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Evidence for tumor necrosis factor receptors (TNFRs) in human MRC5 fibroblast cells.

Abstract

Previous reports have shown that tumor necrosis factor alpha (TNF-a) controls the functions of fibroblasts cells, such as proliferation, cell migration and secretion of factors. The signaling for the biological effects of this and other cytokines is usually exerted through cell surface receptors. In this study, we demonstrated the presence of a surface and soluble TNF receptor in MRC5 fibroblasts. The presence of TNFRI and TNFRII mRNA was demonstrated by reverse-transcriptase-PCR (RT-PCR). Both surface TNFRI and TNFRII are

expressed and the number of both membrane receptors is 9,251 sites per cell. Using TNF receptor specific antibodies and flow cytometry, we showed that MRC5 cells express mainly TNFRI.

Full text

INTRODUCTION

Tumor necrosis factor alpha (TNF-alpha), an inflammatory cytokine primarily produced by activated macrophages, exerts a wide range of both beneficial and detrimental biological effects. Among the beneficial effects is the critical role played by TNF-alpha in the host defence against micro-organisms, in particular against fungi *(Candida albicans, Cryptococcus neoformans)* [1], intracellular bacteria *(Listeria monocytogenes, Mycobacterium tuberculosis)* [2-4] and parasites (*Trypanosoma cruzi* and *Toxoplasma gondii*) [5, 6]. TNF-alpha has also been reported to control various biological functions of fibroblasts, including cell migration, cell proliferation and release of factors and mediators (IL-6, IL-8, GM-CSF) [7].

Like other cytokines, TNF-alpha acts by activating cell surface receptors. Two distinct receptors have been identified, the 55 kDa (TNFRI, CD120a) and the 75 kDa (TNFRII, CD120b) [8, 9]. Both receptors belong to the larger TNF receptor family. Most cells express both types of TNFR, however the relative ratio of TNFRI to TNFRII varies depending on the cell type and tissue of origin [10-12]. Both receptors have been shown to exist in soluble forms (sTNFR), derived by proteolytic cleavage of the membrane extra-cellular domain receptor and are capable of acting as inhibitors of TNF-alpha [13]. These forms can be detected in various body fluids and have been shown to be enhanced in a number of pathological disorders [14].

TNF receptors (TNFRs) have been implicated in a variety of biological functions including cytotoxicity [15], mediation of apoptosis, up-regulation of several adhesion molecules [7], induction of NF-kappaB [16], as well as immune reactions to infectious organisms. Investigations on mice deficient in one of the receptors for TNF-alpha have established that the protection against *L. monocytogenes, M. tuberculosis, Leishmania major* and *T. gondii* depends on TNFRI [17-21]. Thus, modulation of the cell surface expression and shedding of TNFRs are likely to have an important role in the control of TNF action.

To the best of our knowledge, no studies have examined the presence of TNFRs on human MRC5 fibroblasts. We previously showed that TNF-alpha enhances *T. gondii* cyst formation in human fibroblasts MRC5 [22] and here we report the identification of the expression of both TNF receptors on MRC5 cells. We studied mRNA, membrane cell and soluble protein expression.

MATERIALS AND METHODS

Cell culture

MRC5 human lung diploid fibroblasts ATCC CCL 171 (BioMérieux, Marcy-L'Étoile, France) were grown in 24-well plates on glass coverslips in Dulbecco's modified Eagle's medium (DMEM) supplemented with 2% synthetic serum Ultroser G (Life Technologies, Eragny, France), 25 mM Hepes, 4 mM glutamine, 500 U/ml penicillin and 250 mug/ml streptomycin at 37° C in a 5% CO_2, humidified atmosphere. Assays were performed in confluent monolayers.

The human monocytic leukaemia line THP1 was obtained from ATCC and grown in RPMI 1640 supplemented with 100 U/ml penicillin, 100 mug/ml streptomycin and 10% fetal calf serum (FCS).

Cytokines and antibodies

Human recombinant TNF-alpha, Mab to TNFRI (mouse anti-human TNFRI : Mab 225) and Mab to TNFRII (mouse anti-human TNFRII : Mab 226) were obtained from R&D Systems Europe (Abingdon, Oxfordshire, UK). ^{125}I-TNF-alpha (500-1,000 Ci/mmol) was from Amersham-Pharmacia Biotech (Orsay, France). Anti-mouse IgG FITC conjugated and mouse IgG were from Sigma Aldrich (L'Isle-d'Abeau-Chesnes, France).

