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Role of Complements C5, C6 in the Pathogenecity of Psoriasis

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Abstract

Background: Psoriasis lesion/scale contains C5a des Arg and C5b-9 (Takematsu *et al*., 1992; Terui *et al*., 2000 and Uyemura *et al.*, 1993). These activation products may have arisen from C5-C9 produced supposedly by keratinocytes (KC). In this work we have started with C5 and C6 to prove our hypothesis. Since psoriatic lesions contain several pro-inflammatory cytokines, it is important to find out which pro-inflammatory cytokines can differentially regulate the expected synthesis of C5 and C6 by keratinocytes.

Methods: Human KC have been cultured in the absence and the presence of varying concentrations of pro-inflammatory cytokines and the synthesis of C components C5 and C6 have been measured by ELISA at the protein level and RT-PCR at the mRNA level. To test whether KC also secrete these C components, the same measurements have been performed to find out if these late components are present in the supernatant of the medium where these KC were cultured. The keratinocytes cell-line A431 was also used and the monocytes cultures were considered as the positive control.

Results: The results showed that resting KC synthesize C5 mRNAs in detectable amounts. C5 mRNA which is synthesized by resting KC is not translated into detectable amount of protein. Although resting KC did not produce C6 mRNA in detectable amounts the levels of C6 protein were detectable. However, These C6 protein levels were minimally secreted by resting KC, into the culture medium. TCGF induced the secretion of C5 and C6 while TGF-β induced only the secretion of C6.

Conclusion : Normal KC synthesize their own C5 and C6. The synthesis of them is activated by TCGF. While TGF- ²ج activated the synthesis of C6 other factors might be responsible for activating the synthesis of C5. These factors could be secreted from other cell types than KC in human skin

Introduction

Psoriasis is a T-cell immune-mediated dermatitis (Tagami *et al*., 1997). Deposition of complement (C), has been seen in very early of psoriasis (Terui *et al*., 1987). This C system was found in an active state in psoriasis skin since C fragments (C3a, C4a, C5a and C4d), factors (Bb) and late complex (C5b-9) were present in high levels in the lesions (Takematsu *et al*., 1992; Terui *et al*., 2000 and Uyemura *et al.*, 1993). It has been shown that C3, factor B and factor H are produced by interferon (IFN)-γ-stimulated cultured human epidermal keratinocytes (KC) (Basset-Seguin *et al*., 1990; Ozawa *et al*., 2005 and Timar *et al*., 2006). It has also been demonstrated that IFN-γ and tumour necrosis factor TNF-α can augment the production of C3 by human cultured epidermal KC (Kaneko *et al*., 1980; Purwar *et al*., 2006 and Terui *et al*., 1997). Therefore, it is postulated that an abnormal production of C components in lesional skin is involved in the acute inflammation occurring in the epidermis in psoriasis, under the influence of various cytokines including IFN-γ and TNF-α (Kaneko *et al*., 1980; Miura *et al*., 1985; Purwar *et al*., 2006 and Takematsu *et al*., 1986). However, while KC are known to produce

C3, factor B and factor H, the origin of C5b-9 in the psoriatic lesion is not known. Tissue cells other than KC (i.e. glomerular epithelium, neurons, endothelial cells of human umbilical vein and alveolar epithelial cells) have been shown to produce and release the late components of C (Johnson *et al*., 1991; Strunk *et al*., 1988; Thomas *et al*., 2000 and Zhou *et al*., 1993). Therefore, it is interesting to know whether KC are able to synthesize and release the late components of C under the effect of different pro-inflammatory cytokines.

Keratinocytes in normal skin do not express MHC class II molecules but do express them in psoriatic lesion. We suggest that superantigens present in the psoriatic skin (Telfer *et al*., 1992) may interact with MHC class II molecules on MHC class II^+ KC and cause the release of different cytokines/chemokines in psoriatic lesion.

