUVEC were detached with trypsin-EDTA under conditions described below (see flow cytometry section), and washed twice in PBS supplemented with 100 U/ml aprotinin (Trasylol; Bayer, Leverkusen, Germany). Cells were resuspended in 0.75 ml PBS containing 1 mM PMSF (Sigma Chemical Co.), 100 U/ml aprotinin, 10 mM benzamide (Sigma Chemical Co.), 1 mM orthophenanthroline (Fluka Chemie AG), 5 μ M leupeptin (Fluka Chemie AG), 1 μ M pepstatin (Fluka Chemie AG), and 0.1% sodium azide (lysis buffer). 0.75 ml of lysis buffer containing 2% Triton X-100 were added and cells were gently agitated 90 min at 4°C. The lysate was centrifuged 15 min, 70,000 rpm at 4°C in an ultracentrifuge (TL 100; Beckman Instrs., Inc., Fullerton, CA). The supernatant was filtered through a 0.2- μ filter (Acrodisc, Gelman Sciences, Ann Arbor, MI) and incubated 1 h at 4°C under gentle agitation with 50 μ l of protein G-Sepharose FF beads (Pharmacia LKB Biotechnology). The beads were washed sequentially with lysis buffer containing 1% and 0.1% Triton X-100, respectively, and transferred to an ultrafree filter unit (Millipore Products Division, Bedford, MA) to drain the buffer. The beads were resuspended in 20 μ l of Laemmli sample buffer (40), heated for 2 min at 95°C, and centrifuged briefly, and the supernatants subjected to SDS-PAGE. The proteins were transferred to Immobilon-P membranes (Millipore Products Division) and incubated with 20 μ g/ml of RaTNF-R75 or RaTNF-R55, respectively, in 50 mM Tris-HCl, pH 7.4, 140 mM NaCl, 5 mM EDTA, 0.02% Na₃N, and 1% dry milk powder at ambient temperature over night, washed, and incubated with ¹²⁵I-labeled goat anti-rabbit antiserum (New England Nuclear, Boston, MA) for 3 h at ambient temperature. The membranes were then repeatedly washed with PBS, dried, and exposed to an autoradiographic film.

Flow Cytometry. HUVEC were detached with trypsin-EDTA (Gibco Laboratories) according to the following calibrated protocol. When 80% of the cells were detached, the trypsin treatment was stopped with 10 volumes of medium containing 20% FCS, and the cells were washed twice with PBS, 0.05% Na₃N, 1% BSA (FACS[®] buffer [Becton Dickinson & Co., Mountain View, CA]). Cells were incubated 30 min on ice with the respective antibodies at 2 μ g/ml in FACS[®] buffer, washed once in FACS[®] buffer, and incubated 30 min on ice with PE-conjugated goat anti-mouse Ig at 5 μ g/ml in FACS[®] buffer. Cells were then washed twice with FACS[®] buffer and analyzed on a FACSscan[®] cytofluorimeter (Becton Dickinson & Co.). Cells were gated using forward vs. side scatter to exclude dead cells and debris.

Cell Adhesion Assay. 100 μ l of a HUVEC suspension (2.5–3 $\times 10^5$ cells/ml) was seeded in a microtiter plate (MTP; Costar Corp., Cambridge, MA) in the presence of different concentrations of TNF- α , antibodies, or Trp32 Thr 86 TNF. The MTP was in-

cubated 6–96 h at 37°C. Then the wells were gently washed twice with RPMI 1640 supplemented with 10% FCS, 100 U/ml penicillin, 100 µg/ml streptomycin, and 2 mM glutamine. The cells to be used for adherence were prelabeled overnight with 1 µCi/10⁶ cells of methyl-³H]thymidine (Amersham, Amersham, England), washed twice in medium, and added to the endothelial cells in 100 µl medium per well (1–5 × 10⁶ cells/ml). The MTP was further incubated from 30 min to 2 h at 37°C. Nonadherent cells were removed using a calibrated centrifugation of the inverted MTP in PBS at 50 g for 5 min at ambient temperature (41). Radioactively labeled cells specifically bound to the HUVEC were harvested with cell harvester (LKB Instruments, Inc., Gaithersburg, MD). The radioactivity was measured in a Betaplate liquid scintillation counter (Pharmacia LKB Biotechnology). Data are expressed as mean and standard deviation of triplicates.