Analysis of mRNA by reverse transcription-PCR

1) *RNA extraction*

Total RNA was extracted from the cells according to the RNA-Plus^{TM} extraction Kit protocol (Quantum Biotechnologies SA, Montreuil-sous-Bois, France). Briefly, cells (10^6) were lysed with 200 mul of RNA-Plus▒. RNA was extracted by adding 1/5 volume of chloroform and shaking vigorously for 15 sec. After chilling on ice

for 15 min, the mixture was centrifuged at 12,000 g (4° C) for 15 min, and the top aqueous phase was precipitated by the addition of an equal volume of isopropanol. This was then incubated for 15 min in ice, then centrifuged at 12,000 g for 15 min at 4° C. The obtained pellet was washed with 75% cold ethanol by vortexing and then centrifuged for 8 min at 7,500 g (4° C). The pellet was dried and dissolved in RNase-free water. RNA concentration was determined by spectrophotometry at 260 nm and RNA was kept at - 70° C.

2) *cDNA preparation using reverse transcriptase* (ProSTAR First-Standard RT-PCR Kit, Clontech, Saint-Quentin-en-Yvelines, France)

Most of the steps were performed according to the manufacturer's specifications. First-strand cDNA was synthesised from 5-10 mug of total RNA by using oligo (dt) primers : 10x reaction buffer (100 mM Tris-HCL, 500 mM KCl, 15 mM $MgCl_2$) 5 mul, RNase inhibitor (40 U/mul) 1 mul, 100 mM dNTPs 2 mul, reverse transcriptase MMLV-RT (50 U/mul) 1 mul, oligo (dt) (100 ng/mul) 3 mul, and DEPC water to a final volume of 50 mul. The tubes were incubated for one hour at 37° C. Samples were heated at 94° C for 5 min to terminate the action of the RT.

The RT products were then stored at - 20° C and subsequently used for PCR amplification.

3) *PCR*

Samples of the cDNA preparation were analysed for specific cDNA of TNFRI, TNFRII and beta-actin, by PCR amplification using specific primers (Table 1). Five microliters of reverse transcription product were added to 95 mul of PCR reaction mixture containing 2 mul of 10 muM 5' primer, 2 mul of 10 muM 3' primer, 0.8 mul of 100 mM dNTPs, 10 mul of reaction buffer, 0.5 mul of Taq DNA polymerase (5 U/ml) and sterile H_2O to a final volume of 100 mul.

A negative control consisting of the reaction mixture without cDNA was included in each run. Amplification was carried out in a Perkin Elmer-480 thermocycler after initial denaturation at 94° C for 5 min. The PCR was run for 30 cycles (90° C 45 sec, 60° C 45 sec, 72° C 2 min) followed by 10 min at 72° C. Aliquots from the reaction were electrophoresed through a 2% agarose gel, stained with ethidium bromide and photographed under UV fluorescence.

TNF membrane receptor detection on MRC5

1) *^{125}I-TNF-a saturation binding assay*

Saturation binding experiments were conducted at 0-4° C to block the release (shedding) or internalisation of the receptors. After washing with cold medium, 2 x 10^5 cells were incubated with various concentrations of ^{125}I-TNF-alpha (0.0625-2 nM) in a binding buffer for 3 hours. The unbound ligand was removed by two washes with ice-cold medium, and cells were detached from plastic with EDTA 0.05% in PBS (phosphate-buffered saline). Radioactivity was determined in a gamma counter (Cobrall-Packard). The number of cells was calculated using parallel monolayer cultures in which cells were detached and counted.

Non-specific binding was determined in the presence of a 100 fold excess of non-radioactive TNF-alpha. Specific binding was obtained by subtracting the non-specific binding from the total binding. All determinations were performed in triplicate and results are expressed as the mean density of specific binding site and apparent affinity. Saturation binding studies were analysed and Scatchard plots constructed as previously described [23].

2) *Competition binding assays using monoclonal antibodies*

To calculate the relative proportion of the two receptor molecules, competition studies with radiolabelled ^{125}I-TNF-alpha and the respective receptor-specific monoclonal antibodies were performed. The proportion of each receptor type was calculated by the degree of specific ^{125}I-TNF-alpha binding which was blocked by pretreatment with the corresponding receptor-specific monoclonal antibody. For this, 2 x 10^5 cells were preincubated at 37° C for 1 hour with 4 mug/ml of specific monoclonal antibodies against TNFRI or against TNFRII. Cells were then washed with ice-cold medium, incubated with 1 nM ^{125}I-TNF-alpha for 3 hours at 4° C. Cells treated with phorbol 12-myristate, 13 acetate (PMA) 10^{-6} M were used as controls.

