55-kd Tumor Necrosis Factor Receptor Is Expressed by Human Keratinocytes and Plays a Pivotal Role in Regulation of Human Keratinocyte ICAM-1 Expression

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Tumor necrosis factor α (TNFα) is a potent modulator of human keratinocyte intercellular adhesion molecule-1 (ICAM-1) expression. TNFα is known to exert its biologic effects by binding to specific cell-surface receptors. Two distinct TNF binding molecules, the 55-kd and the 75-kd TNF receptor (TNFR) recently have been found to be expressed by human cells. These two receptor types are independently regulated and differ markedly in their intracellular regions, indicating functional dichotomy. In order to gain further insight into the mechanisms underlying ICAM-1 regulation in human keratinocytes, in the present study, the receptor molecules mediating TNFα induced ICAM-1 upregulation in human keratinocytes was defined. Human keratinocyte TNFR expression was assessed using monoclonal antibodies that specifically recognize the 55-kd or the 75-kd TNFR. Using FACS analysis, normal (HNK) as well as transformed (KB) human keratinocytes were found to react with anti-55-kd TNFR, but not anti-75-kd TNFR antibodies. These immunofluorescence data were confirmed by Northern blot analysis revealing clearly detectable amounts of mRNA specific for the 55-kd TNFR in KB cells. Incubation of human keratinocytes with anti-55-kd TNFR antibodies at 37°C for 24 h increased ICAM-1 expression in a TNFα-like fashion. Moreover, the well known synergistic effect of IFNγ plus TNFα on keratinocyte ICAM-1 induction could be mimicked by stimulation of cells with IFNγ plus anti-55-kd TNFR antibodies. Synergistic ICAM-1 induction was not associated with increased expression of the 55-kd TNFR in IFNγ-stimulated human keratinocytes. These studies indicate that human keratinocytes express the 55-kd TNF receptor and that this surface molecule may play an important role in regulation of human keratinocyte ICAM-1 expression. *J Invest Dermatol 97:911–916, 1991*

Upon appropriate stimulation, human keratinocytes express the adhesion molecule intercellular adhesion molecule-1 (ICAM-1), which serves as an important ligand for leukocyte-function-associated antigen-1 (LFA-1) [1–4]. Because ICAM-1/LFA-1-mediated adhesion appears to contribute to a large number of skin diseases by mediating leukocyte-keratinocyte binding, it is of major importance to study regulatory mechanisms affecting human keratinocyte ICAM-1 expression [2–4].

Tumor necrosis factor (TNF) recently was found to be a potent inducer of human keratinocyte ICAM-1 synthesis and expression [5–7]. Moreover, human keratinocytes themselves may be capable of secreting TNFα [8], and TNFα, under certain circumstances, may therefore affect ICAM-1 expression in an autocrine fashion.

Tumor necrosis factor is known to exert its biologic effects by binding to specific cell-surface receptors. Recent work indicates that human cells express at least two molecular species of TNF receptors (TNFR), the 55-kd TNFR (type 1 TNFR, type B TNFR) and the 75-kd TNFR (type 2 TNFR, type A TNFR) [9–13]. Both receptor types were found to be regulated independently and in a cell-specific manner [14]. Molecular analysis revealed that the cytoplasmic domain of the two TNFR molecules markedly differ [9–13], suggesting functional dichotomy of these two receptor molecules. In the present study TNFR expression by human keratinocytes and its functional relevance for ICAM-1 regulation was examined.

