Functional Activities of Receptors for Tumor Necrosis Factor-α on Human Vascular Endothelial Cells

By Ewa M. Paleolog, Sally-Anne J. Delasalle, Wirm A. Buurman, and Marc Feldmann

Tumor necrosis factor-α (TNF-α) plays a critical role in the control of endothelial cell function and hence in regulating traffic of circulating cells into tissues in vivo. Stimulation of endothelial cells in vitro by TNF-α increases the surface expression of leucocyte adhesion molecules, enhances cytokine production, and induces tissue factor procoagulant activity. In the present study, we have examined the relative roles of the two cell surface receptors for TNF-α (p55 and p75) on endothelial cells, using antibodies with both agonistic and antagonistic activities. We report that anti-p55 receptor agonistic antibody Htr-9 induces the expression of tissue factor antigen and the release of interleukin-8 (IL-8) and granulocyte-macrophage colony-stimulating factor (GM-CSF). In contrast, there is very little or no activation of endothelial cell responses by an anti-p75 agonist. TNF-α-induced expression of tissue factor and adhesion molecules, and release of IL-8 and GM-CSF, are decreased by antibodies with antagonistic activities for either receptor, although the effect of anti-p55 antibodies is markedly greater than that of anti-p75 antibodies. The responses of endothelial cells to lymphotoxin/TNF-β are significantly decreased by anti-p55 antagonists alone. Our data suggest that endothelial cell responses to TNF-α, such as expression of tissue factor and adhesion molecules for mononuclear cells, which may be important in the pathogenesis of atherosclerosis, are mediated predominantly, but not exclusively, by the p55 TNF receptor.

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As the lining of the vasculature, endothelium controls the trafficking of cells and molecules into underlying tissues, and is therefore a potential primary target for the action of inflammatory mediators. Cytokines such as tumor necrosis factor-α (TNF-α) and interleukin-1 (IL-1) dramatically alter many responses of cultured endothelial cells in vitro, which may be relevant to the key role of the endothelium in vivo contributing to the development of atherosclerosis, inflammation, and septic shock.

After activation by TNF-α, human umbilical vein endothelial cells (HUVEC) become adhesive for leukocytes through increased cell surface expression of molecules such as E-selectin, which is known to mediate the early phase of neutrophil binding, as well as binding monocytes and certain T-cell subsets. Two other endothelial antigens are intercellular adhesion molecule-1 (ICAM-1), which also binds neutrophils, and the mononuclear cell receptor vascular cell adhesion molecule-1 (VCAM-1). Endothelial cells are themselves a source of inflammatory mediators, such as prostaglandin I₂ (PGI₂), IL-1, IL-6, and granulocyte-macrophage colony-stimulating factor (GM-CSF), as well as the neutrophil chemotaxin IL-8 and monocyte chemotactic protein-1 (MCP-1). Finally, stimulation of HUVEC by TNF-α converts the quiescent endothelium into a procoagulant surface, through the concomitant downregulation of antithrombotic functions and the upregulation of prothrombotic mechanisms such as tissue factor expression and von Willebrand factor secretion.

The involvement of endothelial antigens and secretory products in the pathogenesis of atherosclerosis has been documented in a number of studies. Immunohistochemical analysis of human atherosclerotic arteries has shown increased expression of E-selectin, ICAM-1, and VCAM-1 in areas of intimal thickening and atheromatous plaques. The contribution to plaque generation of endothelial-derived mediators, such as the chemotactic cytokines IL-8 and MCP-1, has been postulated, and expression in arterial lesions of tissue factor, which could contribute to atherosclerosis through the formation of an adhesive platelet/fibrin network, has also been reported. One of the earliest recognizable lesions of atherosclerosis is the "fatty streak," made up of lipid-rich macrophages and T lymphocytes within the arterial intima. These cells are likely to have migrated across the endothelial lining after initial adhesion to endothelium, and such mononuclear cell adhesion and migration could be enhanced by cytokines released from both endothelial and subendothelial cells. Both TNF-α and interferon-γ (IFN-γ) have been detected in atherosclerotic plaques, with immunoreactive TNF-α observed both in the cytoplasm and attached to the cell membrane of endothelium in atherosclerotic, but not normal, arteries.

For TNF-α to transmit a signal and hence exert an effect on endothelial cells, it must initially bind to specific high-affinity cell surface receptors. The TNF receptor family has been studied extensively, in our own and other laboratories, and two high-affinity surface receptors for TNF-α and TNF-β/lymphotoxin, of molecular weights 55 kD and 75 kD (termed p55 or CD120a, and p75 or CD120b, respectively) have been cloned and expressed. Their relative importance on endothelial cells is at present unclear. It has been suggested that the predominant receptor is the p55 type, although both receptors have been detected on the surface of HUVEC. Stimulation of HUVEC by Htr-9, an antibody with agonist activity for p55, has been shown to induce release of IL-6 and rapidly increase the adhesiveness of endothelial monolayers for leukocytes. It was subsequently reported that Htr-9 increased the surface levels of E-selectin, ICAM-1, and VCAM-1, with time courses similar to those
observed for TNF-α, actions not mimicked by anti-p75 agonists. Both p55 and p75 receptors have been detected in experiments measuring the binding of 125I-labeled TNF-α to HUVEC. It has also been shown that monoclonal antibodies (MoAbs) against both p55 and p75 TNF receptors substantially inhibit production of IL-6 from endothelial cells and adhesion of HL60 cells. Maximal reduction of IL-6 secretion was obtained with a combination of the two antibodies.

In addition to the apparent discrepancies with regard to the relative roles of p55 and p75 in the response of endothelial cells to TNF-α, there have also been reports of disparate effects of TNF-α and TNF-β on HUVEC. It was observed that, whereas both TNF-α and lymphotoxin/TNF-β induced adhesion of neutrophils to endothelial monolayers after 4 hours of incubation, only TNF-α, but not TNF-β, increased secretion of GM-CSF. Similarly, TNF-β was found to be only a weak agonist for induction of IL-1 release from endothelial monolayers relative to TNF-α, although binding of 125I-TNF-α to HUVEC was fully inhibited by TNF-β.