Nuclear Extracts and Electrophoretic Mobility Shift Assay (EMSA). Nuclear extracts were prepared as described by Digman et al. (42) with minor modifications. Briefly, cells were harvested and washed twice with PBS. 15–20 × 10⁶ cells were resuspended in a hypotonic lysis buffer (10 mM Hepes, pH 7.9, 1.5 mM MgCl₂, 10 mM KCl; buffer A) at 4°C. After a 20-min incubation on ice, cells were homogenized by 20 strokes with a loose-fitting Dounce homogenizer. Nuclei were collected by centrifugation (4 min, 6,500 rpm, 4°C) and nuclear proteins were extracted with three pellet volumes of high salt buffer (20 mM Hepes, pH 7.9, 25% [vol/vol] glycerol, 0.42 M NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, pH 7.5; buffer B). After a 1-h incubation on ice, samples were centrifuged (5 min, 6,500 rpm, 4°C) and the supernatants were diluted in 3 vol low salt buffer (20 mM Hepes, pH 7.9, 20% [vol/vol] glycerol, 0.1 M KCl, 0.2 mM EDTA, pH 7.5, 1% NP-40; buffer C). The samples were used immediately in EMSA or stored at –80°C. Immediately before use, buffers A, B, and C were supplemented with 1% of a 1 M 1,4-dithio-DL-threitol (DTT; Fluka Chemie AG). For EMSA, a synthetic oligonucleotide containing the NF-κB binding site was used as a probe (5'CTCAACAGAGGGGACTTCCGAGAGGCCATCT3'). A corresponding mutated oligonucleotide was included as control in the assay (5'CTAACAGAAATTACTTCCGAGAGGCCATCT3'). About 60,000 cpm of the labeled synthetic sequence was incubated with 5 µl of each nuclear extract for 45 min at ambient temperature in presence of 2 µg of poly(dI-dC) (Pharmacia LKB Biotechnology) and 30 µg BSA (43, 44).

Results

TNF Receptor Expression on HUVEC. The expression of the two TNF receptor types, TNF-R55 and TNF-R75, by HUVEC was investigated first by flow cytometry, using the htr and utr series of mAbs, respectively (26, 39). The analysis was performed at a stage when cultures were 90% confluent after the second passage. The cells were detached by trypsin-EDTA treatment, which had been shown in separate experiments not to detectably affect the surface proteins of the HUVEC. Control experiments showed that the cells analyzed were entirely Von Willebrand factor positive, which confirmed identity and homogeneity of the HUVEC culture. Fig. 1 A shows that unstimulated HUVEC expressed both receptor types, TNF-R75 and TNF-R55. The level of expression of both receptors was relatively low, as revealed by the parallel staining of U937 cells, which express both TNF receptor types with a total of a few thousand TNF binding sites per cell (39).

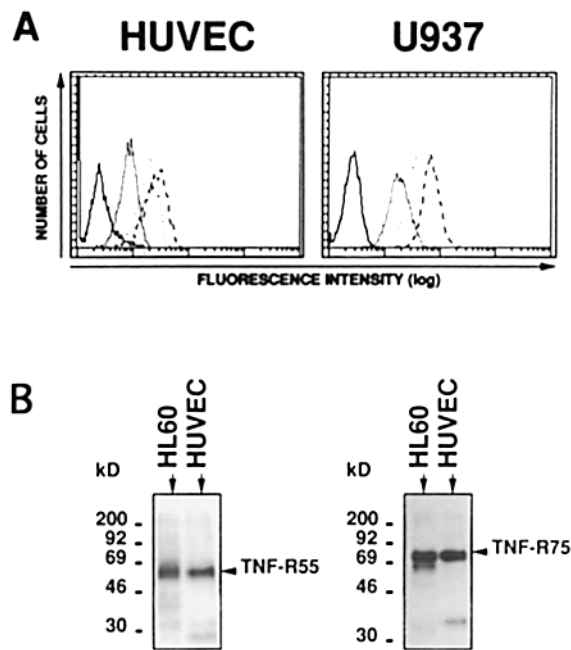


Figure 1. TNF receptor expression on HUVEC and U937 cells. (A) Cytofluorimetric analysis of HUVEC and U937 cells stained with anti-TNF-R55 mAbs htr-5 (· · · ·), htr-1 (— · — ·), and anti-TNF-R75 mAb utr-1 (— — —) followed by PE-conjugated goat anti-mouse Ig. An irrelevant mAb was used as negative control (—). (B) Western blot analysis of TNF receptor expression by HUVEC and HL60 cells. Cells were incubated with the respective utr and htr mAb and solubilized by detergent. Immune complexes were purified with protein G from the cell lysate, subjected to electrophoresis on nonreducing SDS-PAGE (12% gel) and transferred to filter membranes. The utr (right) and htr (left) blots were treated with RaTNF-R75 and RaTNF-R55, respectively, followed with ¹²⁵I-labeled goat anti-rabbit antibody and exposed to x-ray film. Specific receptor bands in the autoradiogram are indicated with arrows. The contaminating lower molecular mass band is due to protein G.