MATERIALS AND METHODS

Cytokines and Antibodies Recombinant human (rh) IFNγ was purchased from Genzyme Corporation, Boston, MA. Recombinant human TNFα was kindly provided by Boehringer Mannheim, Mannheim, FRG. Monoclonal antibody (MoAb) 84H10 (anti-ICAM-1, mouse IgG1, Balb/c spleen cells X myeloma MOPC 315), which is purified from ascites by affinity chromatography on protein A sepharose, was purchased from Immunotech, Marseille, France. The generation of MoAb htr-1 (mIgM), htr-5 (mIgG1), htr-9 (mIgG1) against the 55-kd TNFR and utr-1 (mIgG1) against the 75-kd TNFR has previously been described [9]. These antibodies were affinity purified from hybridoma supernatants by using rabbit-anti-mouse Ig linked to Sepharose 4B (Pharmacia, Freiburg, FRG). Mouse IgG1 and mIgM isotype control antibodies, which

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Abbreviations:
HNK: long-term cultured, normal human keratinocytes
ICAM-1: intercellular adhesion molecule-1
IFNγ: interferon gamma
MoAb: monoclonal antibody
rh: recombinant human
TNF: tumor necrosis factor
TNFR: tumor necrosis factor receptor
UV: ultraviolet radiation

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were purified from mouse ascites by affinity chromatography on protein A sepharose, were obtained from Immunotech (Marseille, France) and Sigma Chemicals (St. Louis, MO) and the FITC-conjugated affinity-purified goat anti-mouse Fab_2 (anti-mIgG and anti-mIgM) from Dianova, Hamburg, FRG.

Cell Culture Long-term cultured normal human foreskin keratinocytes (HNK) were obtained from Clonetics Corporation, San Diego, CA and maintained in a defined keratinocyte growth medium (Clonetics, San Diego, CA) in monolayer cultures at 37°C in a humidified atmosphere containing 5% CO_2 as previously described [15]. For all experiments, fourth passage, subconfluent cells were used. For cytokine or anti-TNFR MoAb stimulation, cells were plated at a density of 5×10^5/ml in six-well tissue-culture plates (Costar, Cambridge, MA) in the presence or absence of the indicated cytokine(s) or antibodies. In addition, cells from the human carcinoma cell line KB (ATCC, Rockville, MD) were used that were maintained in EMEM (Gibco, Berlin, FRG) containing 10% FCS (Gibco, Berlin, FRG) as previously described [15]. This cell line was derived from an epidermoid carcinoma in the mouth of an adult man and has previously been used as a model for transformed human keratinocytes [8,15,16]. In addition, in some experiments cells from the human monocytic cell line U937 (ATCC, Rockville, MD) were used; they were maintained in RPMI 1640 medium (Gibco, Berlin, FRG) supplemented with 10% FCS.

Immunofluorescence Flow Cytometry Keratinocytes were collected by a 45-min treatment with pre-warmed PBS containing 10 mM EDTA. Washed cells (5×10^5) were incubated with the primary MoAb or the equivalent amount of a mouse isotype control antibody for 30 min at 4°C. Optimal staining of the 55-kd TNFR on HNK and KB cells was seen with the following antibody concentrations: htr-1 (100 μg/ml), htr-5 (100 μg/ml), htr-9 (100 μg/ml). Monoclonal antibody utr-1 was used for U937 cells at a concentration of 100 μg/ml, and for HNK and KB cells over a concentration range of 10 to 200 μg/ml. Monoclonal antibody 84H10 was used at a 1:40 dilution. Subsequently, cells were washed 3 times in PBS containing 0.05% sodium azide, resuspended, and incubated with a 1:20 dilution of goat-anti-mouse FITC-$(Fab')_2$ for 30 min at 4°C. Subsequently, cells were washed 3 times and either analyzed immediately or fixed with 1% paraformaldehyde and analyzed within 4 d. FACS analysis was performed using a FACScan (Becton Dickinson, Mountain View, CA). Data are given either as histograms of fluorescence intensity versus cell number or as percent reactive cells in comparison to unstimulated control cells as previously described [15].