All assays were performed in triplicate. Results were average specific counts ± standard deviation of the mean.

3) *Flow cytometry analysis*

To study the membrane expression of the two TNFR types, experiments were performed using flow cytometry. Approximately 4 x 10^5 MRC5 cells were detached from plates with 0.05% EDTA in PBS and 10^6 THP1 cells were used as a control for expression of TNFRI and TNFRII [24]. After several washes, the cells were incubated in blocking solution (PBS containing 10% FCS) in ice for 10 min, and then labelled with mouse anti-human TNFRI or anti-human TNFRII antibodies (40 mug/ml) for 1 hour in ice, in PBS containing 5% FCS. Cells incubated with isotype IgG were used as negative control.

Cells were washed twice and stained with FITC conjugated sheep anti-mouse immunoglobulin G (Sigma Aldrich) in PBS containing 5% FCS for 1 hour. The cells were subsequently washed and stored on ice until analysis by FACS Calibur (Becton Dickinson, Pont-de-Claix, France). Flow cytometry measurements were based on 10,000 cells.

Soluble TNF receptor (sTNFR) assays

We tested the presence of type I soluble TNF receptors (sTNFRI) and type II soluble TNF receptor (sTNFRII) in the culture supernatants of 2 x 10^5 MRC5 cells using specific enzyme-linked immunoassay (Quantikine, R&D systems). Assays were performed according to the manufacturer's specifications. Values were calculated from a standard curve based on fresh-ly prepared dilutions. Cells stimulated with PMA 10^{-6} M were used as a control.

All samples were assayed in duplicate. Results are expressed in pg/ml of sTNFRs. The ELISA sensitivity to sTNFRI and sTNFRII in culture medium samples was 1.5 and 1.0 pg/ml, respectively.

Statistical analysis

Results are given as the mean ± standard deviation (SD). The statistical significance of differences between groups was analysed by Student's t-test. A value < 0.05 was considered significant.

RESULTS

Expression of TNFR mRNA in MRC5 cells

To determine whether MRC5 cells could express receptors for TNF-alpha, RNA was analysed by RT-PCR with specific primers. MRC5 RNA was compared with positive control samples from THP1 cells which express constitutively TNFRI and TNFRII. RT-PCR analysis revealed that TNFRI and TNFRII mRNA were constitutively expressed in MRC5 human fibroblast cell line in our culture conditions (Figure 1).

Expression of surface TNF receptor in MRC5 cells

We first established if MRC5 fibroblasts produced TNF-alpha under our culture conditions at 24, 48 and 72 hours using an ELISA Kit (Immunotech, Marseille, France) (data not shown). TNF-alpha was not detectable in the culture supernatants. Thus, it was not necessary to dissociate endogenous TNF bound to TNFR.

The membrane expression of TNFR on MRC5 was studied by flow cytometry. Two specific monoclonal antibodies against TNFRI and TNFRII were used to identify and determine the relative proportion of these receptors. Flow cytometry analysis revealed that 90% of THP1 cells were positive to TNFRI and TNFRII, while 80% of MRC5 cells were positive to TNFRI and 50% to TNFRII. The distribution pattern of TNFRs also apperead to be different in THP1 and MRC5 cells (Figure 2). The intensity of TNFRI expression on MRC5 was greater than that of TNFRII. These results indicate that TNFRI is predominant on MRC5.

To verify the capacity of MRC5 to bind TNF-alpha and determine the dissociation constant and binding sites per cell, binding assays were used. These experiments were performed under saturation conditions. Figure 3A shows that the binding was dose responsive and saturable.

Scatchard analysis yields a linear plot (Figure 3B). Scatchard plot analysis of the data indicates a dissociation constant (K_d) of 0.34 ± 0.036 10^{-9} M and an average of 9,251 ± 574 binding sites per cell.

Since two distinct isoforms of TNFR have previously been identified by flow cytometry, and binding studies detected only one site, competition binding with specific antibodies for each isoform was used. The competition binding study revealed that both TNFRs are coexpressed on MRC5, with a preference for TNFRI

(Figure 4). In particular, preincubation of the cells with TNFRI-specific Mab reduced the ^{125}I-TNF-alpha specific binding by 60%.

PMA (10^{-6} M) stimulation induced a significant reduction in the binding (about 70%) as compared to paired control cultures (Figure 4). No evidence of PMA-mediated toxicity at 24 hours was demonstrated by viability determination, as compared to control cultures. In addition, no differences in cell numbers were present between control and PMA-treated cultures.

