FcγRIII Expression on Cultured Human Keratinocytes and Upregulation by Interferon-γ

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Keratinocytes of human epidermis are actively involved in inflammatory and autoimmune reactions of the skin and interact with resident or infiltrating immunocompetent cells via cytokines, chemokines, and intercellular adhesion mechanisms. Most immunocompetent cells have been reported to express Fcγ receptors (FcγR), which are important for immunoregulatory functions. In this study we investigate FcγRIII expression on cultured human keratinocytes and upregulation by interferon-γ. By real-time polymerase chain reaction, we show basal mRNA expression of both subclasses FcγRIIIA and FcγRIIIB, but after interferon-γ treatment mRNA of FcγRIIIA and FcγRIIIB is increased 4.4 and 6.5 times, respectively. FcγRIII protein expression and its increase after interferon-γ treatment were shown on cultured human keratinocytes by indirect immunofluorescence. In immunoblotting experiments, a bonified anti-CD16 antibody revealed reactivity to a polypeptide of 50–65 kDa on lysates of treated and untreated keratinocytes. In summary, we demonstrate expression of mRNA specific for the FcγRIIIA and FcγRIIIB subclasses and their upregulation by interferon-γ on human keratinocytes *in vitro*, and confirm FcγRIII protein expression by indirect immunofluorescence and by immunoblotting experiments. ***Key words: epidermal cells/Fcγ receptors/real-time PCR. J Invest Dermatol 119:1074–1079, 2002***

Keratinocytes are the major structural cells of the human epidermis, but have also been shown to play an important pathophysiologic role in inflammatory and immunologic processes of human skin (Stingl *et al*, 1999). Influenced by certain stimuli like allergens or cytokines derived from leukocytes and monocytes, keratinocytes by themselves are able to produce a wide range of cytokines (Nickoloff *et al*, 1990; Barker *et al*, 1991b) and chemokines (Morales *et al*, 1999; Traidl *et al*, 2000) that may activate resident dendritic cells (Barker *et al*, 1991a) or contribute to lymphocyte recruitment into the epidermis (Morales *et al*, 1999). Under certain circumstances, keratinocytes may express surface molecules necessary for interaction with T cells and leukocytes like intercellular adhesion molecule 1 (Griffiths *et al*, 1989) or major histocompatibility complex class II proteins playing a key role in the control of immune response (Albanesi *et al*, 1998). Immunocompetent cells present in inflammatory skin like macrophages, T lymphocytes, and dendritic cells express receptors for the Fc part of IgG molecules, Fcγ receptors (FcγR) (Kiekens *et al*, 2000), which play an important role in immune responses as they link the humoral immune system with cellular effector functions (Kimberly *et al*, 1995; Ravetch, 1997). These cell surface glycoproteins mediate forms of phagocytosis, antibody-dependent cell-mediated cytotoxicity, as well as inhibitory or activating signal transduction (Heijnen and van de Winkel, 1997; Billadeau and Leibson, 2002), depending on associated peptide chains or membrane proteins involved. The leukocyte receptor family represents three major classes: FcγRI (also known as CD64), FcγRII (CD32), and FcγRIII (CD16), differing in molecular structure, binding properties, and cellular distribution (Heijnen and van de Winkel, 1997; Ravetch, 1997). Various genes encode subclasses of the hetero-oligomeric receptor proteins forming transmembrane molecules, with the exception of FcγRIIIB, which is linked to the surface membrane via its glycosylphosphatidylinositol (GPI) anchor (Simmons and Seed, 1988; Ravetch and Perussia, 1989; Scallon *et al*, 1989). Most FcγR proteins consist of the ligand binding α-chain with an extracellular region composed of disulfide bonded Ig-like domains (Heijnen and van de Winkel, 1997) and form hetero-oligomeric receptor complexes with additional subunits such as γ-, ζ-, β-chains functioning in FcγR assembly and, especially via their cytoplasmic tails, signal transduction. FcγRI is a high affinity receptor that binds to monomeric and immune-complexed IgG (Ravetch and Kinet, 1991; Heijnen and van de Winkel, 1997) and is expressed on macrophages, monocytes (Anderson, 1982; Looney *et al*, 1986), interferon-γ (IFN-γ) altered neutrophils (Perussia *et al*, 1983b), and mast cells (Okayama *et al*, 2000). FcγRII is a widely distributed low affinity receptor for immune complexes and found predominantly on hemopoietic cells (Mantzioris *et al*, 1993; Metes *et al*, 1994), dendritic cells (Schmitt *et al*, 1990; Heijnen and van de Winkel, 1997), and endothelial cells (Gröger *et al*, 1996). The FcγRIII subclasses exhibit either a low or medium affinity for monomeric or complexed IgG (Heijnen and van de Winkel, 1997) and have been shown on natural killer cells (Perussia *et al*,

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Abbreviation: FcγR, Fcγ receptor.

