Regulation of expression of complement components, complement regulatory proteins, and chemokines in keratinocytes
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Transforming Growth Factor-β isoforms regulate the surface expression of membrane cofactor protein (CD46) and CD59 on human keratinocytes

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Abstract

We studied the regulation of the expression of complement regulatory proteins, membrane cofactor protein (MCP), decay accelerating factor (DAF) and CD59, on human keratinocytes by supernatant of activated mononuclear cells and by some individual cytokines present therein. Cultured keratinocytes expressed MCP, DAF and CD59. Supernatant of activated mononuclear cells and recombinant forms of transforming growth factor (TGF)-β variants (β1, β2 and β3) up-regulated MCP and CD59 but not DAF. Recombinant IL-1α, IL-2, IL-6, TNF-α and IFN-γ had no influence. TGF-β present in the supernatant was likely responsible for up-regulation of MCP and CD59. A monoclonal anti-TGF-β antibody, which neutralized TGF-β1, -β2 and -β3 did not inhibit the up-regulation of MCP and CD59 by the supernatant. These results indicated that TGF-β and an additional factor(s) present in the supernatant may be responsible for up-regulating the expression of MCP and CD59 on keratinocytes; both may be acting non-synergistically.
Chapter 3

INTRODUCTION

The complement system is constantly activated in vivo and may potentially damage autologous cells. The complement-mediated damage of autologous cells is, however, prevented by complement-regulatory proteins that are expressed on their surfaces. These proteins include decay accelerating factor (DAF), membrane cofactor protein (MCP) and CD59. They protect cells on which they are expressed by inhibiting different steps of complement activation. DAF combines with C4b and C3b bound to the cell membrane and interferes with the interaction of C2 with C4b and of factor B with C3b. DAF also accelerates the dissociation of C2a from C4b and of Bb from C3b within C3-convertases of classical and alternative pathway, respectively. Thus, DAF interferes with the formation of C3- and C5-convertases of the classical and alternative pathways and accelerates their decay. MCP combines with C4b and C3b bound to the cell membrane and then acts as a cofactor for the enzyme factor I. This enzyme cleaves C4b and C3b and generates the inactive derivatives iC4b and iC3b. Thus, MCP interferes with the assembly of C3- and C5-convertases of both pathways. CD59 binds to an epitope on the α-chain of the α-γ-subunit of C8. This epitope is exposed when C8 is incorporated into the C5b-7 complex on the cell membrane. CD59 also binds to C9 in which a CD59 binding site is exposed after surface adsorption. Thus, CD59 inhibits the transmembrane channel formation by late components by binding to C8 and C9. DAF, MCP and CD59 may be coexpressed on a self cell and act synergistically to inhibit complement activation on it.

Activation of complement on or around keratinocytes occurs in many inflammatory conditions of the skin (e.g. pemphigus, pemphigoid) and poses particular risk for keratinocytes to be damaged. Since complement-regulatory proteins are endowed with the task of protecting keratinocytes during complement-mediated inflammation, there should exist a mechanism(s) which can up-regulate their expression on the keratinocyte surface during complement attack. This mechanism could be as follows. complement attack can release several cytokines from keratinocytes as has been shown with many cell types and some of these can up-regulate complement-regulatory proteins which can provide increased protection to keratinocytes against complement attack. Cytokines capable of up-regulating complement-regulatory proteins on keratinocytes may also be released by inflammatory cells that infiltrate the epidermis during complement-mediated inflammation.

In the present study we investigated the regulation of expression of DAF, MCP and CD59 on human keratinocytes by supernatant of activated peripheral blood mononuclear cells and by several individual cytokines known to be present therein. In our laboratory TGF-β was also found to be present in the supernatant. We therefore included recombinant forms of TGF-β
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variants, i.e. TGF-β1, TGF-β2 and TGF-β3, in this study. Some artificial stimuli of protein kinase C (PKC) and protein kinase A (PKA) were also studied to see if their effects mimic the effects of TGF-β in the supernatant of mononuclear cells.