We propose that an understanding of the relative contributions of p55 and p75 receptors on cultured endothelial cells and the mechanisms of their regulation is an important step towards further elucidating the function of endothelium in the pathogenesis of atherosclerosis. In the present study, we examined the relative roles of p55 and p75 in the induction of endothelial activation by TNF-α, using antibodies with both agonistic and antagonistic activities for the TNF receptors.

MATERIALS AND METHODS

Endothelial cell culture. Human endothelial cells were isolated from umbilical veins by digestion for 10 minutes with 0.05% collagenase (from Clostridium histolyticum; Boehringer Mannheim UK, Lewes, East Sussex, UK) and cultured in medium RPMI-1640 plus 25 mmol/L HEPES and 1-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, 10% fetal calf serum (FCS), and 10% newborn calf serum. At confluence, cells were detached with 0.05% trypsin/0.02% EDTA in phosphate-buffered saline (PBS) and subcultured at a 1:3 split ratio in the above endothelial cell growth medium containing, in addition, 50 U/mL heparin (Leo Laboratories Ltd, Princes Risborough, Bucks, UK) and 15 μg/mL endothelial cell growth supplement (ECGS; Sigma, Poole, Dorset, UK). Endothelial cell preparations were confirmed to be ≥98% pure by staining with two markers for endothelial surface antigens, ie, antibody CLB-HEC75 specific for CD31 (Eurogenetics Ltd, Teddington, UK) followed by goat antibody specific for mouse IgG conjugated to phycoerythrin, and Ulex europaeus-1 lectin coupled to fluorescein isothiocyanate (Sigma).

Experimental protocol. For the experiments, cells between the second and fifth passages were removed by trypsinization and seeded onto 12-well culture trays (area, 400 mm²/well) that were precoated with 1% gelatin, at a density of approximately 2 × 10⁴ cells per well. After 48 to 72 hours, culture medium was replaced with prewarmed RPMI-1640 containing 5% heat-inactivated FCS.

To determine the effects of antireceptor antagonistic antibodies, cells were pretreated for 1 hour in the absence or presence of the appropriate antibody, before the addition of TNF-α, to give a final volume of 200 μL per well, and incubated for 4 to 24 hours at 37°C in a 5% CO₂ humidified atmosphere. The antireceptor antibodies used were either anti-p55 antibody H398 (gift from Prof K. Pfizenmaier, Stuttgart, Germany) or anti-p75 antibody Utr-1 (gift from Dr M. Brockhaus, Hoffmann-La Roche, Basel, Switzerland). In certain experiments, endothelial cells were preincubated with antireceptor antibodies as described above and then challenged with lymphotoxin for 4 to 24 hours.

Alternatively, endothelial cell monolayers were stimulated for 4 to 24 hours with agonistic anti-TNF receptor antibodies Htr-9 (specific for p55; gift from Dr M. Brockhaus) or MR2-1 (specific for p75; University of Limburg, Maastricht, Netherlands). Antibody MR2-1 was defined as recognizing the p75 receptor for TNF-α (CD120b) at the 5th International Workshop on Human Leukocyte Differentiation Antigens (November 1993, Boston, MA). Antireceptor antibodies were added either alone at 37°C for the total incubation period, or for 1 hour at 4°C followed by goat antimiouse IgG (Pierce, Rockford, IL) as cross-linker at 37°C for a further 4 to 24 hours.

At the end of the experiment, cell supernatants were removed and frozen at −20°C before being assayed for cytokine production, and the cell monolayers harvested and stained for surface antigen expression. Each experimental point was performed in triplicate. All antibody preparations contained ≤4 pg endotoxin per microgram of antibody, as determined by the LAL assay (sensitivity, ≤10 pg/mL; BioWhittaker UK Ltd, Reading, Berks, UK).

Immunostaining for endothelial cell surface antigens. Expression of adhesion molecules was measured essentially as previously described. After removal of culture supernatants, the endothelial cell monolayers were washed extensively with prewarmed Hank’s Balanced Salt Solution and incubated with 0.02% EDTA for 20 to 30 minutes at 37°C, after which time cells detached readily from the culture plate. Approximately 10⁵ cells per group were washed twice in PBS plus 5% FCS, and adhesion molecule expression was detected by staining for 30 minutes at 4°C with mouse MoAbs specific for either E-selectin (antibody 49B11, 1:100 dilution; gift from Prof M. Vadas, Adelaide, Australia), ICA1 (antibody I4, 1:1,000 dilution of mouse ascites fluid; gift from Dr A. Boyd, Melbourne, Australia) or VCAM-1 (antibody 4B9, 15 μg/mL; gift from Dr J. Harlan, Seattle, WA), followed by 10 μg/mL goat antibody specific for mouse IgG, conjugated to biotin (Southern Biotechnology Associates, Birmingham, AL) for 30 minutes at 4°C. Finally, 10 μg/mL streptavidin conjugated to phycoerythrin (Southern Biotechnology Associates) was added for 30 minutes at 4°C. Cells were subsequently fixed with 1% formaldehyde in PBS.

All cell groups were also stained with second-layer reagents alone (goat antimiouse IgG-biotin and streptavidin phycoerythrin) to determine binding of detection antibodies to anti-TNF receptor antibodies themselves. Under the conditions of our experiments, there was no significant binding of goat antimiouse biotin to either anti-p55 or anti-p75 antibodies (≤5 fluorescence units). Cells were analyzed by FACScan (Becton Dickinson and Co, Mountain View, CA) and analyses were performed on viable cells by gating on forward and side scatter. Results were calculated as mean fluorescence intensity values (mean ± SD from ≥3 determinations). Alternatively, results are expressed as fold increase over unstimulated levels (mean of ≥3 determinations ± SD).