Northern Blot Analysis Northern blot analysis was performed as previously described [15]. Briefly, cultured KB cells were gently detached and total RNA isolated by extraction with an acid guanidinium thiocyanate-phenol-chloroform mixture as described [17]. The concentration of RNA was determined from A260 nm, and A260/A280 ratios were greater than 1.7. RNA (10 μg) was electropheresed in 1.2% agarose gels containing formaldehyde (2.2 M) followed by transfer to nylon membranes (Hybond N, Amersham-Buchler, Braunschweig, FRG). Equivalent loading and uniform RNA transfer were assured by ethidium bromide staining of the gels before and after northern transfer. Membranes were prehybridized (4 h) and hybridized (overnight) at 42°C with 6 × SSPE/5 × Denhardt's solution/200 μg/ml salmon sperm DNA. Fifty-five kilodalton TNFR-specific mRNA was detected using a 2.1-kB cDNA probe as previously described [10]. The cDNA fragments were Biotin-14-dATP labeled by nick translation using the Bio-Nick labeling system (Gibco/BRL, Berlin, FRG) [18]. Northern blot analysis was performed under stringend washing conditions (2 × 5 min using 2 × SSC/0.5% SDS at 60°C and 1 × 40 min using 0.1% SSC/1% SDS at 50°C). Signals were detected using the Photogene detection system (Gibco/BRL, Berlin, FRG). Autoradiography was carried out for 15 min at 25°C by using Kodak XAR films.

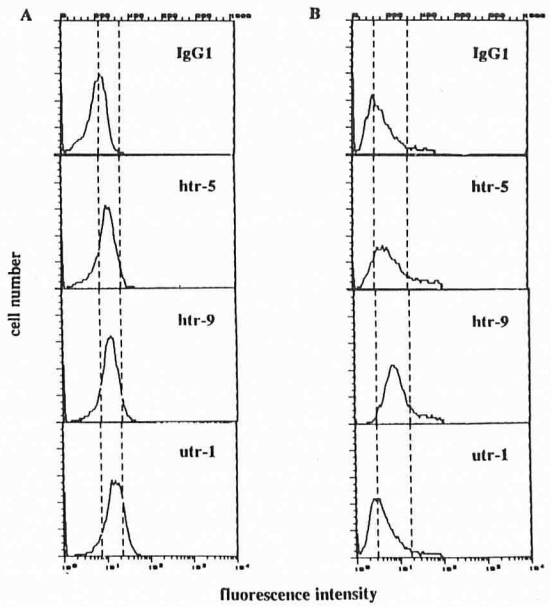

Figure 1. FACS analysis of TNFR expression on transformed human keratinocytes. U937 cells *(A)* or transformed human keratinocytes (KB cells) *(B)* were stained with MoAb directed against the 55-kd TNFR (htr-5, htr-9) or the 75-kd TNFR (utr-1) and TNFR expression determined cytofluorometrically as described in *Materials and Methods*. Data are given as histograms of fluorescence intensity versus cell number. Data represent one of five nearly identical experiments.

Statistical Analysis Statistical significance of the data was calculated using the Mann-Whitney U test.

RESULTS

In order to determine which type of TNFR is expressed on the surface of human keratinocytes, a panel of monoclonal antibodies that specifically recognize either the 55-kd or the 75-kd TNFR was tested for reactivity with HNK or KB cells using FACS analysis. Transformed as well as normal human keratinocytes were found to react with anti-55-kd TNFR (htr-5, htr-9), but not with anti-75-kd TNFR antibodies (utr-1) (Figs 1 and 2). This was not due to the fact that the anti-75-kd TNFR antibodies (utr-1) were incapable of binding to the 75-kd TNFR, because in the same experiment, these antibodies did identify 75-kd TNFR molecules on the surface of U937 cells (Fig 1). Binding of anti-55-kd TNFR antibodies (htr-9) to normal human KC as well as KB cells was highly specific, because preincubation of cells with 5000 U/ml rh TNFα for 1 h at 4°C completely inhibited antibody reactivity (Fig 2). Moreover, when RNA extracted from KB cells was hybridized with a biotin-labeled cDNA probe encoding for the 55-kd TNFR [9], clearly detectable amounts of mRNA specific for the 55-kd TNFR were observed (Fig 3).