Material and Methods

Chemicals and reagents

Human recombinant cytokines IFN-γ, IL-1α, IL-2, IL-6, TGF-β1 and TNF-α were purchased from Boehringer Mannheim (Mannheim, Germany). Polyclonal (goat) anti-human C5, C6, C7 and C9 and monoclonal (murine) anti-human C6 IgG1k, C7 IgG1k antibodies were obtained from Quidel Corporation (San Diego, CA, US). The polyclonal goat anti-human (whole antisera) C5, C6, C7 and C9 were biotinylated as follows: The antibody preparation was diluted in PBS (GibcoBRL) to a final protein concentration 1 mg/ml and dialysed overnight at 4^oC against 0.1 *M* $NaHCO_3$ (pH 8.0, Merck & Co. Inc., NJ, US). Then, 60 µL of 1mg/mL biotin (Sigma, St. Louis, MO, US) in DMSO (Sigma) was added to each milligram of antibody and incubated for 2 h at room temperature with occasional stirring. The unbound biotin was removed by overnight dialysis at 4^oC against PBS. Human complement serum standard was purchased from Sigma. Normal human serum (NHS) was a pool of serum obtained from 6 healthy volunteers, stored in aliquots at -70°C. Supernatant of activated mononuclear cells was prepared from stimulated peripheral blood mononuclear cells as described by (Miltenburg *et al*., 1988). Briefly, peripheral blood mononuclear cells (PBMC; 50 x 10^6/mL) from normal donors were stimulated for 2 h at 37°C with 0.1 µg/ml PMA in IMDM (supplemented with 10% heat-inactivated fetal calf serum (FCS), 100 U/mL penicillin and 100 µg/ml streptomycin). PMA-treated cells were washed extensively and cultured (50 x 10^6/ml) for 48 hr with 15 µg/mL ConA in supplemented IMDM. Con A was neutralized by addition of 50 mM α methyl-mannoside for 30 min at 37°C. Supernatant was obtained by centrifugation. Cytokines, supernatant of activated mono-nuclear cells and neutralizing antibodies were aliquoted in small portions and stored at -20°C and diluted in keratinocyte serum free medium (keratinocyte SFM; GibcoBRL, Breda, The Netherlands) just before use. The sources of other chemicals and reagents are indicated below.

Cells

a) Keratinocyte and A431 cultures

Human keratinocytes were isolated by incubation of foreskin with thermolysin (0.50 mg per ml, Sigma) at 4^oC for 16 hr and subsequent trypsinization (0.025%) for 5 min at 37^oC. Trypsin (Sigma) was then neutralized by an excess volume of heat inactivated fetal calf serum (GibcoBRL, Breda, The Netherlands). Cells were separated from debris by filtering through a nylon mesh, centrifuged, and resuspended in keratinocyte SFM containing supplements (Bovine Pituitary Extract (20-30 µg /ml), rEGF (0.1-0.2 ng/ml)) and gentamicin (100 IU per mL, 50 µg per mL; GibcoBRL). A431 cells (American Type Culture Collection, Manassas, VA, US) and were cultured in keratinocyte SFM medium containing supplements and gentamycin (100 IU per ml, 50 µg per ml). The keratinocytes and A431 cells were plated onto 100 mm plastic Petri dishes at a density of 400,000 cells per Petri dish and were incubated at 37^oC in humidified, 5% CO_2, tissue culture incubator. Medium was

changed every 2-3 d, and at 60-80% confluence, cultures were split after a 5 min exposure to 0.025% trypsin, 1.5 mM EDTA and recultured. In experiments where no stimulus was used, both culture supernatant and lysate were obtained from cultures in passage 1 up to 5 when cells had reached approximately 70% confluence. After collecting the cell supernatant, cell lysate was obtained by adding 5 ml fresh culture medium and freezing and thawing the cells 3-4 times followed by centrifugation. For use in other experiments, keratinocytes were seeded in 6 well tissue culture plates (Costar) at a density of 100,000 cells per well in 2000 µl of medium. Cells in passage 2-5 were used for experiments when 60-80% confluence was achieved. Cells in representative wells were counted by a hemacytometer before the experiment and cells in all wells were counted after finishing the experiment.

b) Monocytes

Monocytes were isolated from the buffy coat (Central Laboratory of blood Transfusion Services (CLB), Amsterdam, The Netherlands) as described (Catharien *et al*., 1997). First, the peripheral blood mononuclear cells (PBMC) were isolated from the buffycoat by density centrifugation on lymphoprep (Nycomed, Torshov, Norway). Subsequently, PBMC were centrifuged on a percoll (Pharmacia, Uppsala, Sweden) gradient, consisting of three density layers (1.076, 1.059 and 1.045 g/ml). The light density fraction contains monocytes. The isolated monocytes were cultured in IMDM/1% FCS medium. Monocytes were plated onto 100 mm plastic Petri dishes at a density of $10/10^6$ cells per Petri dish and were incubated at 37°C in humidified, 5% CO_2, tissue culture incubator for 6 hours. For stimulation, monocytes were treated with LPS at a final concentration of 100 ng/ml.

