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Tumor Necrosis Factor Activates Human Endothelial Cells Through the p55 Tumor Necrosis Factor Receptor but the p75 Receptor Contributes to Activation at Low Tumor Necrosis Factor Concentration

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Tumor necrosis factor-α (TNF-α) interacts with two distinct membrane receptor proteins, p55 and p75, which are variably expressed on different cell types. We have examined the function of p55 and p75 on human endothelial cells (EC). Both receptor types are detected on cultured EC by FACS analysis. A mutagenized recombinant human TNF (R32W-TNF), which binds selectively to p55, is equipotent with human recombinant wild-type TNF (wt-TNF) in upregulating several different leukocyte adhesion molecules as well as class I major histocompatibility complex molecules. R32W-TNF also fully desensitizes EC to wt-TNF, as assessed by inhibition of re-induction of endothelial leukocyte adhesion molecule-1 (ELAM-1). At low wt-TNF concentrations, induction of ELAM-1 is partly inhibited by blocking monoclonal antibodies to either p55 or p75 and to a greater extent by a combination of both monoclonal antibodies. In contrast, ELAM-1 induction by R32W-TNF is only inhibited by anti-p55. We conclude that both TNF receptors (p55 and p75) can contribute to TNF-induced activation of EC, but that signaling through p55 is sufficient. (Am J Pathol 1993, 143:1724–1730)

Tumor necrosis factor (TNF) is a local mediator of inflammation and, at higher concentrations, of systemic tissue injury.[1] In both instances, vascular endothelium is an important target for TNF actions.[2,3] For example, TNF causes the expression of adhesion molecules on the surface of endothelial cells (EC), which contribute to the recruitment of circulating leukocytes to a site of inflammation or injury. Specifically, TNF induces the de novo expression of endothelial leukocyte adhesion molecule-1 (ELAM-1, also called E-selectin), vascular cell adhesion molecule-1 (VCAM-1), and intercellular adhesion molecule-1 (ICAM-1, also called CD54). TNF also increases expression of class I major histocompatibility complex (MHC) molecules, which serve as target structures for recognition by $CD8^+$ cytolytic T lymphocytes. All of these actions of TNF are believed to be initiated by binding to cell surface receptors.

Human TNF has been shown to interact with two distinct membrane receptors of molecular weights 55 and 75 kd (p55 and p75, respectively).[4–10] Cultured human umbilical vein EC, a widely used model for studying TNF actions, have been reported to express only p55 as detected by cross-linking of ^{125}I-TNF.[11] However, it has been observed by others that TNF-induced increases in EC adhesivity for leukocytes can be inhibited by blocking monoclonal antibodies (MAbs) specific for either p55 or p75.[12] Recently, both receptor types were reported to be expressed by indirect immunofluorescence and FACS analysis and by immunoprecipitation, but only p55 appeared to contribute to EC activation.[13] A mutagenized recom-

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binant human TNF molecule (R32W-TNF) has been described, which interacts effectively with p55 but has largely lost its ability to bind to p75.[13,14] This variant TNF molecule provides an opportunity to examine more critically the role of both receptors in human EC responses.