Measurement of endothelial cell tissue factor. Expression of endothelial cell tissue factor was measured using a murine antihuman tissue factor antibody that was a gift from Dr T. Edgington (Scripps Research Institute, La Jolla, CA). Briefly, endothelial cells (10⁴ per sample) were harvested with 0.02% EDTA as described above and washed twice with PBS plus 5% FCS. Anti-tissue factor antibody (10 μg/mL) was added for 30 minutes at 37°C, followed by 10 μg/mL goat antibody specific for mouse IgG conjugated to biotin and 10 μg/mL streptavidin conjugated to phycoerythrin (Southern Biotechnology Associates). Each for 30 minutes at 37°C. Fluorescence analysis was performed by FACScan, and data are presented as mean fluorescence intensity values (mean ± SD from ≥3 determinations).
This assay was found to be comparable to a one-stage clotting assay for tissue factor induction, based on the method of Colucci.20

Assays of cytokine release. Secretion of IL-8 from HUVEC was measured by enzyme-linked immunosorbent assay (ELISA), using murine MoAb 4.1.3 as capture antibody, and biotinylated murine MoAb A5.12.14 as detection antibody, followed by biotinylated streptavidin conjugated to horseshad peroxidase and O-phenylene-diamine hydrochloride as substrate. The results were standardized against human recombinant IL-8 (range, 100 pg/mL to 10 ng/mL). Both human recombinant IL-8 and anti-IL-8 antibodies were a gift from Genentech Inc (San Francisco, CA).

Production of GM-CSF was assessed as the proliferation of the megakaryoblastic cell line Mo7E (gift from Genetics Institute, Cambridge, MA) using 2 x 10⁵ Mo7E cells per well cultured with either serial dilutions of endothelial supernatants or recombinant GM-CSF (specific activity, 9.3 x 10⁶ U/mg, Genetics Institute) in RPMI-1640 plus 10% FCS and 10% newborn calf serum. After 72 hours, the cells were pulsed with 0.5 μCi [³H]-thymidine for the final 4 hours of culture, harvested with a multiwell cell harvester (Pharmacia, St Albans, Herts, UK), and counted using the Beta-Plate System (Pharmacia). All supernatants were assayed in triplicate, and the values of 2 to 6 serial dilutions were computed from a standard curve using human recombinant GM-CSF (assay range, 1 pg/mL to 10 ng/mL).

Similarly, production of IL-6 was assessed as the proliferation of the B9 hybridoma cell line (gift from Dr L. Aarden, Central Blood Transfusion Laboratory, Amsterdam, The Netherlands) using 2 x 10⁵ B9 cells per well cultured with either serial dilutions of endothelial supernatants or recombinant IL-6 (specific activity, 5.65 x 10⁶ U/mg; generous gift from Interpharm Laboratories, Nes Ziona, Israel) in RPMI-1640 plus 10% FCS and 10% newborn calf serum. Cells were pulsed and harvested as described above. Supernatants were assayed in triplicate, and the values of 2 to 6 serial dilutions were computed from a standard curve using human recombinant IL-6 (assay range, 0.1 pg/mL to 2 ng/mL).

Data are expressed as mean release ± SD from at least 3 separate determinations, or as the fold increase over unstimulated levels (mean of ± 3 determinations ± SD). None of the antireceptor antibodies used interfered in the cytokine ELISAs or GM-CSF and IL-6 bioassays.

 Binding of TNF-α to endothelial cells. Binding of TNF-α to endothelial cells was measured using [¹²⁵I]-labeled TNF-α. Cells between the second and fifth passages were cultured to confluence in 75-cm² flasks before removal of monolayers using a nonenzymatic Cell Dissociation Solution (Sigma), added for 5 to 15 minutes at 37°C, after which time cells detached readily. A total of ≥ 5 x 10⁵ endothelial cells per determination was collected and washed twice by centrifugation, resuspended in PBS containing 5% FCS, and incubated for 1 hour at 4°C in the absence or presence of anti-TNF receptor agonistic or antagonistic antibodies as appropriate, at a final antibody concentration of 10 μg/mL. Human recombinant [¹²⁵I]-labeled TNF-α (50 to 110 μCi/μg protein) was added at a final concentration of 50 ng/mL for a further 3 hours at 4°C. Unbound ligand was removed by centrifugation of the cell suspension through 20% sucrose in PBS. Nonspecific binding of [¹²⁵I]-TNF-α was quantified by preincubating endothelial cells in the presence of 160-fold excess of unlabeled human recombinant TNF-α (1 hour at 4°C, 8 μg/mL), before the addition of [¹²⁵I]-TNF-α. Cell pellets were counted using an LKB 1260 Multigamma II γ-counter.

All determinations were performed in triplicate, and results were expressed as mean ± SD. Counts ± SD or as a function of the binding of [¹²⁵I]-TNF-α to cells treated in the absence of antireceptor antibodies.

Endothelial cell proliferation assay. Endothelial cells were plated in 96-well culture plates (area, 30 mm² per well) at a density of 6 x 10⁴ cells per well (50% confluent density) in 200 μL per well RPMI-1640, containing 50 U/mL heparin (Leo Laboratories Ltd), 15 μg/mL ECGS, 10% FCS, and 10% newborn calf serum, and incubated for 24 hours at 37°C. Subsequently, fresh medium, containing the appropriate concentration of either TNF-α or agonistic antibody, together with ECGS, was added and simultaneously cells were pulsed with 0.5 μCi per well of [³H]-thymidine. After a total of 24 hours, endothelial cells were submitted to three cycles of freeze-thawing, harvested with a multiwell cell harvester (Pharmacia), and counted using the Beta-Plate System (Pharmacia). Both human recombinant and anti-TNF receptor antibodies inhibit binding of [¹²⁵I]-TNF-α. Values are means ± SD from at least 3 separate experiments.

Materials. TNF-α (human recombinant, from Escherichia coli; 1 x 10⁷ U/mg; < 0.9 ng endotoxin per milligram of protein as determined by LAL assay) was a gift from Prof. W. Stuc (Centre of Molecular and Macromolecular Studies, Lodz, Poland). Alternately, TNF-α was obtained from Genentech Inc (human recombinant, from E.coli; 5 x 10⁷ U/mg). Genentech Inc (human recombinant, 2 x 10⁷ U/mg). RPMI-1640, penicillin, streptomycin, FCS, and newborn calf serum were purchased from GIBCO (Paisley, UK). Basic FGF was a gift from Synergen (Denver, CO).