ELISA for measurement of C5 and C6

The concentrations of C5 and C6 in culture supernatant and keratinocyte lysate were estimated by an ELISA method that we have developed ourselves.

C5 was assayed as follows. Wells of 96 well flat-bottom micro titer plates were coated with 100 µl of 5.2 µg of polyclonal goat anti-human C5 per mL in carbonate buffer (pH 9.6) overnight at 4°C. After thorough washing with Tween-80 (0.05%) (Sigma) in PBS the wells were blocked for 1 hr at room temperature with 150 µl of PBS containing 2% bovine serum albumin (Sigma) and 0.05% Tween-80. Washing was repeated and wells were incubated with 100 µl of sample, diluted in the same buffer that was used for blocking. Plates were incubated for two hours at room temperature. The wells were then washed and incubated with 100 µl of biotinylated goat anti-human C5 for 1 hr at room temperature. After washing, the wells were incubated for another h at room temperature with 100 µl of streptavidin conjugated to poly HRP (CLB, Amsterdam, The Netherlands). After washing, the wells were incubated with 100 µl 3,3',5,5' tetramethylbenzidine (Sigma) in dimethylsulfoxide (Merck)-citrate buffer for 10 min. The reaction was stopped with 100 µl H_2SO_4 (2 M). Optical density (OD) was measured at 450 nm.

C6 was assayed essentially as C5 except that wells were coated with 100 µl of 2 µg of monoclonal murine anti-human C6. The detecting antibody was biotinylated goat anti-human C6.

Standard curves for C5 and C6 were made using Human Complement serum standards or NHS of predetermined C5 and C6 concentrations.

Isolation of RNA and semi-quantitative reverse-transcriptase polymerase chain reaction

Total RNA was isolated from human keratinocytes, HepG2 and A431 cells grown in 100 mm Petri dishes using Trizol (Life Technologies, Paisley, UK). Total RNA was isolated from frozen pieces of normal human liver (obtained as a gift from Dr. Ruurdje Hoekstra, Lab. Exp. Hep, AMC, The Netherlands) by crashing and using Trizol. The RNA pellet was dissolved in RNAse-free water and the amount of

RNA was determined by a spectrophotometer at 260 nm and 280 nm.

Reverse-transcriptase polymerase chain reaction (RT-PCR) for the detection of C5 and C6 was carried out as follows. Five micrograms of the extracted total cellular RNA was reverse transcribed in a reaction volume of 20 µl and 1 µl of the resulting cDNA solution was used to amplify cDNA by specific PCR. The PCR were performed in 50 µl per well in polyethylene reaction tubes and applying cycles consisting of denaturation step at 94°C for 30 seconds, annealing for 1 min at 59°C for C5 at 57°C for C6 and extension for 1 min at 72°C. The PCR incubation mixture in a total volume of 50 µL contained 50 mM KCl, 10 mM Tris-HCl pH 8.1, 2.0 mM $MgCl_2$, 0.01% gelatin, 1.25 unit Taq polymerase (Gibco), 250 µM dNTP mix (Pharmacia, Uppsala, Sweden), and 140 ng of the sense and anti-sense primer each. The following specific primer sets were used: C5 forward primer 5`-AAATGTTGT GTCGTCGCAAG-3` (nt 2200-2219), and C5 reverse primer 5`-GTCYGCTTTCCTCAAGGGTA-3` (nt 2893-2874) and C6 forward primer 5`-TCTTGCGTCCCAGTCAGTTT-3` (nt 374-393), and C6 reverse primer 5`-GTTAGACCTTTTACAGCCGA-3` (nt 778-759). GAPDH (positive control) forward primer 5'- CTGAGAACGG-GAAGCTTGTC-3' (nt 254-273), and GAPDH reverse primer 5'- TGGTGGTT-GACGAATCGTGG-3' (nt 526-545).