The expression of both TNF receptor types by HUVEC was confirmed using the Western blot technique (Fig. 1 B). Unstimulated HUVEC were incubated for 2 h with either htr-20 (anti-TNF-R55) or utr-1 (anti-TNF-R75) antibodies, detached, and solubilized by detergents. Immune complexes were precipitated with protein G–Sepharose beads and subjected to SDS-PAGE. After electrophoresis, the separated proteins were transferred to filter membranes, which were then incubated with affinity-purified polyclonal rabbit antibodies to TNF-R75 (RaTNF-R75) and to TNF-R55 (RaTNF-R55). After washing, the filters were incubated with ¹²⁵I-labeled goat anti-rabbit Ig and reactive bands were revealed by autoradiography (Fig. 1 B). The specific bands representing TNF-R75 and TNF-R55 of HUVEC were found comigrating with similar bands from HL60 cells, which are known to express both TNF receptor types (26, 39) (Fig. 1 B).

Control of ICAM-1, E-selectin, and VCAM-1 Expression through TNF-R55 but Not through TNF-R75. The modulation of endothelial adhesion molecules ICAM-1, E-selectin, and VCAM-1 by TNF-α is well documented (12, 13, 45). To investigate whether either or both TNF receptors control the expression of these cell adhesion molecules, HUVEC were

stimulated from 0 to 10 h with human TNF- α binding to both receptor types, and with receptor type-specific agonists. The specific activation of TNF-R55 was achieved using three independent reagents: first the agonistic mAb htr-1, second the RaTNF-R55 agonistic antibodies, and third a recombinant mutant of human TNF- α , R32W/S86T-TNF- α (Trp32 Thr86 TNF), which binds exclusively to TNF-R55. TNF-R75 activation was achieved with RaTNF-R75 agonistic antibodies. To investigate ICAM-1, E-selectin, and VCAM-1 expression, HUVEC, activated by the various agents, were detached, immunostained, and analyzed by cytofluorimetry. The cytofluorimetric histograms obtained are shown in Fig. 2. Similar to previous reports (45), ICAM-1 was found to be expressed on unstimulated HUVEC at relatively high levels, whereas E-selectin and VCAM-1 were essentially absent. Stimulation of HUVEC with human TNF- α triggered the upregulation of ICAM-1 and the induction of E-selectin and VCAM-1 expression (Fig. 2). The modulation of ICAM-1 and VCAM-1 expression was detectable after 2 h of TNF- α stimulation and reached a sustained maximum level after 6–8 h, contrasting with the expression of E-selectin where induction was maximal 2 h after stimulation and the expression again had declined after 10 h. This confirmed a previous report showing a tran-

sient induction of E-selectin by TNF- α (15). The upregulation of ICAM-1 and the induction of VCAM-1 in magnitude and kinetic response were essentially the same, when HUVEC were stimulated through TNF-R55 with Trp32 Thr86 TNF or with htr-1 antibodies (Fig. 2), and similar results were also obtained with the RaTNF-R55 antibodies. Minor differences between the different agonists in the extent of induction were seen, and these might be attributed to experimental variation, but it cannot be excluded that the dimeric or trimeric nature of the different ligands may also play a role (27). In contrast, stimulation via TNF-R75 did not lead to any induction in expression of E-selectin and VCAM-1, nor to upregulation of ICAM-1 expression, even under conditions where RaTNF-R75 antibodies in other functional responses of HUVEC displayed full agonistic activity (see below).

TNF- α -induced CD44 Expression Is under TNF-R55 Control. To study the activity of TNF- α on CD44 expression by the endothelium, HUVEC cultures were first stimulated with TNF- α for various time periods, detached as described above, and immunostained with the mouse mAb F10-44-2 directed to a constant epitope of human CD44 (46). The cytofluorimetric histograms obtained are shown in Fig. 3 A.