RESULTS

Anti-TNF receptor antibodies inhibit binding of TNF-α to endothelial cells. To verify that the antibodies used were binding to receptors for TNF-α on HUVEC, we measured binding of [¹²⁵I]-TNF-α after preincubation in the absence or presence of either 10 μg/mL antibody or excess unlabeled TNF-α. Optimal binding was obtained using 50 ng/mL [¹²⁵I]-TNF-α in the absence or presence of 8 μg/mL unlabeled TNF-α (data not shown). All of the antibodies (anti-p55 antibodies H398 and Htr-9, and anti-p75 antibodies Utr-1 and MR2-1) significantly (P ≤ .01) reduced binding of [¹²⁵I]-TNF-α to endothelial cells. In agreement with previously published results, we detected higher levels of p55 TNF receptors relative to p75. However, the addition of a combination of Htr-9 and MR2-1 did not totally inhibit the binding of [¹²⁵I]-TNF-α to HUVEC (inhibition, 79% ± 12%; Table 1).

By counting the number of endothelial cells used in each experiment and knowing the specific activities of the [¹²⁵I]-TNF-α preparations, we were able to estimate the numbers of TNF receptors on HUVEC. In total, we calculate 1,684

<table>
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<th>Addition</th>
<th>Mean ± SD</th>
<th>% Inhibition</th>
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<tr>
<td>None</td>
<td>5,580 ± 494</td>
<td>—</td>
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<tr>
<td>Unlabeled TNF-α</td>
<td>1,973 ± 171</td>
<td>—</td>
</tr>
<tr>
<td>Htr-9</td>
<td>3,640 ± 262</td>
<td>54 ± 7</td>
</tr>
<tr>
<td>MR2-1</td>
<td>4,708 ± 276</td>
<td>24 ± 8</td>
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<tr>
<td>Htr-9 + MR2-1</td>
<td>2,741 ± 434</td>
<td>79 ± 12</td>
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*Calculated as a function of [¹²⁵I]-TNF-α binding loss in the presence of excess unlabeled TNF-α. Values are means ± SD from 3 separate experiments.
± 143 receptors for TNF-α per cell (mean ± SEM, 7 to 10 determinations from 3 separate experiments), which breaks down to 968 ± 48 p55 receptors and 658 ± 88 p75 receptors, irrespective of the anti-p55 or anti-p75 antibodies used.

*Induction of tissue factor antigen on HUVEC by TNF-α is inhibited by antibodies to both p55 and p75 TNF receptors.* Addition of TNF-α to cultured endothelial cells for 4 hours induced the surface expression of tissue factor antigen, measured as the increased binding of a specific anti-tissue factor antibody. Induction of tissue factor antigen expression was dependent on the dose of TNF-α used, and was markedly inhibited when endothelial cells were preincubated for 1 hour in the presence of antagonistic anti-p55 antibody H398 before the addition of TNF-α. Stimulation of tissue factor expression by a submaximal dose of TNF-α (3 ng/mL) induced a 6.8 ± 1.5-fold enhancement in the binding of anti-tissue factor antibody, which was significantly downregulated in the presence of increasing doses of H398 (Fig 1A). Significant inhibition (P < .05 versus response in the presence of TNF-α alone) was observed with as little as 0.3 μg/ml H398, and maximal inhibition was obtained with 10 μg/ml H398 (Fig 1A), which almost totally abolished expression of tissue factor in response to TNF-α (inhibition, 87% ± 8%; P < .001). Addition of a maximal dose of TNF-α (10 ng/mL) after pretreatment with H398 only partially restored the response, and was still significantly less (P < .01) than that induced by TNF-α alone (Fig 1B).

To ensure that binding of H398 to HUVEC did not interfere with subsequent binding of anti-tissue factor antibody, binding of anti-CD31 antibody was also measured in the same experiment and found to be unaffected by anti-TNF receptor antibody treatment (mean fluorescence intensity of unstimulated cells was 147.3 ± 20.1, of cells treated with TNF-α was 146.5 ± 22.4, and of cells pretreated with 3 μg/mL H398 followed by TNF-α was 137.8 ± 2.5).

Anti-p75 antibody Utr-1 was also inhibitory with respect to TNF-α–induced tissue factor expression, although the degree of inhibition was substantially lower than was observed for anti-p55 antibody. For example, in the same experiment, inhibition of tissue factor expression in response to 1 ng/mL TNF-α was 61% ± 5% for H398 (10 μg/mL), as compared with 26% ± 14% for Utr-1 (10 μg/mL; data not shown). As illustrated in Fig 2, concentrations of 1 to 3 μg/mL Utr-1 were effective at reducing tissue factor expression in response to a submaximal dose of TNF-α, although only 3 μg/mL Utr-1 induced significant (P < .05) inhibition. Maximal inhibition was obtained with 10 to 30 μg/mL Utr-1, and did not exceed 70% in any of the experiments.

**Anti-p55 agonist Htr-9 increases tissue factor expression on endothelial cells.** Stimulation of HUVEC through the p55 TNF receptor alone, using the agonistic antibody Htr-9, resulted in a modest but consistent increase in tissue factor antigen expression (Fig 3). This was observed as an increase in the mean fluorescence of cells stained with anti-tissue factor antibody after 4 hours of incubation in the presence of Htr-9 (Fig 3A), although the effect of Htr-9 was substantially less than that of TNF-α. Treatment of HUVEC with a maximal dose of TNF-α (10 ng/mL) increased the mean fluorescence from 5.3 ± 1.4 U for unstimulated cells to 22.8 ± 2.3 U for TNF-α–treated cells (mean ± SD, N = 3). In the same experiment, the mean fluorescence of cells activated with 10 μg/mL Htr-9 was 10.5 ± 1.6 U. The threshold concentration of Htr-9 for significant (P < .05) induction of tissue factor antigen was 1 μg/mL, and maximal tissue factor induction by 50 μg/mL Htr-9 was 47% ± 11% when calculated as a function of the response to TNF-α (Fig 3B).