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1983a), macrophages, monocytes (Fleit *et al*, 1982; Clarkson and Ory, 1988), and granulocytes (Simmons and Seed, 1988; Ravetch, 1997).

Several reports show FcγR expression on human keratinocytes by immunofluorescence experiments using monoclonal antibodies and soluble immune complexes (Tigalonowa *et al*, 1990; 1991; Bjerke *et al*, 1994a; 1994b). These studies suggest that FcγR on human keratinocytes may be mediators for interaction between keratinocytes and Langerhans cells, and, in the stratum granulosum, may contribute to the barrier function against microorganisms and various other antigens (Tigalonowa *et al*, 1990).

In this study, we demonstrate the presence of the subclasses FcγRIIIA and FcγRIIIB mRNA on cultured human keratinocytes and their upregulation after treatment of keratinocytes with IFN-γ using TaqMan real-time polymerase chain reaction (PCR). Indirect immunofluorescence with a monoclonal anti-FcγRIII (anti-CD16) antibody revealed staining of individual keratinocytes. When lysates of IFN-γ-treated and untreated cultured keratinocytes were immunoblotted with the anti-CD16 antibody, protein expression was demonstrated by respective antibody reactivity.

MATERIALS AND METHODS

Cell culture, antibodies, and reagents Normal human keratinocytes were isolated from skin samples of adult skin, obtained from healthy volunteers after cutaneous surgery. Donors gave informed consent. Keratinocytes were grown in culture with modifications, as described previously (Boyce and Ham, 1983). Briefly, epidermis was separated by incubation in 50 caseolytic units per ml dispase (Collaborative, Bedford, MA) for 1–2 h at 37°C. After incubation in 0.25 mg per ml trypsin/ethylenediamine tetraacetic acid (Clonetics, San Diego, CA) for 30 min at 37°C a single cell suspension of keratinocytes was released by gentle pipetting and cultured in keratinocyte growth medium (KGM, Clonetics, San Diego, CA) containing 0.03 mM calcium. Normal human fibroblasts were isolated from skin explant cultures of de-epidermized dermis and cultivated in Dulbecco's modified Eagle's medium (Gibco, Paisley, Scotland, U.K.) containing 10% fetal bovine serum.

Cells were grown to confluence in 100 × 20 mm style Petri dishes using appropriate growth medium to obtain extracts for immunoblotting, in eight-well chamber slides (Becton Dickinson, Franklin Lakes, NJ) for immunofluorescence experiments and in T-150 flasks (Techno Plastic Products, Trasadingen, Switzerland) for mRNA isolation. Keratinocytes and fibroblasts were used without and after incubation with *Escherichia coli* derived human recombinant IFN-γ (HyCult Biotechnology, Uden, The Netherlands; diluted in sterile distilled water to final concentration of 1000 U per μl), at a concentration of 500 U per 1,000,000 cells diluted in culture medium, for 10 h.

For indirect immunofluorescence and immunoblotting experiments monoclonal antibody directed against FcγRIII (CD16), clone HuNK2, was purchased from Neo Markers (Fremont, CA). Isotype-matched murine control antibody (IgG2a) was from Pharmingen (Uppsala, Sweden).

Alexa 488 conjugated goat $F(ab')_2$ antimouse IgG (H + L) from Molecular Probes (Eugene, OR) was used as second step reagent for indirect immunofluorescence.

Alkaline-phosphatase-conjugated goat antimouse IgG (Promega, Madison, WI) was used as second step antibody for immunoblotting.

Human leukocytes as positive control for immunoblotting and real-time PCR assays were obtained from healthy volunteers and prepared using Ficoll-Paque (Amersham Pharmacia, Biotech, Uppsala, Sweden) by density-gradient centrifugation of heparinized venous blood, according to a method described previously (Cassatella *et al*, 1990).

Quantitative real-time PCR (TaqMan) analysis Human keratinocytes and fibroblasts were obtained from four different donors and cultured separately in T-150 flasks as described above.