Shedding of TNF receptors

Modulation of receptor expression represents a central point of the control of TNF action. Shedding of TNFRs is likely to have an important role in the control of this process. So, we investigated the level of both sTNFR (TNFR soluble) in the culture supernatant of MRC5. As shown in Figure 5, sTNFRs are spontaneously shedded from MRC5 at a low rate, the level of sTNFRI in supernatant being much higher than the sTNFRII level (14.82 ± 1.68 and 5.4 ± 0.32 pg/ml respectively). Figure 5 also shows that the levels of TNFRI and TNFRII were increased by PMA treatment.

Treatment of MRC5 cells with TNF-alpha 1 hour before analysis dramatically decreased the release of TNFRI (8.61%), whereas the release of TNFRII was not affected.

DISCUSSION

TNF-alpha is a biological mediator of immune, toxic, and inflammatory responses, the action of which is necessarily mediated by receptors. Two classes of TNF receptors have been detected on a variety of cells, with different concentrations at the peptide or mRNA level [25-27]. To our knowledge, no information is available regarding the ability of MRC5 cells to express TNF receptors. In the present study, RT-PCR analysis revealed that the mRNA of both TNF receptors was expressed in MRC5 fibroblasts. Flow cytometry and binding studies showed additional evidence that this cell line has TNF receptors expressed on the cell surface. The K_d obtained by Scatchard plot analysis is in accordance with previously reported TNFR affinity [28]. According to most previous studies, the TNF receptor has been characterised as a single class

binding site with a K_d in the range of $0.3\ 10^{-9}$ to $3\ 10^{-12}$ M and a relative level from few hundreds to several thousands per cell, depending on the cell type [29].

Furthermore, the TNFR constitutive presence on MRC5 demonstrates a functional role which was predicted by the effects of TNF-alpha on the *in vitro* *Toxoplasma* cyst formation [22].

To ensure precise determination of each of the receptor isotypes, additional experiments using specific antibodies and flow cytometry were performed, and the presence of the two TNFR types on MRC5 cells was revealed. TNFRI was shown to be much more abundant than the TNFRII entity. Our results agree with studies showing that TNFRI is predominant in cells of an epithelial origin and fibroblasts, while TNFRII is predominant in monocytic cell lymphocytes and myeloid cells [30]. Several TNF actions have been reported to be mediated through TNFRI, such as cytotoxicity, anti-viral activity, cell adhesion to endothelial cells and endotoxic shock [18, 31], but little is known about the role of the TNFRII. However, the receptor activity was found to be synergistic or complementary to that of TNFRI in experimental models [7, 32].

Proteolytic cleavage and shedding of extra-cellular domains of both TNFRI and TNFRII cell surface receptors in order to serve as TNF binding proteins, represents one mechanism by which the biological activity can be modulated. As observed in other cells [33], both species of TNF receptors were spontaneously released during MRC5 culture, and the TNFRI soluble form was more abundant than sTNFRII. Our results show that MRC5 treatment with PMA induces a down-regulation of TNF surface receptors and a production of soluble forms. This result is not surprising since the ability of PMA to act as a strong shedding inducer of TNFRs has already been demonstrated in other cell systems by protein kinase C (PKC) activation [33, 34]. In particular, in peripheral blood monocytes and cultured human alveolar macrophages, PMA induces the release of both sTNFRI and sTNFRII, whereas in human airway epithelial cells PMA induces shedding of sTNFRI only [35].

Interestingly, MRC5 cell stimulation by TNF-alpha induced a dramatic decrease in the shedding of TNFRI but did not affect the shedding of TNFRII. The decreased TNFRI shedding induced by TNF may be the result of the receptor internalisation after ligand binding. In fact, in the U937 histiocytic cell line, TNF has been reported to induce sTNFRII shedding and sTNFRI internalisation, with

both effects mediated *via* TNFRI [16]. In some systems, internalisation of the receptor has been shown to be essential for its function.

CONCLUSION

In conclusion, our results demonstrate for the first time, the presence of specific receptors to TNF-alpha on human MRC5 fibroblasts. Thus, MRC5 cells can easily be used as an *in vitro* model to study the role played by TNFRs in the host response to intracellular pathogens, in particular *T. gondii.*