To confirm purity of the keratinocyte cultures, several cDNA samples obtained after reverse transcription of keratinocyte RNA were checked for the presence of non-keratinocyte cDNA using the following primers: Tyrosinase forward primer 5'-AATGAAAAATGGATCAACACCC-3' (nt 976-997), and tyrosinase reverse primer 5'-GTTTCCAGGATTACGCCGTA-3' (nt 1392-1411); HLA-DR forward primer 5'-GCCAACATAGCTGTGGACAA-3' (nt 283-302), and HLA-DR reverse primer 5'-ATAATGATGCCCACCAGACC-3' (nt 706-725); CD18 forward primer 5'-GACTCCATTCGCTGCGACAC-3' (nt 148-167), and CD18 reverse primer 5'-CACGGTCTTGTCCACGAAGG-3' (nt 485-504)(Muller, *et al*. 1994); CD3 forward primer 5'-CTCCATCTCTGGAACCACA-3' (nt 167-186), and CD3 reverse primer 5'-GTTCGCATCTTCTGGTTTGC-3' (nt 362-382) and parathyroid hormone receptor (PTH-R) forward primer 5'-CAATGAG-ACTCGTGAACGGG-3' (nt 553-572), and (PTH-R) reverse primer 5'-AAGTTGA-GCACAATGGAGGC-3' (nt 1133-1152) (Timmerman *et al*., 1996). Each PCR product (12.5 µL) was mixed with 5 µL stop layer mix and run on a 1.7% agarose gel in tris/borate/EDTA buffer. After electrophoresis, the gel was scanned by an Eagle Eye imager (Stratagene Europe, Amsterdam, The Netherlands) and the signal strength was integrated to obtain a densitometric value for each amplification product.

Statistical analysis

Statistical analysis was performed using a Student *t*-test for data from ELISA experiments. A p-value of less than 0.05 was considered significant.

Results and Discussion

Human keratinocytes constitutively release C6 but not C5

Supernatants of keratinocytes cultured for 72 hr were completely devoid of C5 as determined by ELISA. This indicated that keratinocytes probably do not constitutively release C5. Keratinocytes, however, released small amounts of C6 in culture medium as determined by ELISA (Fig 1). When keratinocytes were cultured up to passage five and the release of C6 was monitored in the culture supernatant of each passage, no significant increase with increasing number of passages was observed in cultures derived from three differen foreskins (Fig 1). We used only cultures from passage 2 to 4 in subsequent studies.

Mediators released from activated mononuclear cells up-regulate the release of C5 and C6 from keratinocytes

Keratinocytes were cultured for 72 h in the presence of increasing concentrations of supernatant of activated mononuclear cells and the release of C5 and C6 in culture medium was monitored by ELISA. Three

cultures of keratinocytes were analyzed, each in triplicate. In all cultures, supernatant of activated mononuclear cells induced a dose dependent increase in C5 and C6 (Fig 2 and Fig 3).

The supernatant of activated mononuclear cells is known to contain a number of cytokines, including IL-lα, IL-2, IL-6, TNF-α and IFN-γ (van den Dobbelsteen *et al*., 1994). It also contains TGF-β (). To find out if any of these cytokines mimics the effect of supernatant of activated mononuclear cells on the synthesis of C5 and C6, we tested the effect of recombinant forms of these individual cytokines on the expression of C5 and C6 by keratinocytes in a 72 hour culture in concentrations known to be effective in inducing the synthesis of other complement components (Pasch *et al*., 1999 and 2000). These concentrations were 0-200 U/mL for IL-1α, 50-1000U/ml for IL-2, 01000 U/ml for IL-6, 0-10 ng/ml for TGF-β, 0-1000 U/ml for TNF-α and 0-100 U/ml for IFN-γ. None of these cytokines showed up-regulation of C5 (Fig 3A) while TGF-β seems to be responsible for induction of C6 (Fig 3B).

The batches of FCS, supernatant of activated mononuclear cells, and keratinocyte medium did not show C5 or C6 reactivity in the respective ELISA assays.