Anti-p75 receptor agonist MR2-1 consistently failed to
Fig 2. Inhibition by Utr-1 of TNF-α-induced tissue factor antigen expression. Endothelial cells were preincubated in the absence or presence of Utr-1 for 1 hour, before stimulation with 3 ng/mL TNF-α for 4 hours. Expression of tissue factor antigen was measured by FACS analysis. Data are pooled from two similar experiments. Significant differences versus response in the presence of TNF-α alone: *P < .05.

induce substantial changes in anti-tissue factor antibody binding, although in 2 of 4 experiments a weak, but significant (P < .05), increase in fluorescence after incubation with 10 μg/mL MR2-1 for 4 hours was observed, equivalent to 15% ± 12% of the response induced by 10 ng/mL TNF-α. Such an experiment is illustrated in Table 2. For comparison, induction of E-selectin on the same cells was measured and was also found to be weakly affected by MR2-1 (9% ± 4% of the TNF-α response, P < .01; Table 2). In the same experiment, 10 μg/mL Htr-9 induced tissue factor and E-selectin expression equivalent to 64% ± 7% and 58% ± 10%, respectively. However, in the remaining two experiments, tissue factor expression in response to 10 μg/mL MR2-1 was not significantly greater than that of unstimulated endothelial cells.

To assess whether cross-linking the anti-TNF receptor antibodies would increase the signal through the p75 receptor, HUVEC were equilibrated with either MR2-1 or Htr-9 for 1 hour at 4°C and then incubated for a further 4 hours at 37°C in the presence of goat antimouse IgG as cross-linker. However, no further induction of either tissue factor or E-selectin expression by anti-p75 agonistic antibody could be detected, even in the presence of a 10-fold excess of cross-linker relative to MR2-1 (data not shown).

Anti-p75 and anti-p75 receptor antibodies inhibit endothelial adhesion molecule expression and cytokine release to different degrees. Anti-TNF receptor antibodies were also found to inhibit TNF-α-induced secretion of the endo-

![Image](image-url)

Table 2. Effect of Agonistic Anti-TNF Receptor Antibodies on Expression of E-Selectin and Tissue Factor

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<th>E-Selectin</th>
<th>Tissue Factor</th>
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<tr>
<td>Medium alone</td>
<td>13.0 ± 3.4</td>
<td>6.7 ± 1.9</td>
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<tr>
<td>TNF-α</td>
<td>238.8 ± 3.4</td>
<td>28.5 ± 3.8</td>
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<tr>
<td>Htr-9</td>
<td>143.1 ± 22.5</td>
<td>20.7 ± 4.3</td>
</tr>
<tr>
<td>MR2-1</td>
<td>33.3 ± 9.9</td>
<td>10.1 ± 2.8</td>
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Endothelial cells were incubated in the absence or presence of either 10 μg/mL anti-p75 antibody MR2-1, 10 μg/mL anti-p75 antibody Htr-9, or 10 ng/mL TNF-α for 4 hours, before immunostaining with antibodies to either E-selectin or tissue factor. Values are mean fluorescence ± SD (N = 3 to 5).
TNF RECEPTORS ON VASCULAR ENDOTHELIAL CELLS

that the anti-p55 TNF receptor antibody H398 was consider\
ably more effective than anti-p75 TNF receptor antibody Utr-1 in inhibiting endothelial cell responses.

The threshold concentration of TNF-α for induction of endothelial cell activation as judged by adhesion molecule expression or cytokine production was 0.3 ng/mL, with maximal responses observed at 10 to 30 ng/mL TNF-α. We therefore used 1 to 3 ng/mL TNF-α to measure the effects of antireceptor antibodies. Both Utr-1 and H398 substantially inhibited expression of VCAM-1 by HUVEC stimulated with TNF-α (Fig 4). Maximal inhibitory effects were observed at 10 μg/mL antibody (data not shown) and were 77% ± 9% for H398 and 40% ± 4% for Utr-1 at 3 ng/mL TNF-α. When expression of other TNF-α-inducible adhesion molecules, such as ICAM-1 or E-selectin, was assayed after treatment with antireceptor antibodies, similar data was obtained (data not shown).

Figure 6 illustrates GM-CSF dose-effect curves for TNF-α in the absence or presence of either (■) H398 or (□) Utr-1 (0.3 to 30 μg/mL) for 1 hour, HUVEC were incubated for a further 24 hours with a submaximal dose of TNF-α (1 ng/mL). Release of IL-8 by unstimulated endothelial cells was 5.4 ng/mL (---). Significant differences versus release of cytokine in the presence of 1 ng/mL TNF-α: *P ≤ .05, **P ≤ .01. Values are the means of three determinations ± SD.

In the experiment described in Fig 4, release of IL-8 from HUVEC incubated for 24 hours with 1 ng/mL TNF-α was 102 ± 15 ng/mL greater than unstimulated levels, and was inhibited by antibodies to both TNF receptors. Inhibition was dose-dependent and was greater for H398 than for Utr-1. For example, at a concentration of 10 μg/mL antibody, release of IL-8 from anti-p55-treated cells was 25% ± 12% of that induced by TNF-α alone, in comparison to 58% ± 8% for anti-p75-treated HUVEC. Inhibition of IL-8 secretion was maximal at 10 μg/mL of Utr-1 and 30 μg/mL of H398. In the presence of the highest concentration of H398, release of IL-8 by 1 ng/mL TNF-α was not significantly greater than spontaneous release.

Antibody Concentration (μg/mL)

![Graph showing antibody concentration vs. IL-8 released (ng/mL)](image)

**Fig 5.** Secretion of IL-8 by endothelial cells in response to TNF-α is inhibited by both Utr-1 and H398 in a dose-dependent manner. After preincubation for 1 hour in the absence or presence of either (■) H398 or (□) Utr-1 (0.3 to 30 μg/mL) for 1 hour, HUVEC were incubated for a further 24 hours with a submaximal dose of TNF-α (1 ng/mL). Release of IL-8 by unstimulated endothelial cells was 5.4 ng/mL (---). Significant differences versus release of cytokine in the presence of 1 ng/mL TNF-α: *P ≤ .05, **P ≤ .01. Values are the means of three determinations ± SD.