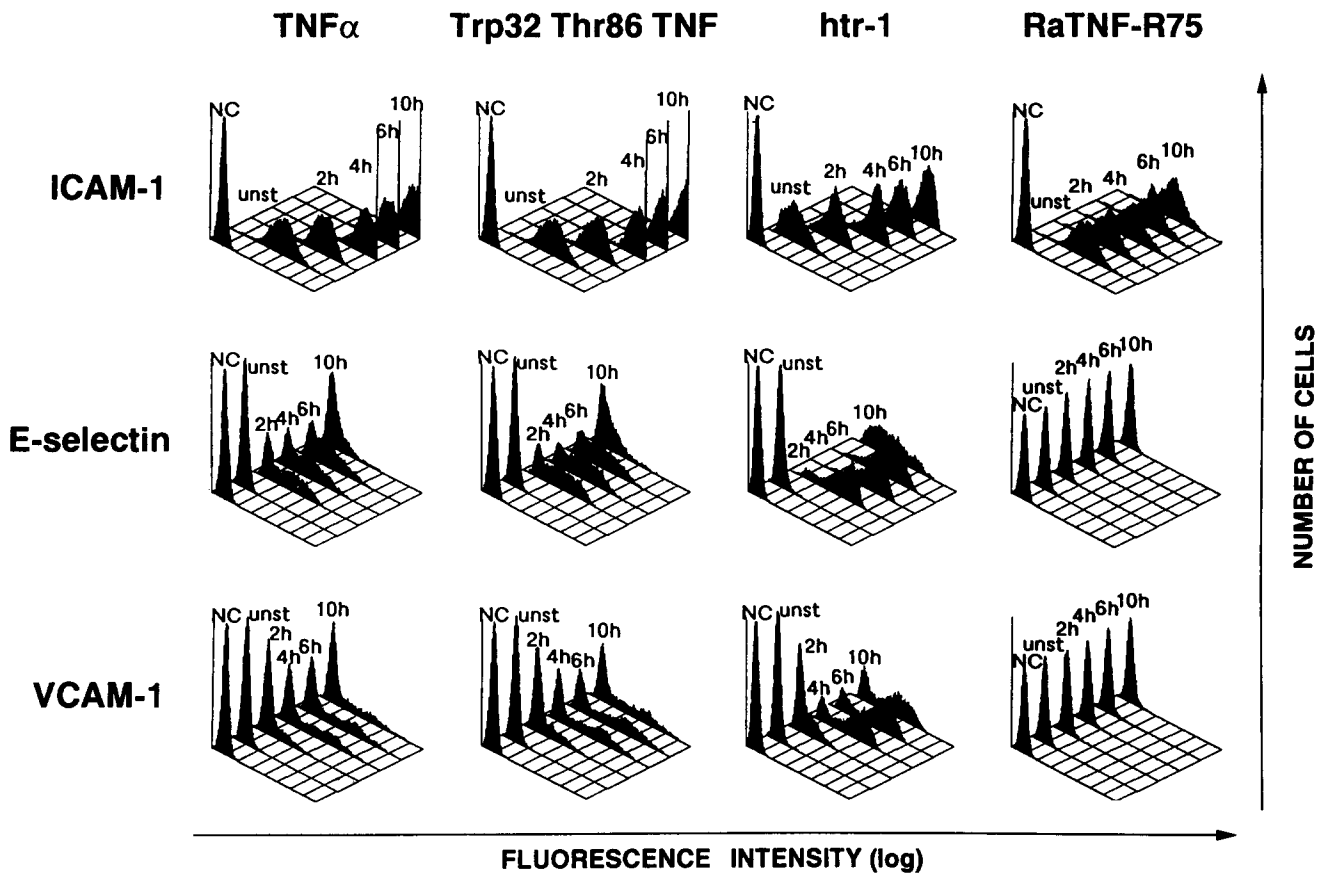


Figure 2. Cytofluorimetric profiles of ICAM-1, E-selectin, and VCAM-1 expression on HUVEC. HUVEC were stimulated in culture for 2, 4, 6, and 10 h with 10 ng/ml TNF- α , 10 ng/ml Trp32 Thr86 TNF, 10 μ g/ml htr-1, and 1 μ g/ml RaTNF-R75 as indicated. HUVEC were then stained for cytofluorimetry with anti-human ICAM-1, anti-human E-selectin, and anti-human VCAM-1 mAbs, followed by PE-conjugated goat anti-mouse Ig. Relative cell numbers (vertical) and fluorescence intensity on logarithmic scale (horizontal) are presented. (NC) Negative control; (unst) unstimulated cells.

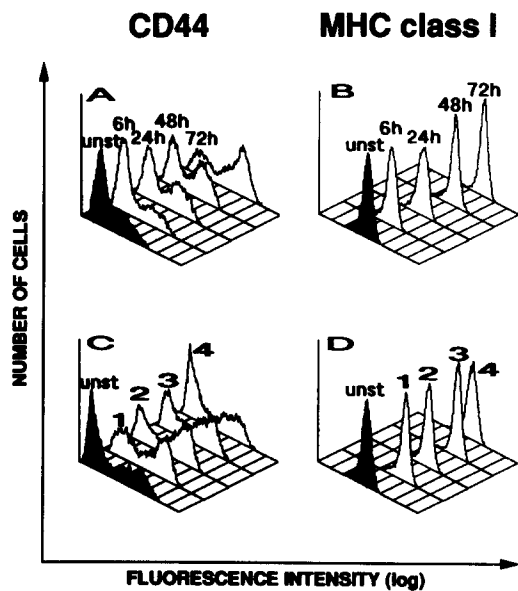


Figure 3. Cytofluorimetric profiles of CD44 and MHC class I expression in unstimulated and activated HUVEC. HUVEC were stimulated 6, 24, 48, and 72 h in culture with 10 ng/ml TNF- α , washed, stained with anti-human CD44 mAb (A), or anti-human MHC class I mAb (B), followed by PE-conjugated goat anti-mouse Ig. HUVEC were also stimulated 72 h with 10 ng/ml Trp32 Thr86 TNF (1), 1 μ g/ml R α TNF-R55 (2), 10 μ g/ml htr-1 (3), and 1 μ g/ml R α TNF-R75 (4), stained as described above with anti-CD44 (C) and anti-MHC class I (D) mAbs. Relative cell numbers (vertical) and fluorescence intensity in logarithmic scale (horizontal) are presented. Unstimulated HUVEC (unst) are in black, stimulated cells in white.