MATERIALS AND METHODS

Chemicals and reagents

Dibutyryl-adenosine-cyclic-monophosphate (cAMP), dibutyryl-guanosine-cyclic-monophosphate (cGMP), phorbol 12-myristate 13 acetate (PMA), calcium ionophore A23187, concavalin A, dimethylmannoside, normal mouse IgG and human recombinant TGF-β3 were purchased from Sigma (St. Louis, MO), Penicillin/streptomycin, keratinocyte serum free medium (keratinocyte SFM), Iscove’s modified Dulbecco’s medium (IMDM) and fetal calf serum (FCS) were obtained from Gibco BRL (Breda, The Netherlands). Human recombinant IL-2 and isotype control mAb (clone 203) were purchased from the Central Laboratory of Blood Transfusion Services (Amsterdam, The Netherlands). Human recombinant cytokines IFN-γ, IL-1α, IL-6, TGF-β1, and TGF-β2 were purchased from Boehringer Mannheim (Mannheim, Germany). Human recombinant TNF-α was purchased from Genzyme (Genzyme, Cambridge, MA). FITC-labeled mouse anti-human MCP IgG1 (clone 122-2) and FITC-labeled mouse anti-human DAF IgG1 (clone Brie 110) were obtained from Instruchemie (Hilversum, The Netherlands). Anti-TGF mAb (clone 2G7) was a gift from Dr. Stevin Schoenberger (Department of Immunohematology, University of Leiden, Leiden, The Netherlands). Mouse anti-human CD59 IgG1 (1F5) was a kind gift from Dr. Noriko Okada (Nagoya City University School of Medicine, Nagoya, Japan) and was biotinylated according to routine technique.

Supernatant of activated mononuclear cells was prepared as described. Briefly, peripheral blood mononuclear cells (PBMC; 50 x 10⁶/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 FCS, 100 U/ml penicillin and 100 μg/ml streptomycin). PMA-treated cells were washed extensively and cultured (50 x 10⁶/ml) for 48 h with 15 μg/ml Con A in supplemented IMDM. Con A was neutralized by addition of 50 mM α-methylmannoside for 30 min at 37°C. Aliquots were stored at -20°C.

Keratinocyte culture

Human keratinocytes were isolated by incubation of foreskins with 0.50 mg/ml thermolysin (Sigma) at 4°C for 16 h and subsequent trypsinization (0.025%) for 5 min at 37°C. Trypsin (Sigma) was then neutralized by FCS. Cells were separated from debris by filtering, centrifuged and resuspended in keratinocyte SFM supplemented with 100 IU/ml penicillin/100 μg/ml streptomycin. The keratinocytes were plated onto 100 mm plastic Petri dishes at a density of 400,000 cells per Petri dish and were incubated at 37°C in a humidified, 5% CO₂, tissue culture incubator. Medium was changed every 2-3 days, and at 70% confluency cells were split with trypsin (0.25%) and recultured. For use in flow cytometry experiments, cells were seeded in 6-well tissue culture plates at a density of 100,000 cells per well. Cells in passage 2-5 were used for experiments as soon as 60-80% confluency was achieved. For studying the effect of various additives, cells were incubated in 1500 μl medium alone or 1500 μl medium containing the additive.

Immunohistochemistry

Keratinocytes were grown for 48 h on a microscopic slide, rinsed in PBS and then fixed for 10 min using a 3% paraformaldehyde solution in PBS. Endogenous peroxidase activity was inactivated by 30 min incubation in
0.3% H₂O₂ in PBS. The immunohistochemical staining was then performed by a three-step immunoperoxidase technique²³. The slides were incubated with 20 μg/ml monoclonal anti-DAF, 2 μg/ml anti-MCP or 10 μg/ml anti-CD59 antibody or 20 μg/ml mouse IgG1 (DAKO A/S, Glostrup, Denmark) for 60 min followed by incubation for 60 min with a 1:200 dilution of a biotinylated rabbit anti-mouse Ig (DAKO) in PBS/human AB serum (10%; DAKO). They were then incubated for 30 min with horseradish peroxidase-labeled polystreptavidin (ABC Complex, DAKO). Peroxidase activity was visualized by incubation of the slides with 0.05% 3-amino-9-ethylcarbazole in acetate buffer for 10 min followed by a counterstaining with hematoxylin for 20-30 s. All incubations were at room temperature.