The inhibition of anti-55-kd TNFR antibody binding by TNFα preincubation of HNK strongly indicated that TNFα and anti-55-kd TNFR antibodies may bind to closely related or even identical epitopes. The functional relevance of the expression of the 55-kd TNFR on human KC for regulation of ICAM-1 expression was therefore assessed by stimulation of cells for 24 h at 37°C with anti-55-kd TNFR antibodies followed by FACS analysis of ICAM-1 surface expression. Stimulation of both HNK and KB cells with anti-55-kd TNFR antibody htr-1 (mIgM) and htr-9 (mIgG1), but not htr-5 (mIgG1) significantly increased human KC ICAM-1 surface expression (Table I). In contrast, low constitutive ICAM-1 surface expression was found to be unchanged if cells were incubated with equivalent amounts of the appropriate isotype control antibodies (mIgM and mIgG1). Moreover, increased fluores-

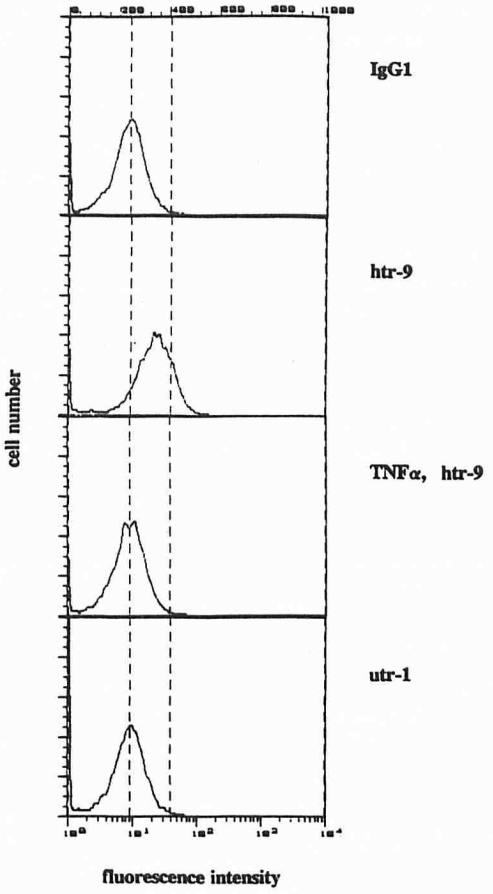

Figure 2. FACS analysis of tumor necrosis factor receptor (TNFR) expression on long-term cultured, normal human keratinocytes. Normal human keratinocytes (HNK cells) were either preincubated with rh TNFα (5000 U/ml) for 1 h at 4°C or left untreated and subsequently examined for TNFR expression by FACS analysis using MoAb specifically recognizing the 55-kd TNFR (htr-9) or the 75-kd TNFR (utr-1). Data are given as histograms of cell number versus fluorescence intensity. Data represent one of three essentially identical experiments.