CD44 was found expressed on unstimulated HUVEC in a heterogeneous fashion. The majority of the cells showed no or low CD44 expression, but about a third of the cells showed higher expression. Interestingly, after 48–72 h of TNF- α stimulation, the profile of CD44 expression was significantly changed, since >50% of the cells were shifted to the CD44 highly expressing cell population. As a control, MHC class I expression was analyzed in parallel cultures (Fig. 3 B). MHC class I molecules were expressed on unstimulated HUVEC, and upregulated after 24 h of TNF- α treatment as previously reported (47). It is noteworthy, however, that in contrast to the heterogeneous CD44 staining, the same cells showed homogeneous staining with respect to MHC class I staining (Fig. 3 B). This demonstrates that the wide spectrum of expression levels is a specific property of CD44 and not due to heterogeneity within the HUVEC population. To define the TNF receptor type mediating this CD44 induction, HUVEC were stimulated with predetermined optimal concentrations of the TNF-R55 agonists Trp32 Thr86 TNF, R α TNF-R55, and htr-1, and with the TNF-R75 agonist R α TNF-R75. As shown clearly in Fig. 3 the induction of CD44 (C) as well as the upregulation of MHC class I (D) were mediated exclusively through TNF-R55.

Both TNF Receptors Mediate α 2 Integrin Induction. To search for a role of TNF-R75 in adhesion, the integrin family of adhesion molecules was studied. HUVEC were incubated with

TNF- α for various periods of time and the expression levels of integrin molecules were analyzed by cytofluorimetry. Some of the integrins, such as α 2, α 5, α 6, α v, β 1, and β 3, have previously been reported to be expressed on unstimulated HUVEC (48–50). TNF- α stimulated an upregulation of α 2 integrin expression (Fig. 4 A). A slight increase of α 2 expression was first seen after 24 h of TNF- α stimulation and the expression reached its maximum after 48–72 h. The upregulation of α 2 expression is significant and was reproducibly found in three independent experiments. Confirming previous reports, a similar upregulation was not seen with other integrins (51), which indicated that TNF- α -dependent regulation is α 2 specific. HUVEC were then stimulated with the receptor type-specific agonists Trp32 Thr86 TNF, R α TNF-R55, htr-1, and with R α TNF-R75. Interestingly, both TNF receptor types were similarly found to mediate α 2 induction (Fig. 4, B and C).

HL60 Cell Adhesion on Stimulated HUVEC. Given the different aptitudes of TNF-R75 and TNF-R55 to mediate the induction of the various cell adhesion molecules, we examined whether the two receptor types also differ in their capacity to modulate cell adhesion. HUVEC were stimulated with the various receptor type-specific agonists. HL60 cells that had been prelabeled with methyl- 3 H]thymidine were overlaid on the HUVEC for 30 min at 37°C. Nonadherent HL60 cells were removed in a precalibrated centrifugation step and the bound HL60 cells quantitated. In the initial series of studies (Fig. 5) the optimal concentrations of TNF- α , Trp32 Thr86 TNF, R α TNF-R55, htr-1, and R α TNF-R75 needed to achieve adhesion after 6 h of treatment of HUVEC were determined. It was found that the adhesion induced by

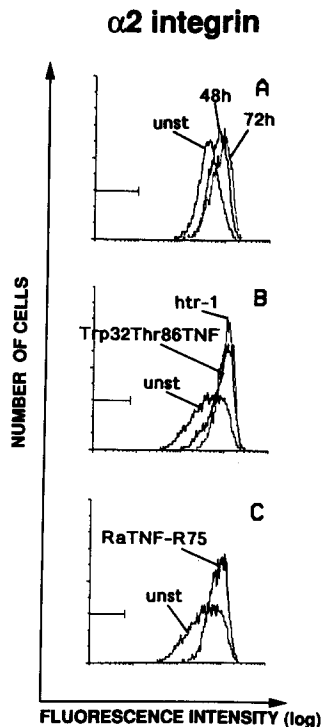


Figure 4. Cytofluorimetric profiles of α 2 integrin expression in HUVEC. HUVEC were stimulated for various periods of time with 10 ng/ml TNF- α and stained with anti-human α 2 integrin mAb, followed by PE-conjugated goat anti-mouse Ig. (A) α 2 integrin expression in unstimulated (unst) HUVEC and in HUVEC after 48 and 72 h TNF- α stimulation. (B) HUVEC stimulated 72 h with TNF-R55 agonists Trp32 Thr86 TNF (10 ng/ml) and htr-1 (10 μ g/ml). (C) HUVEC stimulated with TNF-R75 agonist R α TNF-R75 (1 μ g/ml). Relative cell numbers (vertical) and fluorescence intensity (logarithmic scale, horizontal) are presented. The bar indicates the position of the control peak.