Flow cytometry

Keratinocytes were detached by a trypsin solution (0.025% trypsin, 1 mM EDTA) for 3-5 min. This treatment did not affect DAF, MCP and CD59 as preliminary experiments with combinations of different concentrations of trypsin and EDTA had shown. Trypsin was inactivated by FCS and cells were washed and resuspended in FACS buffer (PBS, 2% FCS, 0.1% sodium azide). Approximately 10⁵ cells were incubated with biotinylated anti-CD59 mAb or the biotinylated isotype control for 30 min at 4°C. Cells were washed twice and incubated for 30 min with streptavidin-RPE-CY5 (DAKO). Cells for analysis of MCP and DAF were incubated with FITC-labeled specific antibodies or isotype control for 30 min at 4°C. Cells were washed twice and 10,000 cells were analyzed immediately using a FACSCalibur (Becton Dickinson, San José, CA).

MFI was calculated with WinMDI software. The MFI values of untreated cells were taken as 100%. The MFI values obtained from cells treated with a test material (e.g., cytokine) are presented in terms of percent of untreated cells.

ELISA for the measurement of TGF-β

The concentration of TGF-β in the supernatant of activated mononuclear cells was estimated by a sandwich ELISA as described²⁴. Briefly, wells were coated with 2 μg/ml mouse anti-human TGF-β (2G7; described above) in PBS by incubating the plates for 1 h at 37°C and then overnight at 4°C. After washing, wells were blocked for 1 h at room temperature with PBS, 0.2% Tween-20, 2% bovine serum albumin (BSA). Washing was repeated and samples were added in triplicate to the wells for 2 h at room temperature. After washing wells were incubated with chicken anti-human TGF-β antibodies coupled to digoxigenin (DIG) for 1 h at room temperature. Plates were washed and incubated with anti-DIG F(ab')² fragments conjugated with horseradish peroxidase for 1 h at room temperature. Peroxidase activity was visualized by addition of the peroxidase substrate 2,2-azino-bis-3-ethylbenzthiazolin (Sigma) prepared in a 100 mM citrate/100 mM phosphate buffer, pH 4.2. Optical density was measured at 415 nm. A standard curve for TGF-β was made using human rTGF-β1 as standard for every plate. TGF-β was activated by pH.

Statistical analysis

Statistical analysis was performed using the Student's t-test for paired samples and a p value of less than 0.05 was considered significant.
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RESULTS

Cultured human keratinocytes express MCP, DAF and CD59

Previous in situ immunohistochemical studies in our laboratory have demonstrated that MCP, DAF and CD59 are present on several structures on human skin including keratinocytes. When the expression of MCP, DAF and CD59 on cultured keratinocytes was studied immunohistochemically, all the three proteins were found to be present on their membranes (Figure 1). Further flow cytometric analysis confirmed that all these complement-regulatory proteins were expressed on nonstimulated keratinocytes (Figure 2).

Expression of MCP and CD59 on human keratinocytes is up-regulated by mediators released from activated mononuclear cells

Complement-mediated inflammation is almost invariably associated with infiltration by inflammatory cells. Therefore it was considered likely that mediators released by inflammatory cells may have profound effects on the expression of complement regulatory proteins on keratinocytes. We analyzed the regulation of surface expression of MCP, DAF and CD59 by supernatant of mononuclear cells activated as described in ‘Materials and Methods’. As shown in Figure 2, supernatant at a final concentration of 10% caused an up-regulation of MCP and CD59 but not of DAF. Time-response studies with 10% supernatant (data not shown) revealed that there was no up-regulation of MCP in the first 24 h and only slight up-regulation of CD59 at 24 h; up-regulation of both was maximal at 72 h and 144 h, respectively.

Figure 1. Surface staining of DAF, MCP and CD59 on cultured human keratinocytes. The cells were stained by an immunoperoxidase technique using primary antibodies to MCP, DAF, and CD59 and IgG1 as isotype control. All views are 40x.
To test if up-regulation of MCP and CD59 in response to supernatant of activated mononuclear cells was due to de novo synthesis, the effect of cycloheximide on the expression and up-regulation of these molecules by the supernatant was studied. Expression of MCP and CD59 in the presence of a predetermined sublethal dose of cycloheximide (2 μg/ml) was lower than in its absence both in supernatant-treated as well as untreated cells (Figure 3). This was probably because cycloheximide not only inhibited supernatant-induced up-regulation but also normal constitutive synthesis of these molecules. The effect of cycloheximide was reversible: expression of MCP and CD59 was restored in cells which after incubation with cycloheximide for 72 h were washed and subsequently cultured for another 72 h (data not shown). These results suggest that up-regulation caused by supernatant was due to de novo synthesis. Cycloheximide also caused a decrease in expression of DAF in absence of supernatant [40% decrease in mean fluorescence intensity (MFI)] and in its presence (50% decrease in MFI). Expression of MCP, DAF and CD59 in the presence of cycloheximide did not reach zero level, likely because pre-existing molecules on the surface of keratinocytes were not catabolized completely during 72 h culture.