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Figures

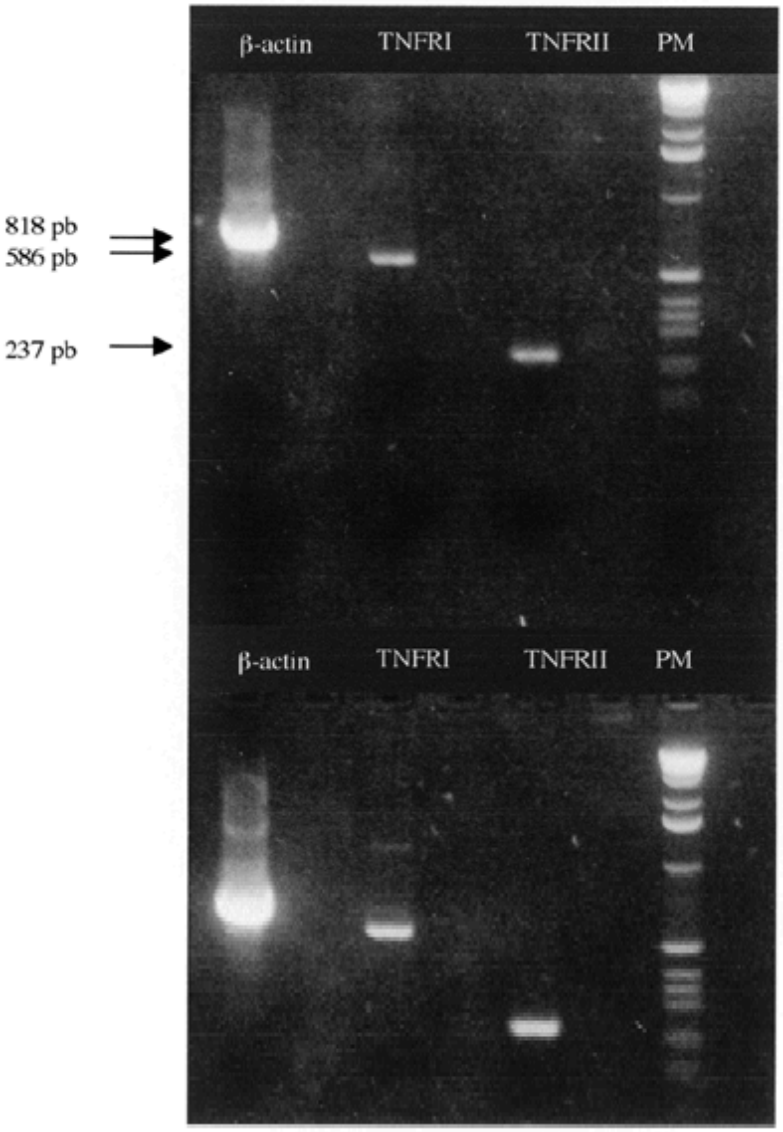


Figure 1

Expression of TNFRI and TNFRII RNA in MRC5 cells and THP1 cells by using RT-PCR.

Total RNA from MRC5 (upper panel) or THP1 (lower panel) cells was reverse transcripted and the resulting cDNA then run in a PCR reaction with upstream and downstream human primers for TNFRI, TNFRII and beta-actin as described in material and methods.