Keratinocytes constitutively express detectable levels of C5 but not C6 transcript

Cultured keratinocytes were tested for their purity by RT-PCR. Primers specific for HLA-DR were used to test the contamination of macrophages, dendritic cells, B-cells; specific for CD3 for contamination of T-cells, specific for tyrosinase for contamination of melanocytes and specific for parathyroid hormone receptor (PTH-R) for contamination of dermal fibroblasts. Using these primers, RT-PCR was performed on different passages of keratinocyte cultures. All cultures in first passage were found to be positive for one or more contaminating cell type but most cultures in third passage onwards were devoid of any contaminating cells (results not shown). Only those keratinocyte cultures that were found to be negative at 36 cycles in a qualitative RT-PCR for tyrosinase, HLA-DR, CD3, PTH-R, and CD 19 were used in subsequent experiments.

As shown in Fig 4, specific transcript for C5 was detected by RT-PCR in unstimulated keratinocytes and A431 cells. This transcript was strongly expressed in nonstimulated monocytes, which were included as positive controls. The size of the product coincided with the predicted base pair (693 bp). Such specific transcripts were found in all samples examined - several cultures of keratinocytes from different foreskins, three different passages of A431 cells, and several preparations of non-stimulated monocytes - demonstrating that these cells constitutively express C5 mRNA.. No signals were found in the negative control water blanks indicating that the results obtained were not due to crossover or DNA contamination. Finally, PCR amplification of GAPDH mRNA (predicted basepair: 292 bp) after 35 cycles is also shown as a positive control for equivalent loading and integrity of the RNA preparations used in the analysis.

As shown in Fig 4, cDNA derived from monocytes which was used as one of the positive controls, after PCR amplification, showed a band of smaller size (300 Bp) than the predicted size (414 Bp). Similar results were obtained with several preparations of RNA from monocytes. Sequencing of the smaller product showed 100% homology with C6 cDNA from nucleotide to nucleotide which was devoid of exon 4 region. Thus smaller product represented region spliced variant of C6. It is not known whether other spliced variants of C6 also exist. C6 RT-PCR product of only smaller size was detected in stimulated and non-stimulated monocytes and A431 cells. C6 RT-PCR product of neither larger nor smaller size could be detected in keratinocytes. Such specific smaller transcripts were found in all samples examined – several preparations -of stimulated and non-stimulated monocytes, three different passages of A431 cells, and several cultures of keratinocytes from different foreskins -

demonstrating that these cells constitutively express region spliced variant of C6 mRNA.. No signals were found in the negative control water blanks indicating that the results obtained were not due to crossover or DNA contamination. Finally, PCR amplification of GAPDH mRNA (predicted basepair: 292 bp) after 35 cycles is also shown as a positive control for equivalent loading and integrity of the RNA preparations used in the analysis.