Several pro-inflammatory cytokines known to be secreted by activated mononuclear cells do not regulate the expression of MCP, DAF and CD59 in human keratinocytes

Supernatant of activated mononuclear cells contains a number of cytokines, including IL-1, IL-2, IL-6, TNF-α and IFN-γ. One or more of these cytokines could have been responsible for up-regulation of MCP and CD59 caused by supernatant of activated mononuclear cells. We tested the effect of their recombinant forms on the expression of MCP, DAF and CD59.

Figure 2. Expression of DAF, MCP and CD59 on cultured human keratinocytes and their up-regulation by supernatant of activated mononuclear cells. Expression of DAF, MCP and CD59 on 10⁴ keratinocytes was analyzed by flow cytometry after culturing in medium containing 10% supernatant of activated mononuclear cells. Histograms for expression of DAF, MCP and CD59 are shown. Bold lines represent expression in the presence and thin lines in the absence of supernatant. Broken lines represent results obtained with isotype controls. The data are representative of three independent staining experiments. Supernatant at 0-5% concentrations had no significant effect on expression of DAF, MCP or CD59.
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Figure 3. Effects of cycloheximide (CHX) on up-regulation of expression of MCP and CD59 by keratinocytes. Keratinocytes were cultured for 72 h in medium alone (Med) or medium containing 10% supernatant of activated mononuclear cells (Sup), CHX (2 μg/ml) or Sup and CHX. MCP and CD59 expression was determined by flow cytometry analysis and results were expressed as a percentage of MFI value obtained with keratinocytes cultured in medium alone.

Keratinocytes (60-80% confluent) were cultured for 72 h in the presence and absence of 2-200 U/ml IL-1α, 10-1,000 U/ml IL-2, 10-1,000 U/ml IL-6, 5-200 U/ml IFN-γ and 10-1,000 U/ml TNF-α and analyzed for the expression of MCP, DAF and CD59 by flow cytometry. None of these cytokines significantly up-regulated any of the three complement-regulatory proteins (three independent experiments with each cytokine; data not shown).

TGF-β is present in the supernatant of mononuclear cells and can up-regulate the expression of MCP, DAF and CD59 on keratinocytes

After having failed to identify any specific mediator, known to be present in the supernatant of activated mononuclear cells responsible for up-regulation of MCP and CD59, we investigated the presence of TGF-β in the supernatant. TGF-β isoforms TGF-β1 and TGF-β2, are produced by activated macrophages. Measurement of TGF-β in the supernatant of activated mononuclear cells by an ELISA, which measures all the three variants combined, showed that TGF-β was present in the supernatant at a concentration of 3.5 ng/ml.

To find out whether TGF-β variants in concentrations present in the supernatant (0.35 ng/ml in 10% supernatant) could be responsible for up-regulating MCP and CD59, the ability of recombinant TGF-β1 and TGF-β2 (0.1-10 ng/ml) to up-regulate MCP and CD59 on keratinocytes was investigated. TGF-β3, which is produced by cells of mesenchymal origin but has 79% homology with TGF-β2, was also included in the study. Keratinocytes (60-80% confluent) were cultured for 72 h in the presence and absence of TGF-β1, -β2, or -β3 and analyzed for the expression of MCP, DAF and CD59 by flow cytometry. Like the supernatant, all the three
variants of TGF-β (at 1.0 and 10 ng/ml) up-regulated the expression of MCP and CD59 (Figure 4) but did not affect the expression of DAF significantly (data not shown). In independent experiments TGF-β1 and TGF-β2, at concentrations 0.35 ng/ml and higher, were found to up-regulate MCP and CD59 significantly (data not shown here). TGF-β3 required higher concentrations than 0.35 ng/ml for significant effect. These results suggest that TGF-β1 and/or TGF-β2 was the factor or factors in the supernatant responsible for up-regulation of MCP and CD59.