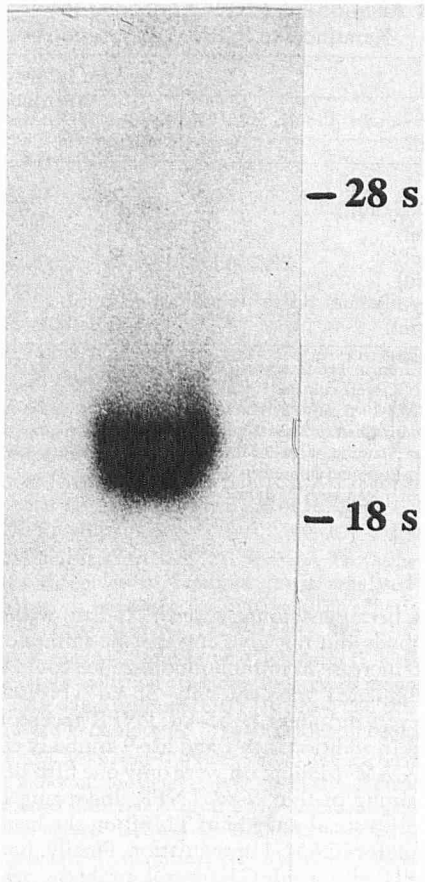

Figure 3. Northern blot analysis for the expression of mRNA specific for the 55-kd TNFR in transformed human keratinocytes. Transformed human keratinocytes (KB cells) were grown to subconfluency. Northern blot analysis of total cellular RNA (10 μg) used a Biotin-labeled cDNA probe encoding for the 55-kd TNFR. KB cells were found to express mRNA for the 55-kd TNFR in three of three experiments.

cence intensity of anti-55-kd TNFR antibody (htr-9, htr-1) -treated cells was not due to binding of the secondary antibody to the 55-kd TNFR antibodies on the cell surface, because staining without the anti-ICAM-1 antibody gave only unspecific background fluorescence (Fig 4*A*, htr-9, FITC). The magnitude of anti-55-kd TNFR antibody (htr-9) -induced ICAM-1 expression on HNK and KB cells strongly resembled that which was observed on cells that had been stimulated with optimal concentrations of rhTNFα (Table I, Fig 4). Moreover, synergistic ICAM-1 upregulation that may be achieved by stimulating KB cells with IFNγ plus TNFα [5,7] could be mimicked by stimulation of cells with IFNγ plus htr-9 antibody (Fig 4).

Several studies indicate that the synergism observed after the combined treatment of cells with IFNγ and TNFα may be due to IFNγ-induced TNF receptor upregulation [19-21]. However, IFNγ stimulation of KB cells did not increase anti-55-kd TNFR antibody (htr-9) reactivity of KB cells after 1, 2, 4, 12, and 24 h, as was determined by FACS analysis (shown in Fig 5 for 2- and 12-h treatments).

DISCUSSION

The present study demonstrates that normal as well as transformed human keratinocytes (KB cells) appear to specifically react with three different MoAb directed against the 55-kd TNFR (htr-1, htr-5, htr-9). In addition, KB cells were found to constitutively express mRNA specific for the 55-kd TNFR. Moreover, selected anti-55-kd TNFR antibodies (htr-1, htr-9) were shown to be capable of upregulating human keratinocyte ICAM-1 expression in a TNFα-like fashion. Taken together these studies strongly indicate the presence of functionally active 55-kd TNFR molecules on the surface of human keratinocytes.

Human keratinocytes exhibited positive immunofluorescence with two anti-55-kd TNFR (htr-5, htr-9), but not an anti-75-kd TNFR antibody (utr-1). Accordingly, keratinocytes previously were shown by Scatchard analysis to express a single class of high-affinity receptors with 1,250 receptors/cell and a K_d of 0.28 nM [22]. Moreover, immunoprecipitation as well as immunofluorescence data indicate that cells from the epithelial cell line HEp2 express the 55-kd TNFR, but not the 75-kd TNFR molecule [9,14]. These data suggest that human keratinocytes exclusively express the 55-kd TNFR, whereas, at least under the conditions tested, the 75-kd TNFR may not be expressed by this cell type. This is in marked contrast to other cell types such as U937 cells, which are known to simultaneously express both receptor types and further supports the concept of cell type specific regulation of the 55-kd and the 75-kd TNFR molecules [9,14].