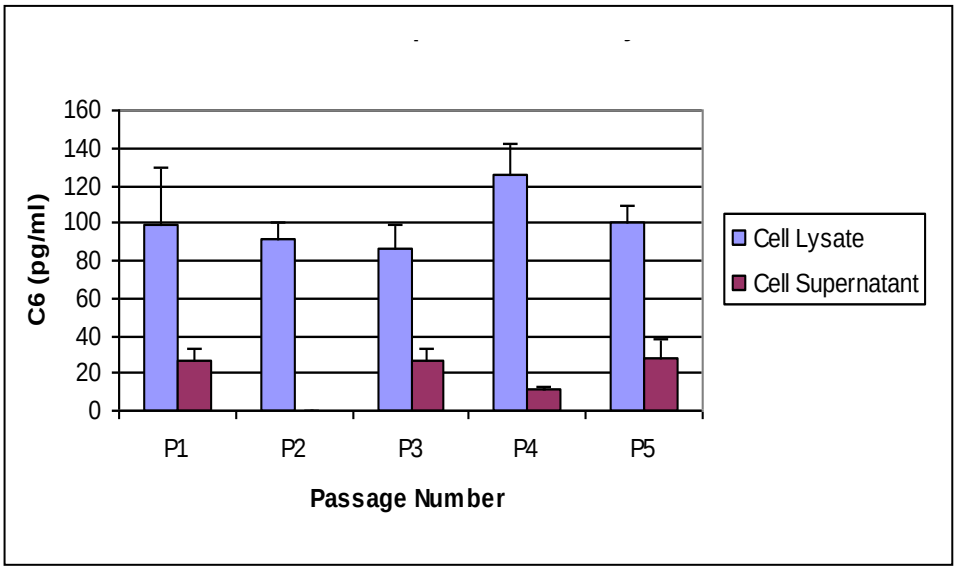


Fig 1. C6 in cell supernatant and lysate

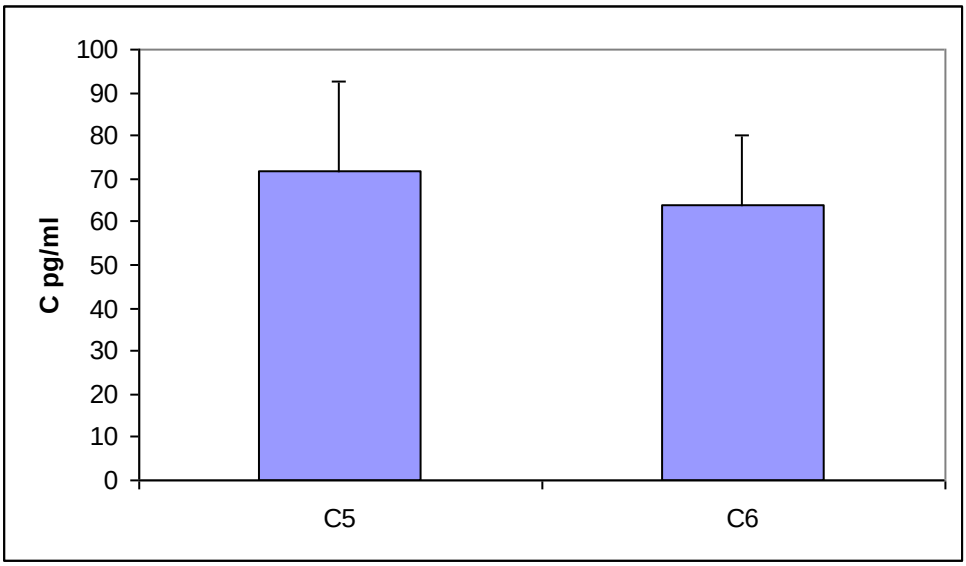