**Fig 4.** Expression of VCAM-1 on HUVEC in response to TNF-α is inhibited by antibodies to both p55 and p75 receptors. Endothelial cells were preincubated in the absence or presence of either 10 μg/mL H398 or 10 μg/mL Utr-1 for 1 hour, before stimulation with either medium alone or 1 to 3 ng/mL TNF-α for 24 hours. VCAM-1 expression was assayed by FACS analysis. Values are means of three determinations ± SD. Significant differences versus response in the presence of TNF-α alone: *P ≤ .05, **P ≤ .01. (■) TNF + 10 μg/mL H398; (□) TNF + 10 μg/mL Utr-1.
In a separate experiment, HUVEC were pretreated with submaximal concentrations of either of the antibodies alone or a combination of both antibodies, before the addition of TNF-α. Final concentrations of antibodies were 3 μg/mL Utr-1 and/or 3 μg/mL H398. Surface levels of E-selectin were measured after 4 hours of incubation with TNF-α, whereas expression of ICAM-1 and IL-8 secretion were measured after 24 hours of treatment with TNF-α. We observed that the effect of a combination of submaximal concentrations of anti-TNF receptor antibodies was additive for all the responses tested (Table 3). Similar data were obtained for secretion of GM-CSF or expression of VCAM-1 and tissue factor (data not shown).

Anti-p55 receptor agonistic antibody increases release of cytokines and adhesion molecule expression on endothelial cells. Secretion of IL-8 and GM-CSF was markedly stimulated by anti-p55 agonist Htr-9, but only very weakly by anti-p75 agonist MR2-1, as has been shown both by other groups for expression of adhesion molecules and IL-6 release18,19 and in the present study for induction of E-selectin and tissue factor.

A concentration of Htr-9 as low as 0.1 μg/mL stimulated significant (P < 0.05) release of both IL-8 and GM-CSF from HUVEC (Fig 7). The response was maximal at a concentration of 10 μg/mL Htr-9, but did not exceed 55% to 65% of that induced by a 24-hour challenge with 10 ng/mL TNF-α. These observations were consistent with the effect of Htr-9 on the expression of VCAM-1 and ICAM-1 in the same experiments (data not shown). The anti-p75 antibody MR2-1 was found to be only a weak agonist for induction of endothelial cell responses. As was observed for expression of E-selectin and tissue factor, MR2-1 was a much less potent stimulus for endothelial activation than was Htr-9, and this agonist activity was not consistently observed. In one experiment, induction of GM-CSF release greater than unstimulated levels after 24 hours with 10 μg/mL antibody was 1.6 ± 0.4 ng/mL for MR2-1 and 18.5 ± 1.1 ng/mL for Htr-9, equivalent to 6% ± 1% and 73% ± 4%, respectively, relative to 10 ng/mL TNF-α. In Table 4, the effects of 1 μg/mL anti-TNF receptor agonists on expression of ICAM-1 and
VCAM-1, and secretion of IL-8 and GM-CSF, are compared with that of a maximal dose of TNF-α. Addition of 1 μg/mL MR2-1 induced significant (P < .05) increases in ICAM-1 levels and release of cytokines. However, VCAM-1 expression in response to MR2-1 was not significantly elevated above unstimulated levels. The effect of MR2-1 was dose-dependent and was observed in only two of four experiments. This weak activity of MR2-1 was unlikely to be caused by endotoxin contamination of this antibody (54 pg endotoxin per microgram of MR2-1), because in our hands at least 100 ng/mL lipopolysaccharide was required to induce adhesion molecule expression and cytokine release.

Anti-p55 agonist Htr-9 inhibits endothelial cell proliferation. In agreement with previous reports,31,37 we observed that in our assay system the addition of TNF-α to subconfluent umbilical vein endothelial cells in the presence of growth factors substantially inhibited endothelial cell proliferation, measured as the incorporation of 1H-thymidine. When endothelial cells were cultured in the presence of ECGS, 1H-thymidine incorporation was enhanced relative to HUVEC cultured in the absence of ECGS. This increase was almost totally abolished when ECGS was added together with 25 ng/mL TNF-α. Inhibition of 1H-thymidine incorporation was dependent on the dose of TNF-α added, and was not due to production of Pg I*, because the addition of 1 μmol/L indomethacin did not restore the proliferative response. Comparable data were obtained when basic FGF (10 ng/mL) was used in place of ECGS (data not shown). Subsequently, results were expressed as mean "stimulation indices" ± SD, relative to 1H-thymidine incorporation by cells cultured in the absence of ECGS.

Replacement of TNF-α by Htr-9 also resulted in significant inhibition (P < .001 versus incorporation in the presence of ECGS alone) of endothelial cell proliferation. This inhibitory effect on 1H-thymidine incorporation was dose-dependent and maximal at 10 ng/mL Htr-9. At this concentration, the addition of Htr-9 reduced the stimulation index from 2.1 ± 0.2 in the presence of ECGS alone to 1.6 ± 0.1 in the presence of ECGS plus Htr-9. In the same experiment, incorporation of 1H-thymidine was reduced to a stimulation index of just 1.3 ± 0.1 by the addition of 10 ng/mL TNF-α (P < .001; Fig 8). However, the inhibitory effect of 10 μg/mL Htr-9 was still less than that of TNF-α (P < .01). In a similar experiment, the p75 agonistic antibody was without significant effect on endothelial cell proliferation in the presence of ECGS. Incorporation of 1H-thymidine in the presence of 10 μg/mL MR2-1 was 92% ± 8% relative to ECGS alone, as compared with 45% ± 4% in the presence of 10 μg/mL Htr-9 in the same experiment (data not shown).

Endothelial cell responses to lymphotoxin are significantly inhibited by anti-p55 but not by anti-p75 antibody. We compared the effects of anti-TNF receptor antibodies on expression of adhesion molecules and tissue factor antigen in response to both TNF-α and lymphotoxin. Lymphotoxin/TNF-β induces a similar pattern of endothelial cell responsiveness to TNF-α, although substantially higher concentrations of lymphotoxin are required to elicit the same magnitude of response.40,41 In our own experiments, the addition of 3 ng/mL TNF-α increased ICAM-1 expression by 592.0 ± 86.8 fluorescence units over unstimulated levels, whereas 3 ng/mL and 30 ng/mL lymphotoxin increased ICAM-1 by only 168.9 ± 101.0 and 489.9 ± 102.4 fluorescence units, respectively (Fig 9). Indeed, addition of up to 100 ng/mL lymphotoxin increased the expression of ICAM-1 and VCAM-1 to only 70% and 50% of the response induced by a maximal dose of TNF-α (10 ng/mL; data not shown).