Stimulation of HNK or KB cells with anti-55-kd TNFR antibodies htr-1 or htr-9 was found to significantly increase anti-ICAM-1 antibody reactivity. Increased immunofluorescence was not due to unspecific binding of the secondary antibody to receptor-bound htr-1 or htr-9 on anti-55-kd TNFR antibody stimulated

Table I. Anti-55-kd TNFR Antibodies Increase Human Keratinocyte ICAM-1 Expression[a]

Stimulated with	Percent ICAM-1-Positive Keratinocytes	
	KB	HNK
Medium	6 ± 2	2 ± 2
rh TNFα (500 U/ml)	28 ± 6[b]	21 ± 6[b]
htr-1 (20 μg/ml)	27 ± 7[b]	25 ± 4[b]
htr-9 (20 μg/ml)	24 ± 4[b]	19 ± 6[b]
htr-5 (20 μg/ml)	ND[c]	3 ± 2
mIgG1 (20 μg/ml)	4 ± 2	5 ± 3
mIgM (20 μg/ml)	3 ± 1	4 ± 2

[a] Transformed human keratinocytes (KB) or normal, long-term cultured human keratinocytes (HNK) were cultured for 24 h with rhTNFα or the indicated antibodies. Percentage of ICAM-1-positive keratinocytes was assessed cytofluorometrically using MoAb 84H10 as described in *Materials and Methods*. Data are given as mean ± SD of three experiments. Staining of htr-9 MoAb-stimulated keratinocytes with FITC-Fab_2 only resulted in background fluorescence.

[b] $p < 0.01$ as compared with medium.

[c] ND, not done.

keratinocytes, because staining with FITC-Fab_2 without the anti-ICAM-1 antibody did not give any specific immunofluorescence. Moreover, no increase in immunofluorescence could be detected if cells were stimulated with the anti-55-kd TNFR MoAb htr-5, which, however, did identify 55-kd TNFR receptors on human keratinocytes. In addition, htr-1 and htr-9 antibody concentrations required for ICAM-1 induction were only one fifth of that required to achieve staining of the 55-kd TNFR, indicating that antibody binding to only a small portion of TNFR on the keratinocyte surface is sufficient for ICAM-1 upregulation. Finally, staining of htr-9 stimulated cells with a mIgG1 control antibody, which was prepared and purified in a manner identical to that used for preparing the anti-ICAM-1 antibody, gave only background fluorescence, thus corroborating that the observed increase in fluorescence intensity was indeed due to ICAM-1 induction and not caused by the presence of heterospecific antibodies contaminating the anti-ICAM-1 antibody preparation.

The magnitude of anti-55-kd TNFR antibody -induced ICAM-1 upregulation strongly resembled that observed on cells that had been treated with optimal concentrations of rhTNFα. Moreover, synergistic ICAM-1 induction, which may be achieved by stimulating human keratinocytes with TNFα in combination with rhIFNγ [5,7], could be mimicked by the combined stimulation of cells with anti-55-kd TNFR antibodies (htr-9) and rhIFNγ. Taken together, these experiments indicate that binding of selected anti-55-kd TNFR antibodies (htr-1, htr-9) to the 55-kd TNFR on the surface of human keratinocytes leads to upregulation of ICAM-1 surface expression in these cells via a TNFα-like mechanism. Accordingly, htr-1 and htr-9 but not htr-5 antibodies recently were found to exert TNFα-like activity on U937 cells, Fs4 fibroblasts, and human umbilical vein endothelial cells [23]. Moreover, the transcription factor NF-kB may be activated by stimulation of HEp2 or HL50 cells with either TNFα or MoAb htr-9 [14]. Recent studies indicate the presence of consensus binding sites for NF-kB in the transcriptional regulatory region of the human ICAM-1 gene [24,25]. It is thus tempting to speculate that htr-9 binding to TNFR on the surface of HNK and KB cells may induce ICAM-1 upregulation via activation of NF-kB.