Confluent keratinocytes in T-150 flasks were washed in DPBS (Dulbecco's Phosphate Buffered Saline-0,0095 M(PO4), BioWhittaker Europe, Verviers, Belgium), scraped from the bottom of tissue flasks, and pelleted by centrifugation at 1600 rpm for 10 min. Cell pellets were stored at −70°C for later use. Total RNA was extracted using Qiagen RNeasy Midi Kit (Qiagen, Valencia, CA) according to the manufacturer's protocol (RNeasy Midi/Maxi Handbook, first edition, October 1999, Qiagen, Valencia, CA).

About 700 ng total RNA in solution with sterile distilled water were reverse transcribed using TaqMan Reverse Transcription Reagents Kit (Applied Biosystems, Foster City, CA) according to the manufacturer's instruction (TaqMan Universal PCR Master Mix Protocol, Applied Biosystems). Briefly, the reaction was performed in the presence of 10 μl of 10 × TaqMan RT buffer, 22 μl of 25 mM magnesium chloride (5.5 mM), 20 μl deoxyNTPs mixture (500 μM of each dNTP), 5 μl of random hexamer (2.5 μM), 2 μl of RNase inhibitor (0.4 U per μl), 2.5 μl of MultiScribe reverse transcriptase (1.25 U per μl), and 38.5 μl of RNA (700 ng in sterile distilled water). The final solution (100 μl) was incubated for 10 min at 25°C, another 30 min at 48°C, and finally for 5 min at 95°C. The resulting cDNA was stored at −70°C for later use. 5 μl of cDNA solution (about 35 ng cDNA) were used as PCR template in the presence of 12.5 μl of TaqMan universal master mix (Applied Biosystems, Foster City, CA), 0.5 μl (10 pmol per μl) of gene-specific TaqMan probe, 2.25 μl (10 pmol per μl) of gene-specific forward and reverse primers, and 2.5 μl of water. cDNA was also amplified in the presence of 12.5 μl TaqMan universal master mix, 1.25 μl β-actin (Applied Biosystems, Foster City, CA) as endogenous control, and 6.25 μl of water. Specific primers and probes for FcγRIIIA and FcγIIIB were obtained from TIB Molbiol, Berlin, Germany. The sequences for primers and probes used for cDNA amplification were designated using Primer Express 1.0 software (supplied by Applied Biosystems) with DNA and the appropriate mRNA sequence of FcγRIIIA and FcγRIIIB gene (Peltz *et al*, 1989; Gessner *et al*, 1995) from Entrez Nucleotide's search tool on the World Wide Web (National Center of Biotechnology Information (NCBI), Bethesda, MD: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=Nucleotide). The following primers were used: TGGCATGCGGACTGAAGAT (forward) and AAGCACGCTGTACCATTGAGG (reverse) for FcγRIIIA; and TGGCATGCGGACTGAAGA (forward) and AAGCACGCTGTACCATTGAGG (reverse) for FcγRIIIB. The sequences for specific fluorogenic probes were TCCCAAAGGCTGTGGTGTTCCTGGT for FcγRIIIA and CCCAAAGGCTGTGGTGTTCCTGGA for FcγRIIIB. The specificity of the chosen primers was tested using NCBI BLAST Search Database to exclude possible cross-reactivity with genomic DNA. Gene-specific probes were labeled with FAM as reporter whereas the probe for endogenous control was VIC associated. The samples underwent the following stages: stage 1, 50°C for 2 min; stage 2, 95°C for 10 min; stage 3, 95°C for 15 s followed by 60°C for 1 min. Stage 3 was repeated 45 times. Gene-specific products were measured by means of an ABI PRISM 7700 Sequence Detection System (Perkin-Elmer Applied Biosystems, Foster City, CA) continuously during 45 cycles.

Relative quantification of mRNA expression was performed according to the manufacturer's protocol using the comparative C_T (threshold cycle) method (User Bulletin ABI PRISM 7700 Sequence Detection System). The relative quantity of mRNA FcγRIIIA and FcγIIIB in IFN-γ-treated cells is given by $2^{-\Delta\Delta C_T}$, where $\Delta\Delta C_T$ = mean ΔC_T (mRNA from treated cells) − mean ΔC_T calibrator (mRNA from untreated cells). Mean ΔC_T is the average ΔC_T value of four different donors. C_T of β-actin subtracted from gene-specific C_T gives the ΔC_T for each donor.