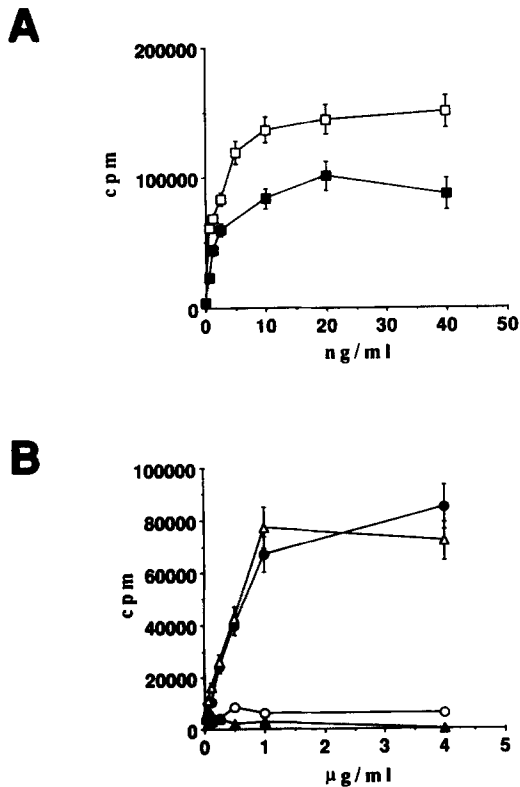


Figure 5. HL60 cell adhesion on HUVEC. HUVEC in culture were incubated 6 h with various concentrations of (A) TNF- α (□) or Trp32 Thr86 TNF (■), and (B) htr-1 mAb (●), R α TNF-R55 (Δ), R α TNF-R75 (○), and the nonagonistic utr-1 mAb (▲). After incubation, HUVEC cultures were washed with medium and overlaid with 200 μ l methyl- 3 H]thymidine-labeled HL60 cells (10^6 cells/ml). Adhesion was performed for 30 min at 37°C. Nonspecifically bound cells were removed by calibrated centrifugation. Bound HL60 cells were harvested and bound radioactivity was counted. The mean of triplicates and standard deviation are given.

TNF- α could be mimicked by Trp32 Thr86 TNF despite a small difference in specific activity (Fig. 5 A). These findings were also confirmed when HUVEC were stimulated with R α TNF-R55 and with htr-1 (Fig. 5 B). No significant differences were seen among these TNF-R55 agonists. In contrast, TNF-R75 triggering resulted in little or no induction of adhesion after 6 h stimulation. These findings were confirmed by microscopic inspection of the various cell culture adhesion assays. Similar results were obtained when U937 or MOLT-4 cells were used instead of HL60 cells for the adhesion assays (data not shown).

To investigate the time dependence of the induction of cell adhesion, HUVEC were treated for up to 96 h with the same optimal concentrations of TNF- α , Trp32 Thr86 TNF, R α TNF-R55, htr-1, and R α TNF-R75, and cellular adhesion assays were performed as before with HL60 cells. It was found consistently in three independent experiments (Fig. 6) that a first phase of adhesion occurred within 24 h stimulation when HL60 adhesion was strongly induced through TNF-R55, whereas stimulation through TNF-R75 did not lead to any increased adhesion above background levels. In a later

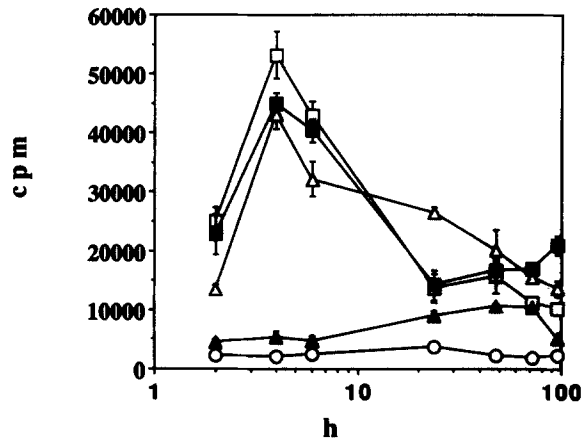


Figure 6. Time dependence of HL60 cell adhesion on activated HUVEC. HUVEC in culture were stimulated for various periods of time with optimal concentrations (see Fig. 5) of TNF- α (□), Trp32 Thr86 TNF (■), htr-1 mAb (Δ), R α TNF-R75 (▲), and the nonagonistic utr-1 control mAb (○). HL60 adhesion assay was performed as described in Fig. 5. The mean of triplicates and standard deviation are given.

phase, beginning 24 h after the start of cell stimulation, weak adhesion of HL60 cells also was induced. This late adhesion was at a much lower but still significant level. Interestingly, this late adhesion was observed after stimulation through both TNF-R55 and TNF-R75.