TGF-β1, TGF-β2 and TGF-β3 mediated up-regulation of MCP and CD59 was blocked by the anti-TGF-β mAb 2G7 (Figure 5). This showed that up-regulation caused by these TGF-β variants was specific. The anti-TGF-β could not block up-regulation of MCP and CD59 induced by supernatant of activated mononuclear cells, even at a concentration of anti-TGF-β as high as 200 μg/ml (Figure 5). This indicated the presence, in addition to TGF-β1 and TGF-β2, of a factor(s) in the supernatant responsible for causing up-regulation of MCP and CD59 on keratinocytes in a non-synergistical fashion.

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*Figure 4. Effect of TGF-β variants on the expression of complement-regulatory proteins by keratinocytes. Expression of DAF, MCP and CD59 on 10^4 keratinocytes after culturing in medium containing the indicated concentrations of TGF-β1, TGF-β2 or TGF-β3 was analyzed by flow cytometry. Bold lines represent MCP or CD59 expression in the presence of TGF-β isoforms. Broken lines show isotype controls. None of the TGF-β isoforms had significant influence on expression of DAF (data not shown). The results are representative of three staining experiments carried out in duplicate.*
Expression of MCP, DAF and CD59 on human keratinocytes is up-regulated by activators of PKC and PKA

Since TGF-β is known to activate PKC, it was interesting to investigate whether activators of PKC (PMA and calcium ionophore A23187) can mimic the effects of TGF-β variants on expression of MCP, DAF and CD59. An activator of PKA, dibutyryl-cAMP, was also included. Keratinocytes (60-80% confluent) were stimulated with these activators for 72 h and analyzed by flow cytometry. PMA (0.5-5.0 nM), calcium ionophore A23187 (5.0-50 mM) and dibutyryl-cAMP (0.1-1.0 mM) caused a dose-dependent increase in surface expression of MCP, DAF and CD59 (Figure 6; data with highest concentrations are shown here). To rule out the effect of dibutylryl part of dibutyryl-cAMP, we used dibutyryl-cGMP as a control. Dibutyryl-cGMP had no effect on the surface expression of MCP, DAF and CD59 (data not shown).

These results indicate that PKC and PKA activation can up-regulate the expression of MCP, DAF and CD59 on human keratinocytes.

Figure 5. Neutralization of MCP- and CD59-up-regulating capability of TGF-β1, TGF-β2 and TGF-β3 but not that of supernatant of activated mononuclear cells by an anti-TGF-β mAb. Keratinocytes were cultured in medium containing 1.0 ng/ml of TGF-β1, TGF-β2 or TGF-β3 or 10% supernatant (Sup) of activated mononuclear cells in the presence or absence of an anti-TGF-β mAb or a control mouse-IgG (M-IgG). Keratinocytes cultured in medium alone were also examined (Med). Expression of MCP and CD59 was determined using flow cytometry analysis. Up-regulation of MCP and CD59 by 1.0 ng/ml of TGF-β1, TGF-β2 or TGF-β3 could be completely neutralized with 20 μg/ml, 100 μg/ml and 200 μg/ml anti-TGF-β antibody, respectively. Up-regulation of MCP and CD59 by 10 % supernatant could not be neutralized by a final concentration as high as 200 μg/ml of the anti-TGF-β antibody. M-IgG did not show neutralizing effects on TGF-β induced up-regulation of MCP and CD59.
DISCUSSION