For control RNA from human leukocytes (four different donors), untreated and IFN-γ-treated fibroblasts were extracted and reverse transcribed, and cDNA was used for amplification with the same primers and probes as described above for keratinocytes. Statistical analysis was performed using the paired Student's *t* test, and p-values < 0.05 were considered as statistically significant. Variables are described by mean ± standard deviation.

Cell proliferation and cytotoxicity assay To determine the cytotoxic effects of IFN-γ on cultured human keratinocytes *in vitro* a nonradioactive cell proliferation and cytotoxicity assay (Biomedica, Vienna, Austria) was performed (Mosmann, 1983). For this purpose, keratinocytes were grown to confluence and incubated with human recombinant IFN-γ (500 U for 1,000,000 cells) for 10 h in 96-well plates. Lyophilized dye substrate solution was dissolved in activator solution and warmed up to 37°C prior to use. 20 μl of this solution was then added to each well containing 200 μl KGM and incubated 4 h at 37°C. The plate was mixed and the absorbance was read at 450 nm with 630 nm as reference. The metabolic capacity of cells is a result of their ability to convert the yellow-colored tetrazolium compound to its red formazan derivate. The reference absorbance at 630 nm is used to correct for nonspecific background values. Absorbance from a substrate blank in assay medium without cells was subtracted from all values.

Indirect immunofluorescence on cultured cells Normal human keratinocytes were grown to near confluence in eight-well chamber slides, fixed with 3% paraformaldehyde for 30 min, and incubated with mouse monoclonal anti-CD16 antibody (Neo Markers, clone HuNK2), diluted 1:50 in PBS, for 1 h. The cells were then washed with PBS and incubated

in appropriate second step antibody, diluted 1:100 in PBS, for 1 h, followed by final washing and embedding in fluoprep (bioMérieux, Marcy l'Etoile, France). Alternatively, keratinocytes were incubated with IFN-γ for 10 h (HyCult Biotechnology; 1000 U per μl stock solution; 500 U per 1,000,000 cells) and fixed, and immunofluorescence was performed.

Human fibroblasts were cultured in appropriate medium in eight-well chamber slides, fixed with 3% paraformaldehyde for 30 min, incubated with anti-CD16 antibody (Neo Markers, clone HuNK2, diluted 1:50 in PBS), washed, and incubated with the respective second step antibody. The same procedure was also performed with IFN-γ-treated fibroblasts (10 h).

Embedded cells were examined using an OLYMPUS BH 2-RFCA fluorescence microscope.

Immunoblotting Immunoblotting was performed according to methods described previously (Hashimoto *et al*, 1990). Keratinocytes were grown to confluence in Petri dishes. A portion was treated with human IFN-γ (HyCult Biotechnology; 1000 U per μl stock solution; 500 U per 1,000,000 cells) for 10 h. After washing in PBS, cells were scraped from the Petri dish into a 1.5 ml Eppendorf tube (Eppendorf-Netheler-Hinz, Hamburg, Germany), homogenized in 2% sodium dodecyl sulfate (SDS, Bio-Rad, Hercules, CA) in 0.0625 M Tris-buffered saline, pH 6.8, supplemented with 5% 2-mercaptoethanol (Bio-Rad, Hercules, CA) and 2 mM phenymethylsulfonyl fluoride (Sigma Chemical, St. Louis, CA), boiled, and centrifuged. Alternatively, cultured human fibroblasts, with and without IFN-γ treatment, and normal human leukocytes were lyzed accordingly. Extracted proteins were separated by SDS polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto nitrocellulose membranes (Towbin *et al*, 1979). Strips of nitrocellulose were exposed to mouse monoclonal anti-CD16 antibody (Neo Markers, clone HuNK2) or isotype control antibody (Pharmingen), each diluted 1:20 in blotting buffer overnight, washed, and incubated with alkaline-phosphatase-conjugated goat antimouse IgG (Promega). Bound antibody was visualized by nitroblue tetrazolium/bromochloroindolyl phosphatase (Kirkegaard and Perry Laboratories, Gaithersburg, MD) enzyme reaction (Blake *et al*, 1984).

RESULTS

FcγRIIIA and FcγRIIIB mRNA expression in cultured human keratinocytes is upregulated by IFN-γ To demonstrate unalterated viability of IFN-γ-treated cultured keratinocytes and fibroblasts, a nonradioactive cell proliferation and cytotoxicity assay was performed. The cells exhibited no altered metabolic capacity, untreated or after treatment with IFN-γ for 10 h at the concentrations described (data not shown).