TNF-R55 Controls NF- κ B Activation. TNF- α is a strong activator of the transcription nuclear factor κ B, NF- κ B (52, 53). Furthermore, as a common feature, κ B elements are known to be present in the regulatory flanking sequences of the ICAM-1, E-selectin, VCAM-1, and MHC class I genes (54–59). NF- κ B has been found to be involved in MHC class I expression (59). The possible correlation between the control of the adhesion molecules by TNF-R55 and NF- κ B activation was therefore investigated. HUVEC were stimulated for 15 min under predetermined optimal conditions with TNF- α , and with the receptor type-specific agonists. Nuclear extracts were prepared from these cells and analyzed in an EMSA, performed using a labeled NF- κ B-specific oligonucleotide probe. As shown in Fig. 7 A, NF- κ B was activated exclusively through TNF-R55 and not through TNF-R75, which parallels the ICAM-1, E-selectin, and VCAM-1 induction through TNF-R55. As a specificity control, a similar experiment was performed using the HL60 cell line. The HL60 cells were stimulated 15 min with TNF- α and various receptor type-specific agonists. The results presented in Fig. 7 B confirmed the findings with the HUVEC, since TNF-R55 controls NF- κ B activation also in HL60.

Discussion

Among the factors that control the binding of leukocytes to activated endothelium, TNF- α may be one of the most important. The role of TNF- α in cell adhesion has been investigated in a number of studies. For example, melanoma cell adhesion to HUVEC has been reported to be induced by

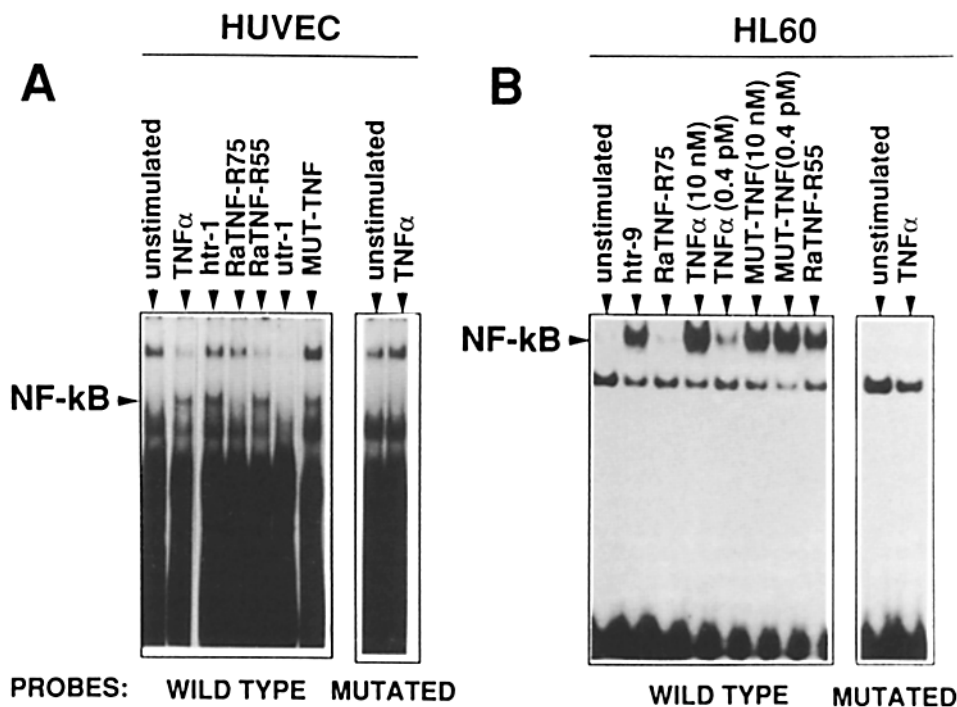


Figure 7. NF- κ B activation in HUVEC and HL60 cells analyzed by electrophoretic mobility shift assay. (A) HUVEC in culture were stimulated 15 min with 10 ng/ml TNF- α , 10 ng/ml Trp32 Thr86 TNF (*MUT-TNF*), 10 μ g/ml htr-1 mAb, 1 μ g/ml RaTNF-R75, 1 μ g/ml RaTNF-R55, and 1 μ g/ml utr-1 mAb. Nuclear extracts were incubated 45 min with a 32 P-labeled oligonucleotide containing the NF- κ B binding site. As control, nuclear extracts were also incubated with a labeled oligonucleotide containing a mutated NF- κ B binding site. (B) HL60 cells were incubated 30 min with 5 μ g/ml htr-9 mAb, 1 μ g/ml RaTNF-R75, 10 and 0.4 nM TNF- α , 10 and 0.4 nM Trp32 Thr86 TNF, and 1 μ g/ml RaTNF-R55. Nuclear extracts were incubated 45 min with the same oligonucleotide probes as used for the HUVEC. The specific NF- κ B bands are indicated by arrows in the autoradiographs.