Materials and Methods

Cytokines and MAbs

The wild-type TNF (wt-TNF) used in this study (Biogen, Cambridge, MA) is a purified recombinant protein, expressed in *Escherichia coli*, with a specific activity of 5.3×10^6 U/mg measured in an L929 cell cytotoxicity assay without actinomycin D. The mutagenized recombinant TNF protein, R32W-TNF[14] has a specific activity of 4×10^7 IU/mg as assayed by cytotoxicity of HEP2 cells in the presence of cycloheximide. The wt-TNF and R32W-TNF are essentially equipotent as competitive inhibitors of ^{125}I-TNF binding to HeLa cells, a line that expresses almost exclusively p55 receptors.[15] Interleukin (IL)-1β was purchased from Genzyme (Cambridge, MA) and has a specific activity of 5×10^8 U/mg as measured in an EL4 thymoma bioassay. All of the cytokines used in this study contained less than 1 pg/ml of lipopolysaccharide by Limulus assay, a concentration that does not induce endothelial cell adhesion molecule expression.[16] Three anti-TNF receptor MAbs were used: a nonblocking mouse anti-human p55 (called anti-p60) and a blocking rat anti-human p75 (called anti-p80), both purchased from Genzyme, and Htr-5, a blocking mouse anti-human p55, which has been previously described.[4] Additional antibodies used for FACS analysis include the mouse MAbs anti-ELAM-1 (H4/18, IgG1),[16] anti-VCAM-1 (E1/6, IgG1, gift of M.P. Bevilacqua, University of California at San Diego, San Diego, CA),[17] anti-ICAM-1 (RR1/1, IgG1, gift of T.A. Springer, Center for Blood Research, Boston, MA),[18] anti-class I MHC (W6/32, IgG2a), anti-gp96 (E1/1.2, reactive with an unmodulated positive control surface protein, IgG2), K16/16 (nonbinding IgG1 negative control, gift of D.L. Mendrick, Brigham and Woman's Hospital, Boston, MA), and rat MAb, anti-human EC (1F10, reactive with an unmodulated positive control surface protein, IgG, gift of S. Goerdt, Wilhelms-Universitat, Munster, Germany).[19] Normal rat serum (Sigma, St. Louis, MO) was used as a negative nonbinding rat Ig control.

Cells and Experimental Protocols

Human EC were isolated by collagenase treatment of three to five human umbilical veins pooled and serially cultured as previously described.[20–22] All of the EC used in these experiments were grown to confluency on gelatin-coated 6-well plastic tissue culture plates at passage level two to five.

Cytokine responses were assessed by adding indicated amounts of cytokines to the culture wells in complete media for the indicated times, after which EC were analyzed for surface molecule expression (see below). In the desensitization experiments, EC were treated with 6 ng/ml of either R32W-TNF or wt-TNF or mock treated for 24 hours and then retreated with control medium or with 6 ng/ml R32W-TNF, 6 ng/ml wt-TNF, or 50 U/ml IL-1β for an additional 4 hours before analysis. Anti-receptor antibody blocking studies were conducted by preincubating EC for 20 minutes in the presence of blocking or control MAb (10 μg/ml) before adding R32W-TNF or wt-TNF for an additional 4 hours.

Indirect Immunofluorescence and FACS Analysis

Cells were suspended for TNF receptor staining with Hanks/EDTA and scraping, whereas trypsin/EDTA was used to harvest cells for all other experiments. Staining was performed as previously described.[16] Fixed cells were then analyzed by FACS using a FacSort (Becton Dickinson, San Jose, CA). Data are presented as histograms of cell number (y axis) vs fluorescence intensity (log scale, x axis).

Results

Expression of TNF Receptors on EC

Indirect immunofluorescence and FACS analysis was used to assess the expression of TNF receptors on cultured human umbilical vein EC. As shown in Figure 1 both the p55 and p75 receptors are present on EC. Although anti-p75 staining seems slightly brighter, the relative intensities of p55 and p75 staining cannot be accurately compared because the primary anti-receptor MAb are from different species and are detected with different secondary antibodies. The cells used in Figure 1 were nonenzymatically harvested before staining because preliminary experiments revealed that both TNF receptors on EC are sensitive to trypsin.