This study shows that human keratinocytes express complement regulatory molecules DAF, MCP and CD59 on their surface. The co-expression of all these molecules suggests that keratinocytes may possess a high degree of resistance against autologous complement. Indeed, human keratinocytes\(^{38}\) and a squamous carcinoma cell line, SCC-12F\(^{39}\), are remarkably resistant to complement-mediated lysis. In diseases such as pemphigus in which there is a strong complement attack directed against keratinocytes\(^{287}\), keratinocyte death is not a predominant feature. Thus, keratinocytes are strongly protected against autologous complement attack. They need strong protection against complement because epidermis is under a continuous threat of exposure to foreign microbes and other materials which can activate complement and cause bystander complement attack on keratinocytes.
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The protective role of complement-regulatory molecules against autologous complement demands that their expression on keratinocytes be up-regulated in inflammatory conditions of epidermis in which complement attacks keratinocytes. The mechanism of increased expression could be the up-regulation by cytokines produced keratinocytes attacked by C3 or by infiltrating cells at the site of complement-mediated inflammation. To test this, we used supernatant of activated mononuclear cells as a source of inflammatory cell-derived cytokines and investigated its ability to up-regulate the expression of complement-regulatory proteins on keratinocytes. The expression of MCP and CD59 was indeed up-regulated by this supernatant but the expression of DAF was not appreciably altered. Results of experiments with cycloheximide (Figure 3) showed that increased expression of MCP and CD59 was likely due to increased de novo synthesis. If these in vitro findings could be extrapolated to the in vivo situation, this would mean that mediators released from mononuclear cells in inflammatory conditions in vivo will up-regulate MCP and CD59 on keratinocytes. Thus, keratinocytes in areas of inflammation could be better protected from complement-mediated lysis than those present in areas free of inflammation.

Attempts were made to identify the factor(s) present in the supernatant responsible for causing up-regulation of MCP and CD59. IL-1, IL-2, IL-6, TNF-α, and IFN-γ are known to be present in the supernatant of activated mononuclear cells. Recombinant forms of none of these cytokines up-regulated any of the three complement-regulatory proteins on keratinocytes. This suggested the presence of some other active principle in the supernatant. We argued that TGF-β (TGF-β1 and TGF-β2), which is produced by activated macrophages, might be present in the supernatant of activated mononuclear cells and may be responsible for up-regulation of MCP and CD59 on keratinocytes. Further experiments showed that recombinant TGF-β1, TGF-β2 and TGF-β3, like the supernatant, up-regulated MCP and CD59 but not DAF. The concentration of TGF-β found in the supernatant, and the concentrations of TGF-β1 and TGF-β2 required for up-regulation, suggested that TGF-β present in the supernatant may be causing the up-regulation of surface expression of MCP and CD59 on keratinocytes.

Addition of anti-TGF-β in amounts in excess of those needed to neutralize all the TGF-β present in the 10% supernatant did not abolish the up-regulation of MCP and CD59 caused by the supernatant (Figure 5). This suggests that an additional factor other than TGF-β may also be present in the supernatant of activated mononuclear cells which can up-regulate MCP and CD59 in a non-synergistical manner. This may be postulated to be a member of the TGF-β superfamily.

TGF-β variants perform a variety of functions. They are involved in proliferative,
inductive and regulatory effects on a wide variety of cell types. For instance, they regulate the production of acute phase proteins. TGF-β inhibits inflammatory cytokine-induced C3 production in astrocytes. It up-regulates MCP and CD59 in keratinocytes as has been demonstrated in this study. Further studies may show similar effects of TGF-β in other cell types. If so, TGF-β may be assigned the new function of protection of cells against complement attack by inhibiting complement production and up-regulating complement-regulatory proteins on cells.

Activators of PKC (PMA and A23187) of PKA (dibutyril-cAMP) can up-regulate MCP, DAF and CD59. This raises questions such as (1) why IL-1 and TNF-α, which can also activate PKC and PKA, cannot up-regulate any of the three complement-regulatory proteins and (2) why TGF-β, which is known to activate PKC can up-regulate only MCP and CD59. These observations provide rationale to investigate up-regulating and down regulating factors in pro-inflammatory cytokines and TGF-β mediated signalling in keratinocytes.

In conclusion, the expression of MCP and CD59 but not DAF on human keratinocytes is up-regulated by the supernatant of activated mononuclear cells. This supernatant was found to contain TGF-β. TGF-β (β1, β2 and β3) also up-regulates MCP and CD59 but not DAF. TGF-β present in the supernatant could be responsible for up-regulation of MCP and CD59. An additional factor(s) appears to be present in the supernatant which can up-regulate MCP and CD59 on keratinocytes in a non-synergistical manner. Expression and up-regulation of complement-regulatory proteins are important for protection of keratinocytes during complement-mediated inflammation.