We performed real-time PCR experiments to investigate the mRNA expression of FcγRIII in cultured human keratinocytes and to determine possible upregulation of the two heteromeric isoforms FcγRIIIA and FcγRIIIB after IFN-γ treatment using specific primers and probes to distinguish FcγRIIIA and FcγRIIIB transcripts. TaqMan real-time PCR assay is based on measuring threshold cycle (C_T) values associated with the increase of detection signal indicating exponential growth of the PCR product. β-Actin was used as endogenous control. Using the comparative C_T method we obtained the relative quantity of upregulated mRNA (**Tables I, II**). C_T mRNA values of FcγRIIIA and β-actin from untreated and IFN-γ-treated keratinocytes of four different donors were measured and used to determine the relative quantity of FcγRIIIA mRNA. Based on this calculation, cultured human keratinocytes treated with IFN-γ resulted in about a 4.4-fold higher expression of FcγIIIA mRNA compared with that of untreated keratinocytes (**Table I**).

The relative quantity of FcγRIIIB mRNA is determined by measured C_T mRNA values of FcγRIIIB and β-actin from untreated and IFN-γ-treated keratinocytes. As a result, the expression of FcγRIIIB mRNA is about 6.5-fold higher in treated than in untreated keratinocytes (**Table II**).

Upregulation of FcγRIIIA and FcγRIIIB mRNA after treatment with human IFN-γ was significant when a paired Student's *t* test was performed (p-values: 0.024 for FcγRIIIA and 0.022 for FcγRIIIB). RNA from human leukocytes obtained from four healthy volunteers was used as positive control. The leukocytes contained 5595 times as much FcγRIIIA mRNA and 1217 times as much FcγRIIIB mRNA compared to untreated human keratinocytes (data not shown). In contrast, mRNA isolated from cultured IFN-γ-treated and untreated human fibroblasts after reverse transcription was used for real-time PCR analysis. In 45 performed cycles we could not detect specific cDNA ampflication, either in untreated or in treated

Table I. FcγRIIIA mRNA expression and upregulation with IFN-γ in human keratinocytes

	Untreated cultured human keratinocytes				IFN-γ treated cultured human keratinocytes					
	C_T[a] FcγRIIIA	C_T β-actin	ΔC_T[c]	Mean $\Delta C_T \pm$ SD	C_T FcγRIIIA	C_T β-actin	ΔC_T[c]	Mean $\Delta C_T \pm$ SD	$\Delta\Delta C_T$[b]	$2^{-\Delta\Delta C_T}$ = relative quantity of upregulated mRNA
Donor 1	34.90	19.16	15.74		33.87	21.14	12.73			
Donor 2	33.36	19.23	14.13		32.95	20.24	12.71			
Donor 3	33.18	18.99	14.19		33.50	20.43	13.07			
Donor 4	37.71	20.27	17.44		34.56	20.10	14.46			
				15.38 ± 1.57				13.24 ± 0.83	−2.14 ± 0.83	4.41 (2.48–7.84)

[a] C_T, threshold cycle.
[b] $\Delta\Delta C_T$ = mean ΔC_T (mRNA of treated cells) − mean ΔC_T calibrator (mRNA of untreated cells).
[c] $\Delta C_T = C_T$ FcγRIIIA − C_T β-actin.

Table II. FcγRIIIB mRNA expression and upregulation with IFN-γ in human keratinocytes

	Untreated cultured human keratinocytes				IFN-γ treated cultured human keratinocytes					
	C_T[a] FcγRIIIB	C_T β-actin	ΔC_T[c]	Mean $\Delta C_T \pm$ SD	C_T FcγRIIIB	C_T β-actin	ΔC_T[c]	Mean $\Delta C_T \pm$ SD	$\Delta\Delta C_T$[b]	$2^{-\Delta\Delta C_T}$ = relative quantity of upregulated mRNA
Donor 1	35.24	19.16	16.08		34.94	21.14	13.80			
Donor 2	33.61	19.23	14.38		33.46	20.24	13.22			
Donor 3	34.00	18.99	15.01		32.83	21.69	11.14			
Donor 4	40.76	20.27	20.49		35.10	18.13	16.97			
				16.49 ± 2.76				13.78 ± 2.41	−2.71 ± 2.41	6.54 (1.23–34.78)

[a] C_T, threshold cycle.
[b] $\Delta\Delta C_T$ = mean ΔC_T (mRNA of treated cells) − mean ΔC_T calibrator (mRNA of untreated cells).
[c] $\Delta C_T = C_T$ FcγRIIIB − C_Tβ-actin.