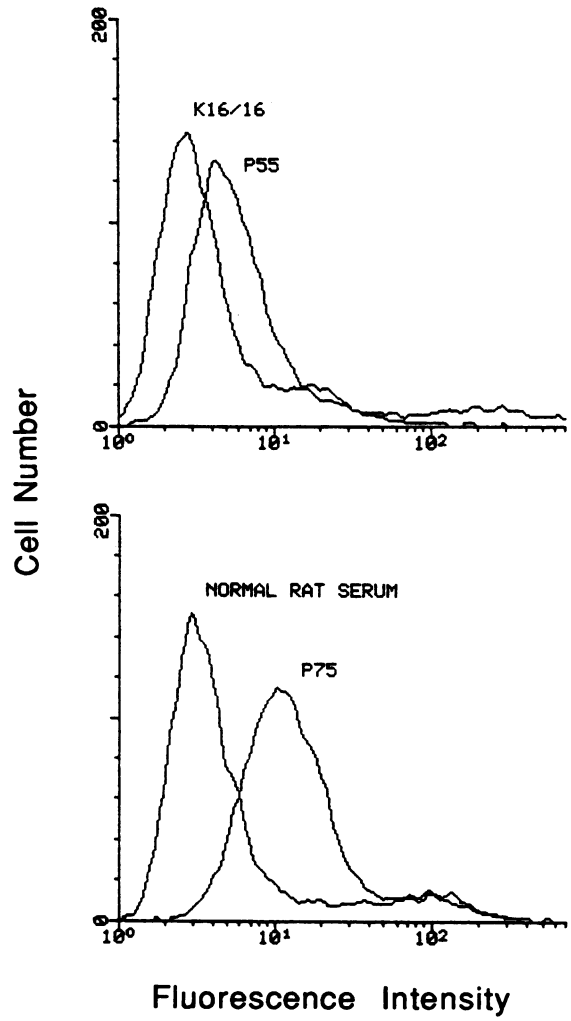

Figure 1. *Expression of TNF receptors by EC. EC were stained with mouse anti-p55 (anti-p60) and rat anti-p75 (anti-p80) and analyzed by FACS. Both MAbs were used at 10 μg/ml. One of four experiments with similar results.*

Wt-TNF and R32W-TNF Both Effectively Activate EC

Van Ostade et al[14] have recently reported the characterization of R32W-TNF that has largely lost the ability to interact with the p75 receptor, but still effectively binds to the p55 receptor. The abilities of R32W-TNF and wt-TNF to induce ELAM-1 expression on EC were compared. Both cytokines appear equipotent at concentrations from 0.2 to 200 ng/ml for inducing ELAM-1 expression (not shown). We next compared the ability of these cytokines to activate EC at various times when administered at equal concentrations. Specifically, EC harvested after 6, 24, and 48 hours of cytokine treatment were evaluated for surface expression of ELAM-1, VCAM-1, ICAM-1, and class I MHC molecules. As shown in Figure 2, both wt-TNF and R32W-TNF gave indistinguishable patterns of upregulation and subsequent loss of surface expression of ELAM-1 and VCAM-1. Both ICAM-1 and class I MHC molecules exhibited sustained upregulation, and again no significant differences were noted between the effects of R32W-TNF and wt-TNF.

Previous studies from our laboratory have shown that TNF not only induces transient ELAM-1 expression on EC, but by 24 hours causes specific desensitization to re-induction of ELAM-1 by TNF but not by IL-1β.[16] We next compared R32W-TNF with wt-TNF for the ability to cause desensitization. EC were pretreated for 24 hours with either R32W-TNF or wt-TNF. In both cases, ELAM-1 expression had significantly declined from peak levels by 24 hours. Replicate cultures were then retreated for an additional 4 hours with either no mediator, wt-TNF, R32W-TNF, or IL-1β. As shown in Figure 3, EC pretreated with wt-TNF were unresponsive to further treatment with either wt-TNF or R32W-TNF but maintained responsiveness to IL-1β. Remarkably, cells pretreated with R32W-TNF were equally unresponsive to further treatment with R32W-TNF or wt-TNF but also retained responsiveness to IL-1β. Thus, activation exclusively via the p55 receptor can fully desensitize EC to activation by wt-TNF as assessed by ELAM-1 re-induction.