The combined stimulation of human keratinocytes with IFNγ and TNFα or TNFβ previously was reported to synergistically upregulate human keratinocyte ICAM-1 expression [5,15]. The basis of this synergistic interaction is not known, although several studies indicate that IFNγ may be capable of inducing TNFR in various human cell lines [19-21]. It has therefore been proposed that TNFR modulation may at least in part account for the observed synergism between these two cytokines. In contrast, IFNγ recently was found to fail to induce TNFR expression on human umbilical

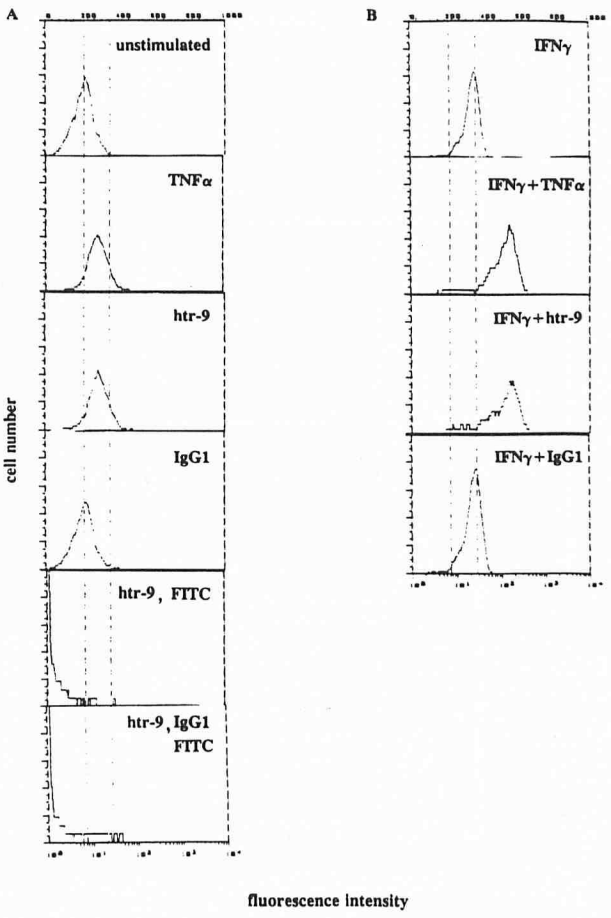

Figure 4. FACS analysis of rhIFNγ- and anti-55-kd TNFR antibody-induced ICAM-1 expression on KB cells. *A* and *B* represent the same experiment. In *A*, the first four *curves* from the top represent KB cells which were assessed for ICAM-1 surface expression by FACS analysis using MoAb 84H10 plus FITC-Fab2. Cells were either left unstimulated or stimulated for 24 h with rhTNFα (500 U/ml), MoAb htr-9 (20 μg/ml), or a mIgG1 isotope control antibody (20 μg/ml). The two bottom *curves* represent htr-9 antibody stimulated cells, which were stained with FITC-Fab2 without the primary antibody (htr-9, FITC) or with a mIgG1 isotope control antibody plus FITC-Fab2 (htr-9, IgG + FITC). Moreover, IgG1 control stainings were performed for each KB cell population tested in this experiment and did not differ from that shown for htr-9-stimulated cells. In *B* all four *curves* represent KB cells assessed for ICAM-1 expression using MoAb 84H10 plus FITC-Fab2. Cells were stimulated for 24 h with IFNγ (500 U/ml), IFNγ plus TNFα, IFNγ plus htr-9, and IFNγ plus a mIgG1 isotope control antibody. Data are given as histograms of cell number versus fluorescence intensity.

vein endothelial cells, although in this cell type a synergistic induction of HLA-class I heavy and light chain genes by combined TNF and IFNγ stimulation could be observed [26]. In the present study, synergistic induction of human keratinocyte ICAM-1 expression was not associated with an increase in TNFR expression on IFNγ-stimulated KB cells as was determined by FACS analysis. In addition, we were previously able to show that IFNγ and TNFβ each independently increase mRNA levels and together further increase steady-state mRNA levels of the ICAM-1 gene in these cells [15]. These data strongly indicate that synergistic ICAM-1 induction in human keratinocytes is not caused by TNFR upregulation in IFNγ-stimulated human keratinocytes. Further studies are currently underway to determine whether synergistic ICAM-1 induction in human keratinocytes is due to the synergistic increase in transcription rates of the ICAM-1 gene as has previously been suggested for HLA-class I gene regulation in endothelial cells [26].