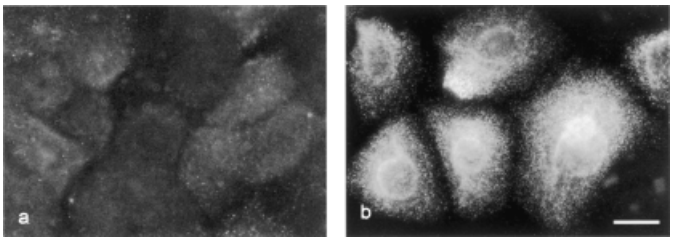

Figure 1. Indirect immunofluorescence on cultured human keratinocytes exhibits expression of FcγRIII. Confluent keratinocytes were fixed with 3% paraformaldehyde, exposed to mouse monoclonal anti-CD16 antibody and Alexa 488 conjugated second step reagent. At the cell surface of untreated cultured human keratinocytes, FcγRIII expression could be detected as a weak granular staining pattern (*a*). When keratinocytes were treated with IFN-γ for 10 h prior to fixation and immunofluorescence with anti-CD16 antibody, a strong, distinct granular staining pattern demonstrates significant upregulation of FcγRIII on the cell surface of keratinocytes. *Scale bar*: 20 μm.

fibroblasts, indicating that human fibroblasts do not express FcγRIIIA and FcγRIIIB mRNA.

Indirect immunofluorescence shows FcγRIII protein expression on cultured human keratinocytes Indirect immunofluorescence was performed on cultured human keratinocytes to examine protein expression of FcγRIII molecules. Using a mouse monoclonal antibody directed against FcγRIII we found a weak but distinct granular fluorescence staining pattern with individual keratinocytes (**Fig 1*a***). We also tested IFN-γ-treated (10 h, 500 U per 1,000,000 cells) keratinocytes. In that case, the fluorescence staining from individual keratinocytes gave a specific and distinct granular pattern (**Fig 1*b***).

Biochemical characterization shows FcγRIII protein expression on cultured human keratinocytes We examined the specificity of FcγRIII protein expression on cultured human keratinocytes by immunoblotting experiments. Protein lysates were prepared from cultured human keratinocytes without and after 10 h treatment with IFN-γ. The extracted proteins were separated electrophoretically and transferred to nitrocellulose. Strips of the blotted proteins were incubated with the monoclonal antibody against FcγRIII. When untreated keratinocytes were probed, weak binding reactivity of the antibody to a polypeptide at 50–65 kDa (**Fig 2**, *lane 1*) was observed. Lysates obtained from keratinocytes after 10 h treatment with IFN-γ were immunoblotted with the same antibody and gave prominent reactivity with the polypeptide of 50–65 kDa indicating a selective increase of protein expression (**Fig 2**, *lane 2*). For control, immunoblotting with the anti-FcγRIII antibody was performed on SDS lysates from human leukocytes giving same reactivity (**Fig 2**, *lane 3*). When protein extracts of fibroblasts with and without IFN-γ treatment were used for immunoblotting, no reactivity could be detected (**Fig 2**, *lanes 4, 5*).

DISCUSSION

In this study, we demonstrate that cultured human keratinocytes express FcγRIIIA and FcγRIIIB genes and FcγRIII protein on their cell surface. As FcγRIII is encoded by two homologous genes (Peltz *et al*, 1989; Gessner *et al*, 1995), we performed real-time PCR experiments using specific primers and probes for FcγRIIIA and FcγRIIIB, to characterize subtypes and also to show the possible induction of gene expression by treatment with IFN-γ. Normal human keratinocytes cultured in selective keratinocyte growth medium show mRNA expression of both isoforms, at a relative low level, however, compared to human leukocyte mRNA, which was used as positive control.