Inhibition of TNF Actions by Anti-Receptor Antibodies

Results reported by Shalaby et al[12] showed that TNF-induced increases in EC adhesivity for leukocytes could be blocked by the presence of MAbs to either p55 or p75. We performed a similar series of experiments to examine the effect of blocking anti-p55- and anti-p75-specific MAbs (Htr-5 and anti-p80, respectively) on the TNF-induced expression of ELAM-1 on EC. As shown in Table 1, MAbs to either p55 or p75 alone cause a significant reduction in the expression of ELAM-1 induced by wt-TNF. Anti-p55 is also effective at inhibiting responses by R32W-TNF but anti-p75 is not. Furthermore, the degree of inhibition of the response to wt-TNF but not to R32W-TNF is greater when both MAbs are present simultaneously. These observations suggest that p75 contributes to wt-TNF- but not R32W-TNF-mediated responses. Of note, the degree of inhibition observed in the presence of both MAbs was never complete, even after treatment with 10 times more antibody, and neither MAb was able to inhibit

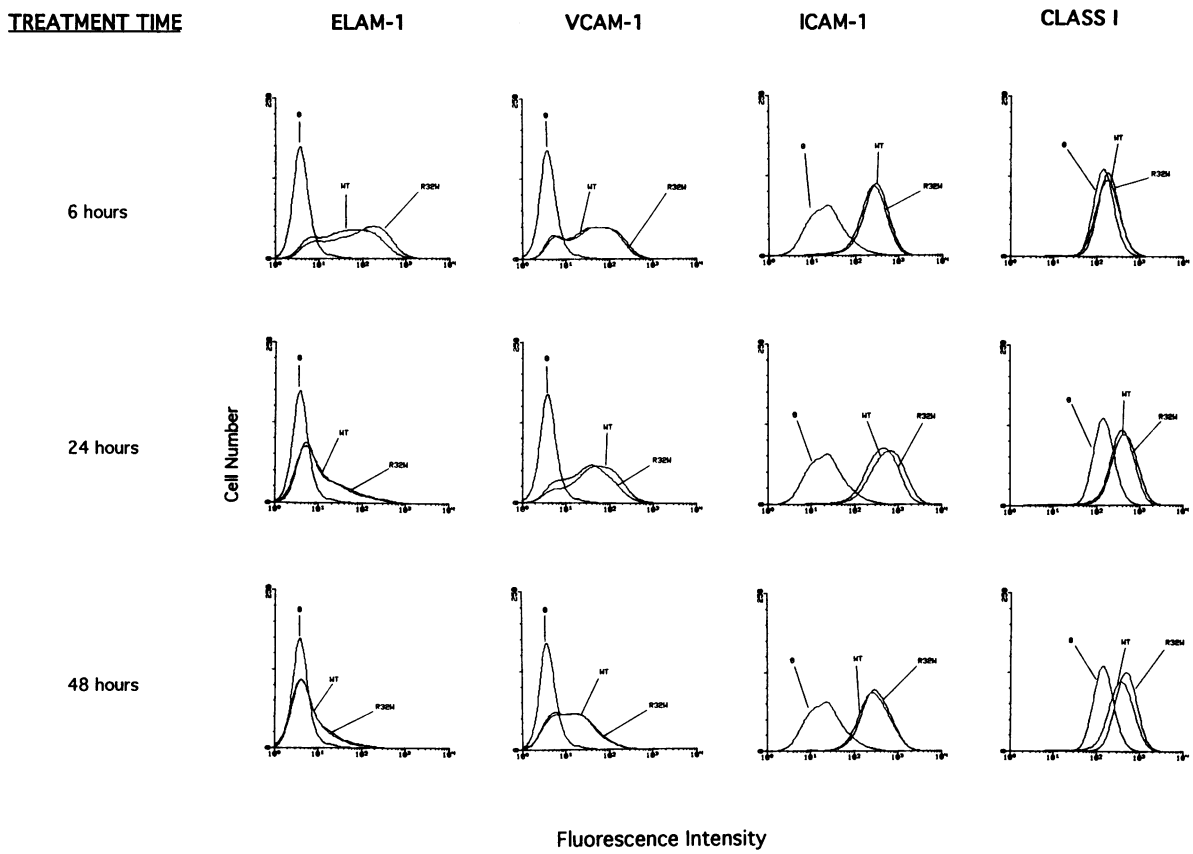

Figure 2. *Time dependence of induction of adhesion molecules and class I MHC molecules on EC by wt-TNF and R32W-TNF. FACS analysis of EC treated with either wt-TNF (WT) or R32W-TNF (R32W) each at 6 ng/ml for 6, 24, or 48 hours and stained with anti-ELAM-1, anti-VCAM-1, anti-ICAM-1, and anti-MHC class I MAbs. One of four experiments with similar results.*