Preincubation of HUVEC with 10 μg/mL anti-p55 antibody H398 decreased expression of ICAM-1 induced by both 3 ng/mL and 30 ng/mL lymphotoxin to levels not sig-
Endothelial cell responses to lymphotoxin are significantly inhibited by anti-p55 TNF receptor antibody alone. Endothelial cells were pretreated for 1 hour in the presence of 10 μg/mL of either H398 or Utr-1 before the addition of either TNF-α (3 ng/mL) or lymphotoxin (3 ng/mL and 30 ng/mL) for a further 24 hours. Expression of ICAM-1 was measured by FACS analysis, and values are means of three determinations ± SD. Significant differences versus response in the presence of TNF-α or lymphotoxin alone: *P < .05, **P < .01. (---) Unstimulated expression. (■) Cytokine alone; (□) +10 μg/mL Utr-1; (△) +10 μg/mL H398.

The vascular endothelial cell lining of blood vessels plays a key role in vivo, both in the control of hemostasis and in the development of pathologic conditions such as rheumatoid arthritis, atherosclerosis, and septic shock. In particular, cytokines such as TNF-α may activate the endothelium, leading to increased adhesion of leukocytes and platelets, expression of prothrombotic activities, and production of a range of proinflammatory mediators. Two surface receptors for TNF-α, termed p55 and p75, have been identified. We have examined the relative roles for these receptors in TNF-α-mediated activation of cultured human vascular endothelial cells, assaying expression of leukocyte adhesion molecules, production of cytokines, and induction of tissue factor antigen.

In the present study, we have shown that inhibition of TNF-α binding to both TNF receptor subtypes on endothelial cells reduces responses to TNF-α. Analysis by flow cytometry of HUVEC incubated with anti-TNF receptor antibodies alone showed very low expression of TNF receptors. The fluorescence intensity of HUVEC stained with, for example, 10 μg/mL H398 (4.4 ± 0.4 fluorescence units above background) was negligible when compared with the fluorescence intensity of cells treated with 3 ng/mL TNF-α and stained with antiadhesion molecule antibodies such as 1H4 anti-ICAM-1 antibody (856.8 ± 94.6 U). This observation of a low level of TNF receptor expression is in agreement with previously published data. Nevertheless, in all our experiments in which HUVEC were pretreated with anti-TNF receptor antibodies before FACS analysis, appropriate controls were included to assess the contribution of any binding of goat antimouse biotinylated detection reagent to cell-bound anti-TNF receptor antibodies. From our experiments using 125I-TNF-α, we calculated a total of 968 ± 48 p55 receptors and 658 ± 88 p75 receptors for TNF-α on cultured endothelial cells.

Antibodies with antagonistic activity at both TNF receptors potently inhibited TNF-α–induced expression of adhesion molecules (E-selectin, ICAM-1, and VCAM-1), induction of tissue factor antigen, and release of cytokines (IL-8 and GM-CSF). The inhibitory effect of anti-p55 antagonist H398 was markedly greater (maximal inhibition, 70% to 100%) than that of anti-p75 antagonist Utr-1 (maximal inhibition, 40% to 70%). We frequently observed that the maximal inhibitory effect of anti-p55 antibody with respect to all of the responses tested was greater than 80%, whereas in the same experiment the maximal effect of Utr-1 exceeded 40%, suggesting a possible cooperative action between the two receptors. A combination of suboptimal doses of anti-p55 and anti-p75 receptor antibodies was additive in effect.

and 30 ng/mL lymphotoxin to levels not distinguishable from those observed on unstimulated cells (inhibition, ≥97%). In the same experiment, E-selectin induction by 3 ng/mL TNF-α was inhibited by 38% ± 7% after preincubation with Utr-1 and by 63% ± 2% after preincubation with H398. In a separate experiment, incubation of HUVEC with 150- to 1,000-fold excess of lymphotoxin completely prevented the subsequent binding of 125I–TNF-α (data not shown).
A theoretical model has been proposed for an interactive effect between p55 and p75, in which the higher-affinity p75 plays an indirect role in p55 responses at low TNF-α concentrations by binding TNF-α and delivering it to the lower-affinity p55 receptor. Subsequent studies using murine L929 cells confirmed that antibodies to p55 could completely inhibit TNF-α–induced cytotoxicity, whereas anti-p75 antagonists blocked the effect of TNF-α only at low cytokine concentrations. Under the same conditions, there were no apparent effects of p75 TNF receptor agonists, suggesting that p75 may indeed facilitate the activation of cells by TNF-α through the p55 receptor subtype. This mechanism would explain certain published observations such as the complete neutralizing activity of p55 antagonists observed with cell lines expressing both p55 and p75 TNF receptors, and our own data are also in agreement with the above model.

Anti-p55 TNF receptor antibody Htr-9 was observed to be a potent endothelial cell agonist, suggesting that the p55 receptor is involved in the signalling pathway for TNF-α. Activation of endothelial cells by Htr-9 increased the expression of leukocyte adhesion molecules such as E-selectin, VCAM-1, and ICAM-1, and enhanced the release of cytokines such as IL-8 and GM-CSF. Additionally, Htr-9 induced tissue factor antigen expression on HUVEC in a dose-dependent manner. The response induced by a maximal dose of Htr-9 was 50% to 70% relative to that elicited by a maximal dose of TNF-α. Despite the significant inhibitory effect of antagonistic anti-p75 TNF receptor antibody on all of the endothelial cell responses assayed, anti-p75 TNF receptor antibody MR2-1 was found to be only a very weak and inconsistent endothelial agonist, inducing negligible levels of GM-CSF and IL-8 release and adhesion molecule expression, as well as very slight induction of tissue factor antigen.

A combination of both agonistic antibodies did not restore the response observed with a maximal dose of TNF-α. However, MR2-1 was not devoid of bioactivity, because in our hands MR2-1 was a potent stimulus in the KYM-1D4 cell line cytotoxicity assay over the range 0.01 to 10 μg/mL (S. Su, personal communication, November 1993). Antibody MR2-1 was defined as specific for the p75 receptor for TNF-α (CD120b) at the 5th International Workshop on Human Leukocyte Differentiation Antigens (November 1993, Boston, MA). We also showed that Htr-9, but not MR2-1, mimicked the activity of TNF-α in inhibiting endothelial cell incorporation of 3H-thymidine. This inhibitory action of TNF-α has been suggested to be caused by a cytotoxic effect on the endothelium, although under the conditions of our experiments we did not observe any significant cytotoxicity (data not shown). A similar inhibition of proliferation has been reported for IL-1, and this action was also not apparently associated with cytotoxicity. Such a pattern of agonistic activity by p55 antibody only, but antagonistic effects by both anti-p55 and anti-p75, is again consistent with the model proposed by Tartaglia and Geeddel.