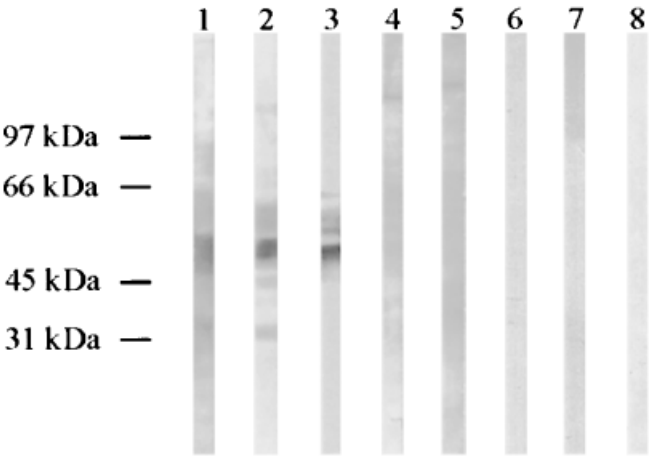

Figure 2. Biochemical detection of FcγRIII on cultured human keratinocytes. Protein lysates of cultured human keratinocytes and fibroblasts and, for control, peripheral blood leukocytes were separated by SDS-PAGE followed by transfer onto nitrocellulose. *Lane 1* shows immunoblotting with monoclonal anti-CD16 antibody using untreated keratinocytes giving reactivity at 50–65 kDa. When keratinocytes were treated with IFN-γ for 10 h before protein preparation, immunoblotting with anti-CD16 antibody gave a more prominent reaction at 50–65 kDa as shown in *lane 2*. This reaction is similar to the labeling of anti-CD16 on blood leukocytes in *lane 3*. No specific reaction was observed when untreated, *lane 4*, and IFN-γ-treated human fibroblasts, *lane 5*, were used. Immunoblotting with isotype control antibody (mouse IgG2a) on keratinocytes, *lane 6*, leukocytes, *lane 7*, and fibroblasts, *lane 8*, gave no binding.

IFN-γ, a multifunctional lymphokine secreted by T lymphocytes, natural killer cells, and Langerhans cells, is known to act as a potent stimulus for keratinocytes (Barker *et al*, 1990; Nickoloff *et al*, 1990; Kalvakolanu and Borden, 1996). According to previous reports on IFN-γ upregulation of FcγRIII proteins on neutrophils (Ravetch and Perussia, 1989) and eosinophils (Hartnell *et al*, 1992), we asked whether these molecules can be upregulated by IFN-γ in cultured human keratinocytes. As it has been reported that IFN-γ induces growth arrest and cell differentiation in epithelial cells within 12 h of treatment (Saunders *et al*, 1996), we incubated the cells for 10 h with IFN-γ and performed additional cell cytotoxicity assays. Real-time PCR results showed that gene expression on human keratinocytes was altered by incubation with IFN-γ. The FcγRIIIA expression was 4.4-fold higher and FcγRIIIB was 6.5-fold higher after treatment with IFN-γ. This upregulation is still much lower than reported for FcγRI and FcγRIII on human mast cells or neutrophils (Perussia *et al*, 1983b; Okayama *et al*, 2000), and eosinophils (Hartnell *et al*, 1992), respectively.

Immunofluorescence experiments with monoclonal antibodies against known structural epitopes exhibited the presence of FcγRIII protein on cultured human keratinocytes. After treatment with human recombinant IFN-γ there was a marked increase in intensity in the form of a granular fluorescence signal on individual keratinocytes indicating upregulated FcγRIII protein expression at the light microscopy level. These findings could be observed on fixed as well as freshly prepared keratinocytes (not shown). Immunoblotting lysates of IFN-γ-treated and untreated cultured keratinocytes using the bonified anti-CD16 antibody yielded reactivity with a polypeptide of 50–65 kDa appearing as a broad band. The reactivity on untreated keratinocytes was low, however, giving a weak band at the expected molecular weight. It is interesting to note that the fluorescence pattern resembles surface binding of immune complexes on keratinocytes as described previously (Tigalonowa *et al*, 1991). The molecular weight of the polypeptide corresponds to that recognized by the anti-CD16 antibody in lysates of keratinocytes as

found when anti-CD16 antibody was reacted with the leukocyte lysates. These findings let us assume that cultured human keratinocytes express the entire functional heteromeric FcγRIII molecule.

The subclasses of human FcγRIII are encoded by two linked genes: a transmembrane form (IIIA) with a 25 amino acid cytoplasmic tail and a GPI-linked form (IIIB) without a cytoplasmic portion (Simmons and Seed, 1988; Ravetch and Perussia, 1989). The multichain transmembranous FcγRIIIA is one of those receptors with an immunoreceptor tyrosine-based activation motif (ITAM; Billadeau and Leibson, 2002) mediating cell activation, endocytosis, and phagocytosis. FcγRIIIB, a human receptor without ITAM, has no triggering capability by itself but appears to transduce signals with additive receptor proteins like FcγRIIA (Vossebeld *et al*, 1995; Heijnen and van de Winkel, 1997). The FcγRIIIA displays medium affinity interacting with monomeric and complexed IgG and is predominantly expressed on natural killer cells (Perussia *et al*, 1983a), macrophages, and monocytes (Fleit *et al*, 1982; Clarkson and Ory, 1988), whereas FcγRIIIB serves for ligand binding on neutrophils (Ravetch and Perussia, 1989).