to any measurable extent when 10-fold higher concentrations of TNF were used (not shown).

Discussion

The data presented in this study make three points about TNF-mediated activation of human EC. First, in agreement with Mackay et al[13] cultured human EC express both p55 and p75 receptors. Second, in contrast to the conclusions drawn by Mackay et al[13] our data suggests that both of the TNF receptors can contribute to TNF-induced activation of EC. Experiments with R32W-TNF provide clear cut evidence for p55 function, which is further supported by MAb blocking data. Unfortunately, there is no mutagenized TNF molecule that exclusively interacts with p75, and therefore, evidence for p75 function rests entirely on MAb blocking. However, the ability of the anti-p75 antibody to inhibit responses to wt-TNF but not to R32W-TNF lends critical support for the specificity of this blocking MAb. As noted in RESULTS, both of our blocking MAbs only effectively inhibit EC responses at low TNF concentration. We attribute the inability to block at higher TNF concentrations to insufficient affinities of the MAb to compete with TNF for binding to the receptor rather than to a third, lower affinity receptor because TNF binding analyses on cultured human EC have not revealed a low affinity binding site.[23] Our current studies do not distinguish between direct signaling through p75 or, as proposed by Tartaglia and Goeddel,[24] a model in which p75 "collects" TNF to pass along to p55. Recent transfection studies have shown that p75 can mediate cytotoxicity.[15] It remains to be whether isolated p75 can activate NF-κB or other transcription factors in cells that lack p55.

The third conclusion of our study is that signaling through p55 appears to be sufficient to completely activate EC in every assay examined. This is of note because human TNF, which fails to interact with murine p75, is less toxic for mice than murine TNF.[25] In