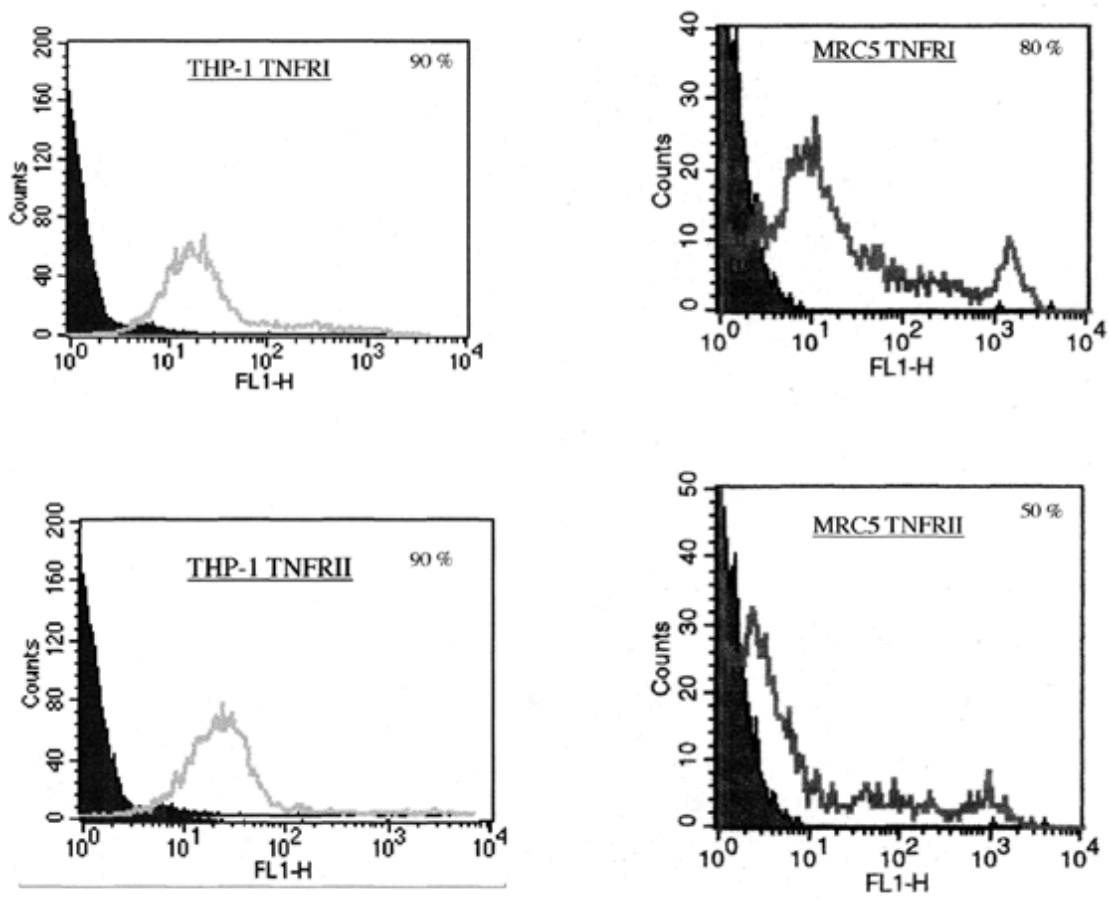


Figure 2

Flow cytometry analysis of TNFRs on MRC5 and THP1 cells.

Cells were labelled by mouse anti-human TNFRI, or mouse anti-human TNFRII antibodies (lines), or an isotype

control IgG antibody (dark areas), then stained with FITC conjugated anti-mouse IgG. Data are from one representative experiment out of 3 performed.

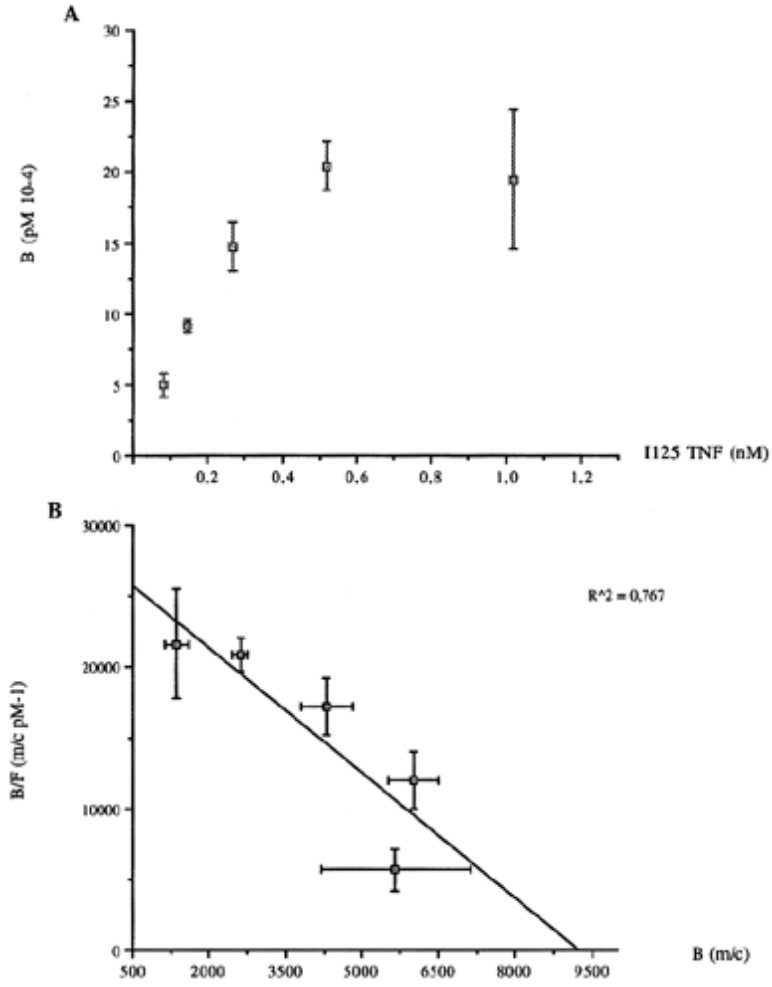


Figure 3

Receptor binding of radiolabelled TNF-alpha **to human MRC5 cells.**

(A) Saturation curve: cells were incubated at 4° C with increesing concentrations of ^{125}I-TNF (0.0625-1 nM). Total and non-specific bindings were determined as described in material and methods. **(B)** Scatchard plot analysis of the binding data shown in the upper panel. Results are

expressed as the mean of triplicates ± SD and are from 1 representative experiment out of 3 performed.