In our study we provide molecular biologic, immunomorphologic, and biochemical evidence that human keratinocytes express FcγRIII, which appears to be significantly upregulated by IFN-γ. Using real-time PCR, we could also observe gene expression for FcγRI and FcγRII subtypes at a low level, with no significant upregulation (data not shown). Human FcγR represent a family of cell surface glycoproteins known to mediate interactions of IgG antibodies with immune effector cells to generate and control an appropriate humoral immune response (Heijnen and van de Winkel, 1997). As most immunocompetent cells express multiple FcγR, each of the expressed proteins acts in concert with the other FcγR families expressed on the same cell. Depending on the cell type or on cytokines that differentially regulate the expression of various FcγR, and the composition of immune complexes they interact with, the FcγR elicit respective immunoregulatory processes (Daeron, 1997).

Only few reports have shown protein expression of FcγR molecules on human keratinocytes (Tigalonowa *et al*, 1990; 1991; Bjerke *et al*, 1994a; 1994b). Their expression on human keratinocytes can still be said to be a matter of controversy. In view of the potentially important immunomodulatory role of keratinocytes in human epidermis, we searched for the presence and investigated the possible upregulation of FcγR molecules at the mRNA and protein level. FcγRIII has been reported to be involved in immune complex mediated inflammation binding to complex and weak monomeric IgG. Mice deficient for the α-chain of FcγRIII lack natural-killer-cell-mediated antibody-dependent cytotoxicity and phagocytosis and exhibit an impaired Arthus reaction (Hazenbos *et al*, 1996), indicating that the class III receptor plays a prominent role in type III inflammatory response. Although FcγR deliver signals when aggregated at the cell surface and low and medium affinity receptors do not bind monomeric immunoglobulins with a measurable affinity (Daeron, 1997), it is possible that human keratinocytes are actively involved in immunoregulatory procedures via their FcγRIII. For example, lupus erythematosus, an autoimmune disease with skin involvement, is caused by immune complexes and characterized by autoantibodies directed against intracellular target molecules (Sontheimer, 1999; Kamradt and Mitchison, 2001). Studies with antinuclear antibodies of patients with systemic lupus erythematosus have suggested that autoantibodies might enter the cells via FcγR (Alarcón-Segovia *et al*, 1979). Considering that these autoantibodies are also targeting intracellularly located antigens in keratinocytes, one could imagine that under certain conditions FcγR are involved in the penetration of antibodies into cells. Like GPI-linked FcγRIIIB, various proteins have been reported to be anchored in GPI-rich plasma membrane domains like, for example, caveolin 1 (Rothberg *et al*, 1992), the protein component of caveolae, small plasmalemmal vesicles involved in clathrin-independent endocytosis. Interaction among those proteins could help antibodies or other molecules to reach the interior of cells via receptor-mediated endocytosis. Moreover, FcγRIIIA has been shown to mediate endocytosis (Strzelecka *et al*, 1997; Hunter *et al*, 1998), leading to the hypothesis that both subclasses could support ligand binding and elicit cellular pathways initializing FcγR-mediated endocytosis via plasmalemmal vesicles. As the nature of a response to FcγR depends primarily on the cell type (Daeron, 1997) one can imagine that FcγR proteins contribute to the immunoregulatory functions of keratinocytes eliciting ligand binding and cell signaling in a cell-specific fashion. Based on our data, the level of FcγRIII expression on human keratinocytes is much lower than its expression on human leukocytes. But IFN-γ treatment induced significant upregulation of FcγRIII on cultured human keratinocytes. So it might be possible that functional FcγRIII contributes to the immunoregulatory capacity of keratinocytes in activating signal transduction and cytokine production in the course of inflammatory and immunologic processes of skin diseases (Barker *et al*, 1991a; Heijnen and van de Winkel, 1997), though its role remains to be clarified.

In summary, we demonstrate expression of FcγRIIIA and FcγRIIIB mRNA in cultured human keratinocytes and its upregulation by IFN-γ. Immunofluorescence and immunoblotting results suggest expression of functional FcγRIII proteins reactive to IFN-γ stimulation. Further *in vitro* and *in vivo* studies are necessary to elucidate the functional relevance of FcγR expression by human keratinocytes.

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