TNF- α (15). In HUVEC culture, the TNF- α -stimulated cell adhesion has been found to correlate with the induction of several cell adhesion molecules such as ICAM-1, E-selectin, and VCAM-1 (reviewed in references 20 and 60). Several studies have reported significant reduction of cell adhesion when endothelial cells were treated with blocking antibodies directed against these three adhesion molecules (61). In our own studies, the adhesion of HL60 cells to HUVEC was strongly induced by TNF- α treatment within a few hours, reached a maximum after 6–8 h, then gradually decreased. This adhesion phenomenon was mediated by TNF-R55 exclusively. After prolonged stimulation through TNF-R55 for >24–48 h, a weak cell adhesion persisted. This latter adhesion phenomenon interestingly also could be triggered through TNF-R75, demonstrating that both TNF receptor types are functional and mediate signals in HUVEC. The exclusive role of TNF-R55 in the early and strong induction of cellular adhesion correlates with the induction of ICAM-1, E-selectin, and VCAM-1 expression through TNF-R55, whereas TNF-R75-mediated signals did not influence the expression of these three molecules. The finding that TNF-R55, but not TNF-R75, mediated TNF- α -induced adhesion is consistent with previous reports describing the modulation of ICAM-1 and E-selectin on HUVEC after stimulation with the TNF-R55-specific htr mAb (62) and in keratinocytes that express only TNF-R55 (31).

It is a common feature of the ICAM-1, E-selectin, and VCAM-1 gene promoter sequences to contain κ B elements that are thought to be important for their regulation (54–58). It is an interesting parallel that NF- κ B in HUVEC is specifically activated through TNF-R55, which is consistent with the exclusive role of TNF-R55 in TNF- α -dependent

NF- κ B activation reported in other cell systems (32, 33). The parallel between NF- κ B activation and regulation of expression through κ B elements suggests that NF- κ B might play a critical role in the control of cell adhesion to HUVEC. However, other control mechanisms in addition to the NF- κ B pathway must exist, because ICAM-1 is already expressed on unstimulated HUVEC, whereas E-selectin and VCAM-1 appear only after TNF- α stimulation. Moreover, these molecules show different patterns of induction kinetics. E-selectin is very transiently induced, whereas ICAM-1 and VCAM-1 have similar induction kinetics. Additional factors therefore must play a role in the TNF- α signal transduction pathway eliciting adhesion. These factors appear to be under TNF-R55 control, because stimulation through TNF-R55 gives similar expression patterns as those seen after TNF- α treatment interacting with both receptor types.

The adhesion molecules involved in the weak late adhesion found equally after TNF-R55 and TNF-R75 stimulation have not been identified. The integrin adhesion molecules, also called very late antigens (reviewed in reference 63) might be candidates. Previous reports described the regulation of α 1, α 6, and β 3 integrins in HUVEC by TNF- α with or without IFN- γ cooperation (51, 64–66). These responses required a minimum of 24 h of TNF- α exposure and reached a maximal level between 48 and 72 h. The α 2 integrin in HUVEC in the present study was reproducibly induced after 48–72 h of TNF- α stimulation. A further parallel induction is provided by the fact that both TNF receptor types similarly mediated the α 2 induction and the weak late adhesion, and integrins thus might be envisioned to play a role in late adhesion. However, while α 2 associated with the β 1 integrin is known to bind to collagen and laminin (63), its role

on the endothelial cell surface is unclear. Furthermore, in a recent study, $\alpha 2$ mRNA and protein in human endothelial cells were found not to be affected by TNF- α (66); the increase in $\alpha 2$ staining upon TNF- α stimulation thus might reflect a change in conformation, or an association with a different cell surface component, leading to an increased reactivity with the antibody rather than a higher expression level. Integrins have been shown, under an appropriate stimuli, to be able to change their conformation (reviewed in reference 63).

Similar to ICAM-1, E-selectin, and VCAM-1, the CD44 adhesion molecule on HUVEC is upregulated after prolonged TNF- α stimulation through TNF-R55. The F10-44-2 anti-

body (63) used to detect CD44 recognizes a constant part of the various differentially spliced forms of CD44 (67). CD44 is known to be a receptor of several ligands including collagen and hyaluronate. It has been shown to play a role in T cell activation (reviewed in reference 68) and metastatic properties of certain tumor cells (67), but its functional role in endothelial cells is largely unknown. However, the present findings establish distinct activities of TNF-R55 and TNF-R75 in HUVEC and point to a pivotal role of TNF-R55 in TNF- α -induced cell adhesion to the endothelial cell layer, and thus in leukocyte migration and extravasation in sites of inflammation and tissue injury.

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