The response of HUVEC to lymphotoxin (measured as expression of adhesion molecules) was only significantly affected by preincubation with the anti-p55 antagonist H398. In contrast, anti-p75 antibody Utr-1 was without effect. This was not caused by any lack of inhibitory activity of the Utr-1 preparation, because in the same experiments the responses of HUVEC to TNF-α were consistently inhibited by ≥30%. Anti-p55 antibody H398 was actually a more potent inhibitor of the response of HUVEC to lymphotoxin as opposed to TNF-α, because inhibition of ICAM-1 expression induced by 30 ng/mL lymphotoxin was totally abolished by H398, whereas the response of a 10-fold lower dose of TNF-α was only inhibited by 68% ± 18%. In a different experiment, the addition of 100 ng/mL lymphotoxin increased ICAM-1 expression to levels comparable to those observed in response to 3 ng/mL TNF-α. However, even at this ratio of TNF-α to lymphotoxin of 1:30, H398 was a more effective inhibitor of lymphotoxin-induced ICAM-1 expression (inhibition, 83% ± 3%) than of TNF-α–induced ICAM-1 expression (inhibition, 53% ± 11%). We also assessed the effect of anti-TNF receptor antagonists on lymphotoxin-induced release of IL-6 and GM-CSF. Release of cytokines in response to lymphotoxin was also not affected by anti-p75 antibody Utr-1, and was almost totally abolished after preincubation with anti-p55 antibody H398. In contrast to a previous report, we consistently found that lymphotoxin was able to stimulate release of GM-CSF from HUVEC. This may be explained by differences in the preparations of lymphotoxin and TNF-α used in these studies. Moreover, in our hands, a significant secretory response required the addition of at least 30 ng/mL (6 × 10^3 U/mL) lymphotoxin, whereas the highest dose used in the published study was 1 × 10^3 U/mL (8 ng/mL) lymphotoxin. However, we observed that even at a concentration of 30 ng/mL, lymphotoxin was less effective than a submaximal dose of TNF-α.

Our data clearly imply that, although only the p55 receptor agonist Htr-9 can mimic the activity of TNF-α on HUVEC, both receptors are implicated in the induction of all of the responses studied. This is shown by the ability of antibodies with agonist activity for both p55 and p75 to substantially inhibit endothelial activation by TNF-α (ie, expression of adhesion molecules and tissue factor antigen, and release of cytokines), although the effects of anti-p55 antagonists were consistently greater than those of anti-p75 antagonists. Despite the marked inhibition by anti-p75 antibody of all TNF-α–induced responses measured, there appeared to be very little, if any, signalling through the p75 receptor, although MR2-1 did bind to endothelial cells, as determined by its ability to reduce 125I–TNF-α binding to HUVEC. We have thus confirmed that both the p55 and p75 TNF receptors are critical in mediating the effects of TNF-α on endothelial cells, although only p55 appears to play an important role in transducing intracellular signals that lead to the observed responses. Previous investigators have shown a major role for only the p55 TNF receptor in the induction of endothelial cell adhesion molecules by TNF-α, measuring expression of E-selectin, ICAM-1, VCAM-1, and CD44, and production of IL-6, using both agonistic and antagonistic anti-TNF receptor antibodies, as well as TNF receptor type-specific agonists that bound exclusively to either p55 or p75. A recent publication has also shown that mutant forms of TNF-α with a selective capacity to bind to p55 alone stimulated endothelial cells to express E-selectin and bind neutrophils.
phils, whereas p75-selective mutants were without effect. These observations are consistent with our findings in the present study with respect to the induction by anti-p55 agonist Htr-9 of endothelial cell GM-CSF and IL-8 release and expression of tissue factor. Interestingly, Slowik et al recently reported that a p55-selective mutant form of TNF-α fully desensitized endothelial cells to human recombinant TNF-α, measured as E-selectin expression, but induction of this adhesion molecule in response to TNF-α was blocked by antibodies to both p55 and p75.

In vivo, vascular endothelial cell activation in response to cytokines such as TNF-α or IL-1 has been proposed to play a key role in a number of pathologic conditions, such as atherosclerosis, rheumatoid arthritis, and gram-negative sepsis. For example, TNF-α-inducible endothelial adhesion molecules for monocytes and lymphocytes, such as VCAM-1 and E-selectin, and endothelial-derived mediators such as MCP-1 and PDGF, have been detected in atherosclerotic plaques in humans. Additionally, TNF-α itself has also been observed in human atherosclerotic vessels. Such enhanced adhesion molecule expression and chemotaxis production may potentially contribute to the generation of "fatty streaks," made up of T lymphocytes and lipid-rich macrophages within the arterial intima, both by mediating the attachment, activation, and migration of leukocytes, and by stimulating lesion progression through release of molecules such as plasminogen-activator inhibitor-1 and platelet-activating factor and through the expression of tissue factor.

We propose that an understanding of TNF-α receptor expression on vascular endothelium is an important step towards determining its role in vivo in the regulation of inflammation and pathologic conditions such as atherosclerosis. Such regulation of expression could be effected by circulating cytokines such as IFN-γ, IL-1, or IL-4. We have shown in an earlier study that both IFN-γ and IL-4 modulate endothelial cell responses to TNF-α. Our own unpublished results indicate that IFN-γ increases total TNF-α binding to cultured endothelial cells, although this is not in agreement with an earlier report. Our data suggest that endothelial responses to TNF-α, such as monocyte adhesion and expression of procoagulant activity, which are important in the pathogenesis of atherosclerosis, are mediated predominantly by the p55 receptor, although the p75 receptor also appears to contribute significantly to endothelial activation by TNF-α. Regulation of TNF receptor expression on vascular endothelium may play a critical role in the development of atherosclerotic lesions in vivo.

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Functional activities of receptors for tumor necrosis factor-alpha on human vascular endothelial cells

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