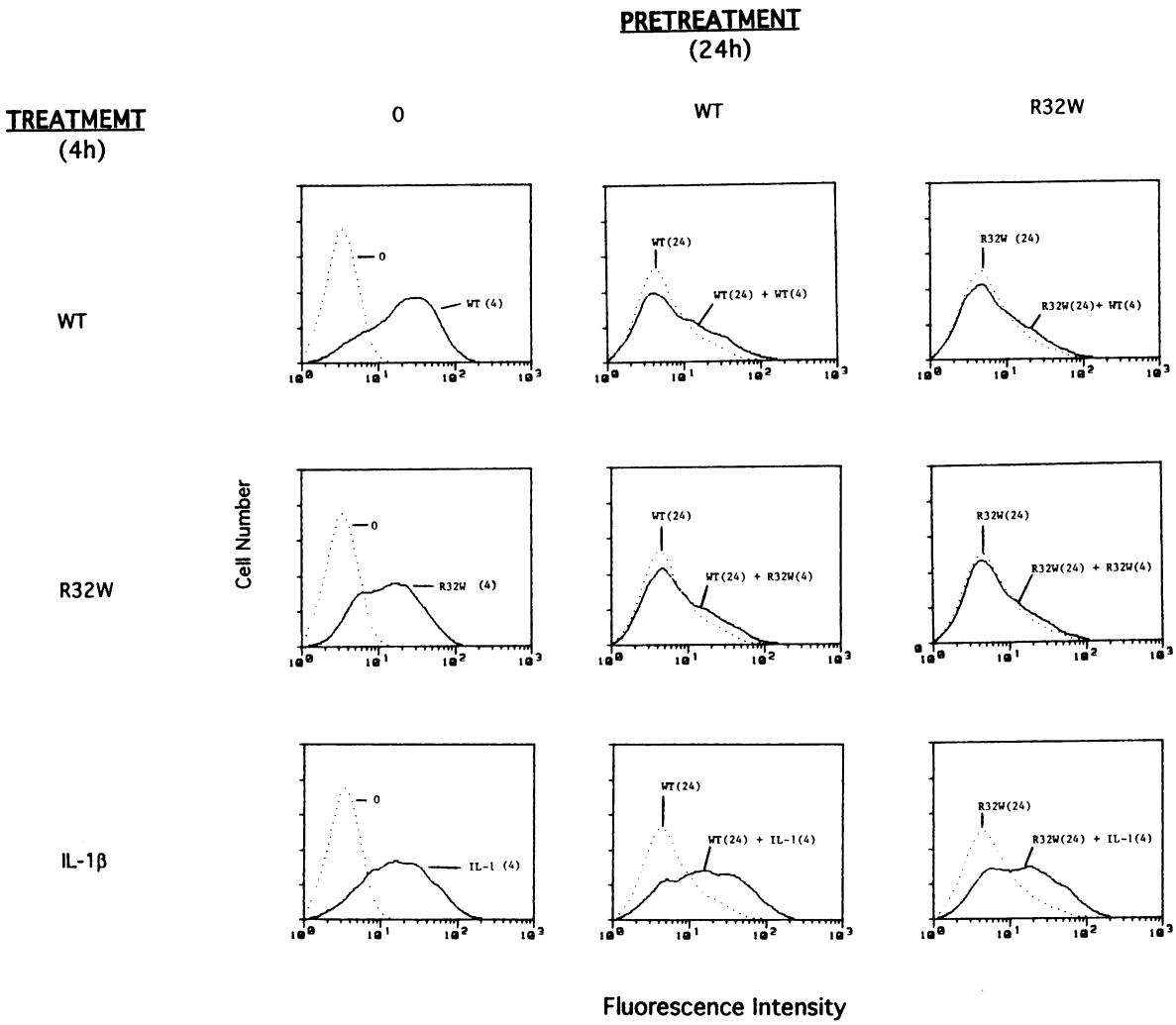

Figure 3. *Desensitization of EC for re-induction of ELAM-1 by wt-TNF or R32W-TNF. EC were pretreated with either wt-TNF (WT) or R32W-TNF (R32W) for 24 hours and then retreated with either wt-TNF, R32W-TNF, or IL-1β for 4 hours. Cells were stained with anti-ELAM-1 and analyzed by FACS. One of five experiments with similar results.*

Table 1. *Inhibition of ELAM-1 expression on EC by anti-TNF receptor MAbs*

	Antibody treatments	ELAM-1 expression (mean fluorescence)	
		WT-TNF	*R32W-TNF*
Experiment No. 1	Rat control	56.60	44.57
	Rat anti-p75	23.56	37.30
	Mouse control	60.08	48.80
	Mouse anti-p55	21.50	25.82
	Rat anti-p75 + mouse anti-p55	11.19	22.77
Experiment No. 2	Rat control + mouse control	283.44	224.90
	Rat anti-p75	189.03	271.90
	Mouse anti-p55	Not determined	166.57
	Rat anti-p75 + mouse anti-p55	115.56	158.31
Experiment No.3	Rat control	126.58	91.57
	Rat anti-p75	51.32	95.35
Experiment No.4	Rat control	163.03	148.91
	Rat anti-p75	76.97	154.48

Antibodies used were: 1F10, rat control MAb; K16/16, mouse control MAb; anti-p80, blocking rat anti-p75; HTR-5, blocking mouse anti-p55. Both wt-TNF and R32W-TNF were used at 0.2 ng/ml.

light of our new observations, it seems unlikely that the lesser toxicity of human TNF for mice is due to inability to activate EC through p75. However, our data do not exclude the possibility that p75 may mediate effects on EC different from those assayed herein. Indeed, the complete range of p75-mediated effects have not been ascertained in any system.

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