Fig 2. C5 and C6 in supernatant of KC treated with 10% TCGF

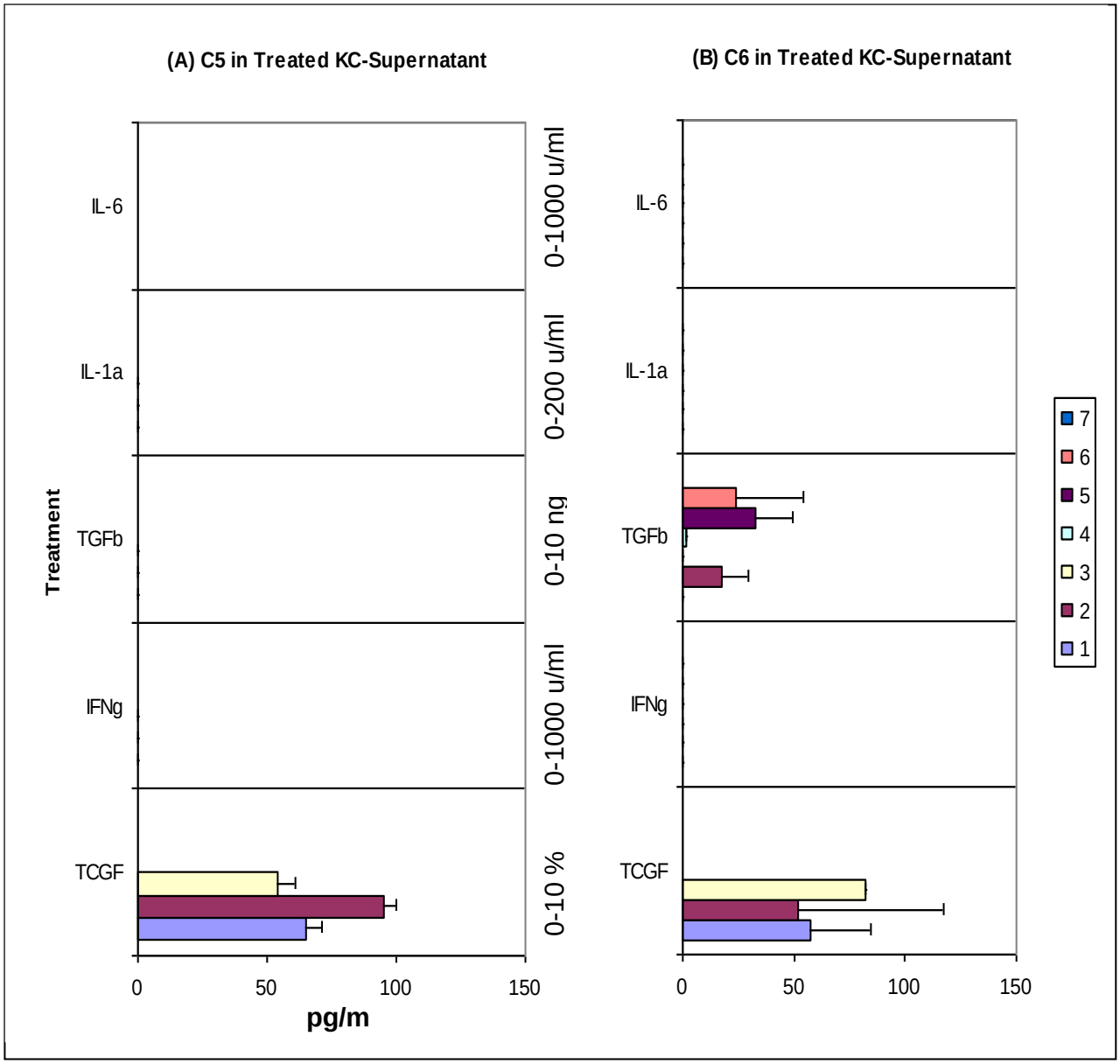
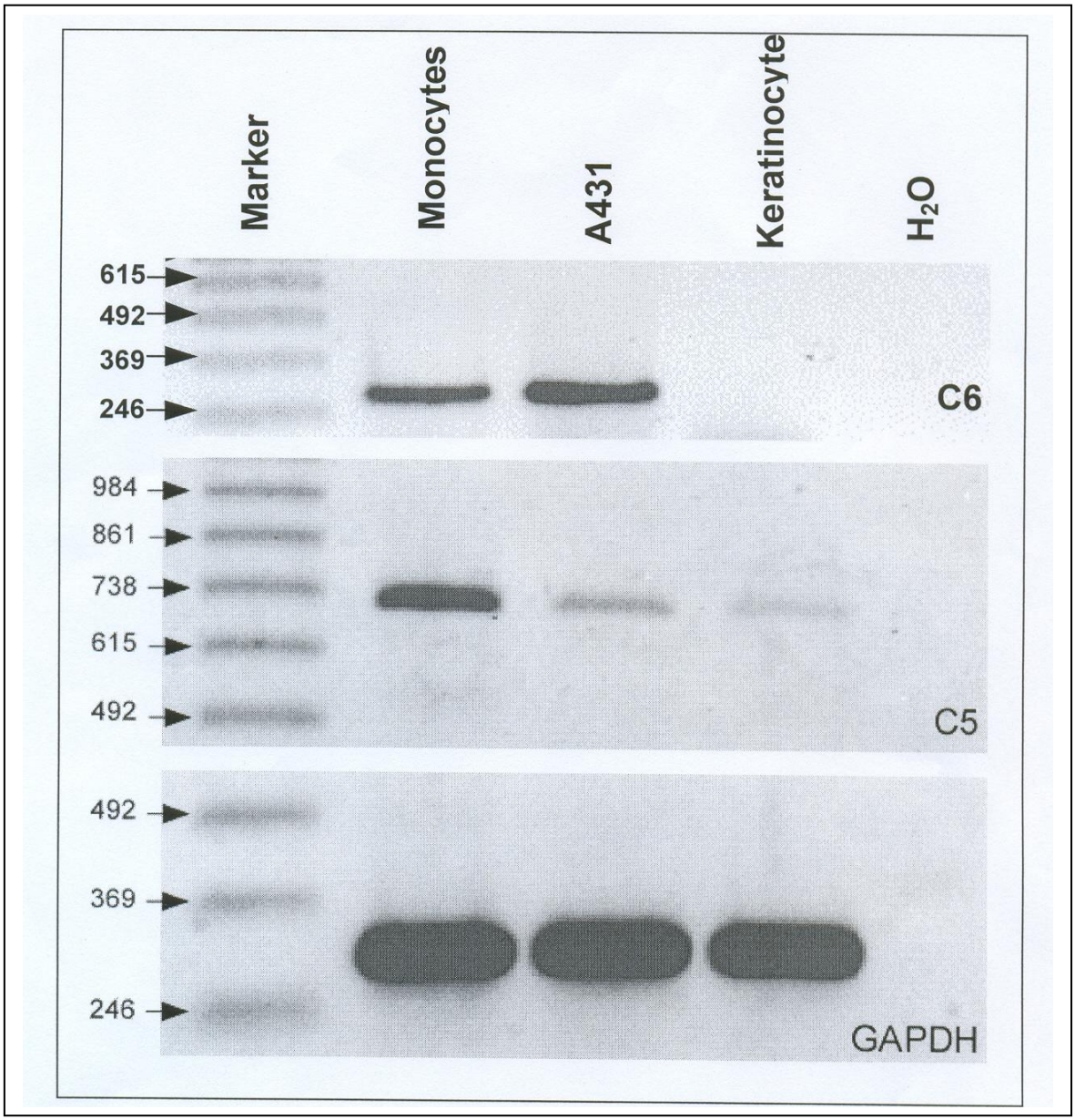