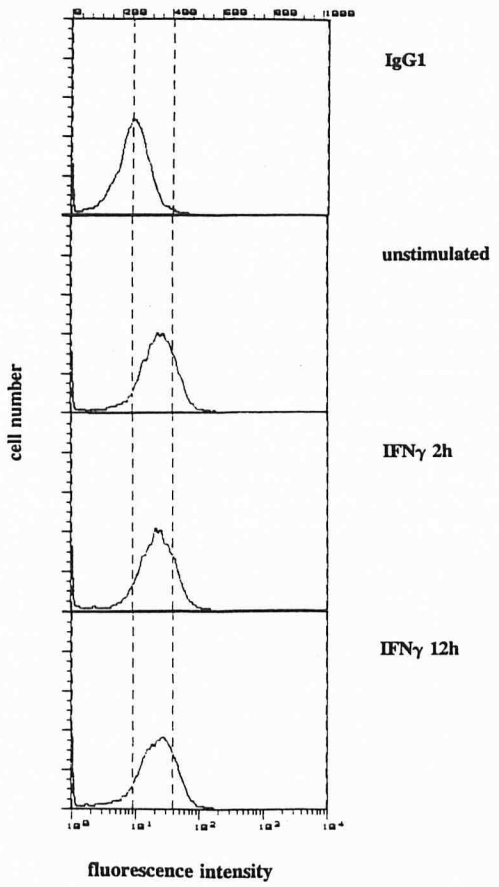

Figure 5. FACS analysis of the expression of the 55-kd TNFR on IFNγ-stimulated KB cells. Transformed human keratinocytes (KB cells) were either left untreated or incubated for 1, 2, 4, 12, and 24 h (data are shown for 2 and 12 h) with rhIFNγ (500 U/ml) and subsequently examined for the expression of the 55-kd TNFR by FACS analysis using MoAb htr-9 as described in *Materials and Methods*. IgG1 controls were done for stimulated KB cells for each time point and did not exceed background fluorescence. Data are given as histograms of fluorescence intensity versus cell number and represent one of three essentially identical experiments.

There is increasing in vitro and in vivo evidence that ICAM-1 as a ligand for leukocyte LFA-1 [27] may play a key role in the pathogenesis of inflammatory skin diseases. As a consequence, extensive studies have been conducted to elucidate the mechanisms by which ICAM-1 expression is regulated [4]. From these studies it appears that selected inflammatory cytokines including TNFα play a pivotal role in ICAM-1 regulation. The present study provides direct evidence that a cytokine receptor, in addition to the cytokine itself, is critically involved in regulation of the expression of this adhesion molecule. This finding points to a previously unnoted level at which ICAM-1 expression may be controlled. Further studies will have to determine whether the capacity to provide activation signals for ICAM-1 regulation is specific for the 55-kd TNFR, or may also be observed upon appropriate stimulation of 75-kd TNFR molecules.

In summary, human keratinocytes were found to express functionally active 55-kd TNFR molecules, which were shown to play a pivotal role in regulation of human keratinocyte ICAM-1 expression. These studies provide the basis to further investigate the regulatory mechanisms relevant for TNFα effects on human keratinocytes. Accordingly, preliminary studies indicate that binding of keratinocyte-derived TNFα to the 55-kd TNFR may occur on the surface of UV-irradiated human keratinocytes, indicating that this receptor molecule may regulate autocrine effects of TNFα relevant to ICAM-1 regulation in UV-irradiated human keratinocytes [28–30].

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