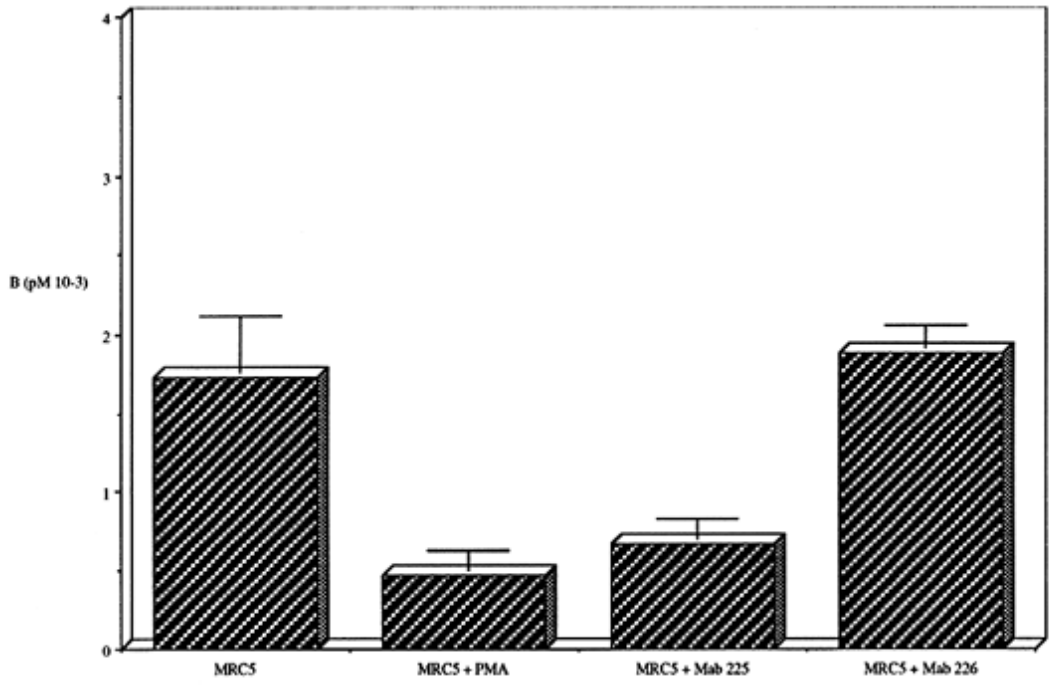


Figure 4

The inhibition of ^{125}I-TNF binding to MRC5 cells in the presence of PMA and specific antibodies against TNFR.

MRC5 cells were treated with medium alone, PMA 10^{-6} M, anti-TNFRI mab (MAB 225), anti-TNFRII mab (MAB 226) for 1 hour before the binding assay. Total and non-specific bindings were measured in the presence of 1 nM of ^{125}I-TNF-alpha, without or with an excess of unlabelled TNF-alpha (100 nM), respectively. Values are means of triplicate samples ± SD.