Fig 3. C5 (A) and C6 (B) in the supernatant of treated KC



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دور المتمم C5, C6 فى نشأة مرض الصدفية

فايز محمد أحمد شلضوم
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يحتوى الجلد المصاب بالصدفية على المركبات المتأخرة النشطة من بروتينات نظام المتمم, وليس هناك من يعلم إذا كان مصدر هذه البروتينات هو خلايا الجلد نفسها أم أنها قد أتت مع الدم من خارج الجلد, هناك دراسات سابقة أثبتت قدرة خلايا جلد الإنسان على تكوين وإفراز البروتينات المبكرة فى نظام المتمم C,

وتهدف هذه الدراسة إلى إثبات ما إذا كانت خلايا جلد الإنسان قادرة أيضا على تكوين وإفراز البروتينات المتأخرة فى نظام المتمم والتى يعتمد عليها هذا النظام فى مهاجمة الميكروبات, وقد بدأنا بالمتمم C5 و المتمم C6 فى هذه الدراسة لإثبات هذه الفكرة,

وكذلك عمدت هذه الدراسة فى حالة اثبات ما سبق, اختبار المحفزات المناعية القادرة على زيادة افراز خلايا الجلد للمتمم,

وقد تم دراسة نسخ جزيئات الحامض النووى الريبوزى الرسول mRNA لكلاً من المتمم C5 والمتمم C6 داخل خلايا الجلد المستزرعة بالمعمل باستخدام تقنية RT-PCR, ودراسة تكوين وافراز بروتينات المتمم C5 و المتمم C6 باستخدام تقنية ELISA,

وقد أثبتت هذه الدراسة قدرة خلايا الجلد على انتاج نسخ mRNA للمتمم C5 بكميات يمكن قياسها على الرغم من أنها لم تترجم إلى كميات كافية من البروتين يمكن قياسها, وعلى العكس من ذلك فإنه لم يمكن قياس نسخ mRNA للمتمم C6 عل الرغم من أن كمية انتاج خلايا الجلد من هذا البروتين كانت واضحة إلا أن كمية افرازه خارج الخلايا إلى وسط الإستزراع كانت قليلة,

كذلك أثبتت هذه الدراسة قدرة TCGF على تحفيز انتاج كلاً من بروتينات المتمم C5والمتمم C6 فى حين أن TGFb كان قادراً على تحفيز انتاج C6 فقط,