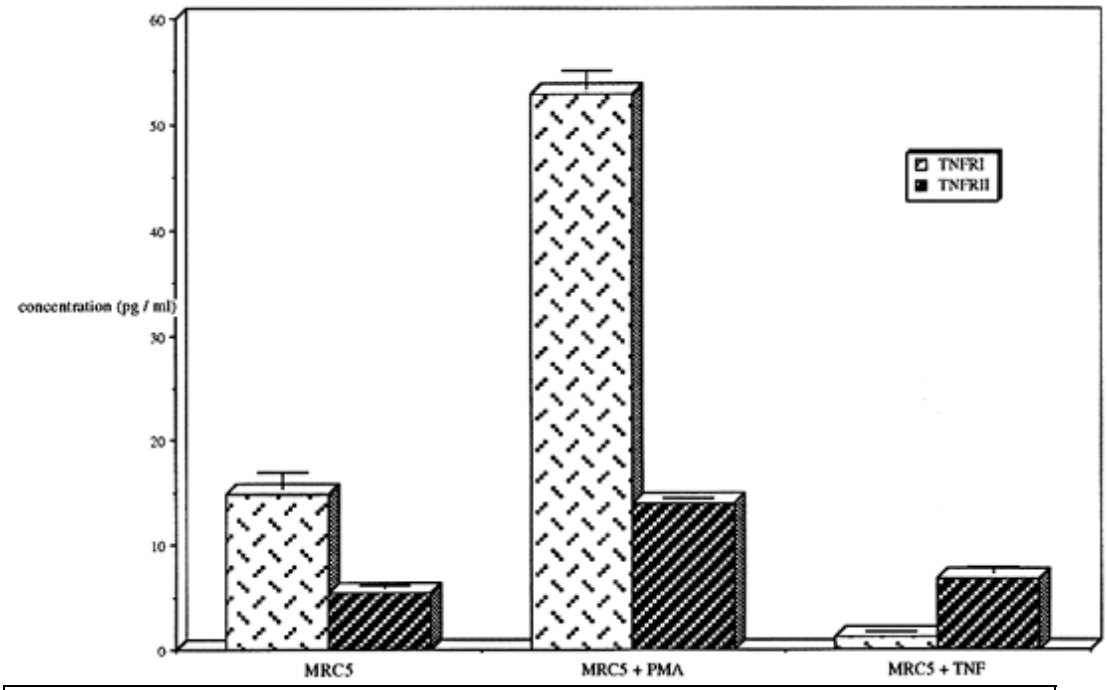


Figure 5

Soluble TNF receptors released by MRC5 cells.

Aliquots of the culture medium of control cells or cells with PMA 10^{-6} M or TNF-alpha (30 ng/ml) for 1 hour were collected and tested for sTNFRI and sTNFRII, as described in material and methods. Data are the mean of two separate determinations performed in duplicate ± SD.

Table 1
Amplification primers
The primer sets used for PCR amplification of specific cDNA derived from TNF receptors and beta-actin mRNAs.

Gene		Oligonucleotide sequence	Product size pb
TNFR I (Clontech)	5' primer 3' primer	ATTTGCTGTACCAAGTGCCACAAAGGAACC GTCGATTTCCCACAAACAATGGAGTAGAGC	586
TNFR II	5' primer 3' primer	CCTGCCCCTTGGAGTGCCTGAT CACAACCCGCTGCCTCTGCTCTT	237
beta-actin (Clontech)	5' primer 3' primer	ATCTGGCACCACACCTTCTACAATGAGCTGCG CGTCATACTCCTGCTTGCTGATCCACATCTGC	818

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In this issue

Regulation of interferon-g receptor (INF-gR) chains: a peculiar way to rule the life and death of human lymphocytes.

P. Bernabei, A. Allione, L. Rigamonti, M. Bosticardo, G. Losana, I. Borghi, G. Forni, F. Novelli
human, IFN-g, IFN-g receptor, signal transduction, cellular proliferation, apoptosis.

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interleukin-6, embryo, blastocyst, endometrium, heart, gp130.

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cyclic AMP, prostaglandins, nitric oxide, interleukin-10, tumor necrosis factor-a, interferon-g.

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TNF-a, idiopathic pulmonary fibrosis, polymorphism.

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interferon-g, cytokines, host defense, Pseudomonas aeruginosa, pneumonia.

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allergic contact dermatitis, p75TNF receptor, p55TNF receptor, Langerhans cells, dendritic cells.

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transforming growth factor-beta1, HIV-RNA, AIDS-dementia complex, interferon-alpha.

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interferon-beta, natural autoantibodies, multiple sclerosis.

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interleukin-6, cord blood, gene polymorphism, inflammatory mediators.

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HCV-RNA, interleukin-10, H9 T cells, CD81, capsid mutation, protein kinase C.

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neuroblastoma, immunotherapy, gene therapy, cytokine, IL-1b, TNF-a.

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intestinal mucosa, Crohn's disease, interleukin-18, caspase-1/ICE, immunohistochemistry, reverse transcription polymerase chain reaction.

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TNF, cytokine, LPS.

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rodent, inflammation, nitric oxide, cytokines, in vivo animal models.

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MUC5AC, MUC5B, mucus-secreting cell line HT29-MTX.

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apoptosis, caspases, Hsp27, Hsp70, T lymphocytes.

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MHC class II antigens, murine macrophages, c-Fos.

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transforming growth factor-b1, interleukin-10, U. 937 cells.

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MRC5, fibroblasts, TNF receptor, RT-PCR, binding, flow cytometry.

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cytokines, lipocalin, IL-9, 24P3.

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cytokines, lipid mediators, macrophages, rat.

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apoptosis, interleukin-1, caspases, polymorphonuclear leukocytes, ICE.

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IL-10, recombinant, rat, cytokines.

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