Regulation of expression of complement components, complement regulatory proteins, and chemokines in keratinocytes
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Chapter Six

CD40 mediated activation of keratinocytes induces the production of chemokines but not of complement components and complement regulatory proteins

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Abstract

Keratinocytes are CD40 expressing immunocompetent cells. In some inflammatory conditions of the skin, keratinocytes express increased amounts of CD40 and epidermis contains activated T cells which transiently express CD40 ligand (CD40L). It is believed that CD40 on keratinocytes may ligate with CD40L on T cells. This ligation may induce the release of some inflammatory mediators and may contribute to inflammation. We tested by ELISA whether CD40 activation of IFN-γ pre-treated cultured human keratinocytes (CD40+ keratinocytes) by means of CD40L transfected cells or soluble CD40L can result in enhanced production of chemokines IL-8, RANTES and MCP-1 and of complement components C3 and factor B by keratinocytes. We also tested the effect of CD40 activation of CD40+ keratinocytes on the expression of complement regulatory proteins, namely membrane cofactor protein (MCP), decay accelerating factor (DAF), and CD59 by flow cytometry.

CD40 activation of CD40+ keratinocytes up-regulated the release of IL-8 and RANTES greatly, and that of MCP-1 moderately. The production of C3 and factor B and the expression of MCP, DAF, and CD59 was not altered. Specificity of the results with CD40L transfected cells was confirmed using untransfected cells as controls, co-culturing CD40+ keratinocytes and transfected cells with and without physical contact with each other in a Transwell system, and inhibiting CD40 activation with neutralizing anti-CD40 monoclonal antibodies.

In conclusion, CD40 activation on cultured human CD40+ keratinocytes up-regulated their release of the chemokines IL-8, RANTES and MCP-1 without affecting the release of complement proteins C3 and factor B and without altering the expression of MCP, DAF, and CD59. If these in vitro results may be extrapolated to in vivo situations they suggest that an interaction of activated T cells with keratinocytes via CD40-CD40L interaction may play a role in inflammatory conditions of the skin.
INTRODUCTION

CD40 is a 50-kD cell membrane glycoprotein expressed on B-cells, monocytes, dendritic cells and T-cells. The ligand of CD40 (CD40 ligand; CD40L; CD154) is a 35-kD glycoprotein which is transiently expressed on activated CD4+ T-cells, mast cells, eosinophils, and basophils. Both CD40 and CD40L belong to the tumour necrosis factor (TNF) receptor superfamily and have been reviewed extensively.

CD40-CD40L ligation between CD40 on B and CD40L on T cells was shown to play a role in T cell dependent B cell activation and in isotype switching of IgM producing B cells. Absence of CD40-CD40L ligation due to mutations in the CD40L gene in humans results in X-linked hyper-IgM syndrome in which there is deficient isotype switching characterized by lack of circulating IgG and IgA and absence of germinal centers. Recent studies have, however, shown that CD40 is also expressed on non-lymphoid cells. These include fibroblasts, endothelial cells, dendritic cells, mesangial cells, and cortical and medullary thymic epithelial cells. Interaction of CD40 expressed on these cells with CD40L has been shown to cause release of mediators of inflammation and induction of expression of pro-inflammatory cell surface molecules on them.

Recently, human keratinocytes have also been shown to express functional CD40. Keratinocytes are the major cell-type in the epidermis which by virtue of their ability to synthesize and secrete inflammatory mediators such as cytokines and complement components have been recognized as initiators of inflammation in the skin. CD40-CD40L ligation has been shown to activate keratinocytes resulting in the release of IL-6 and IL-8 and induction of proinflammatory cell adhesion molecule ICAM-1. This ligation also enhances the differentiation of keratinocytes and inhibits their proliferation. The role which CD40-CD40L interaction plays in regulation of the production of other inflammatory proteins in keratinocytes is not known.

CD40-CD40L interaction on keratinocytes may be involved in the pathogenesis of some inflammatory conditions of the skin. It has been shown that in lesional skin of patients with psoriasis and atopic dermatitis, CD40 expression on keratinocytes is elevated. In psoriatic lesions, increased CD40 expression in epidermal keratinocytes is associated with increased production of the chemokines IL-8, RANTES, and MCP-1, increased accumulation of complement activation products (e.g. C5a des-arg, C5b-C9), dramatic cellular infiltration, and altered expression of complement regulatory proteins DAF and CD59. It is likely that the interaction of CD40L on activated T cells with CD40 on keratinocytes may be one of the factors responsible for inducing these abnormalities in the epidermal compartment. From this point of view we investigated the release of chemotactic cytokines IL-8, RANTES and MCP-
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1 and of complement proteins C3 and factor B by keratinocytes, which were induced to express optimal amounts of CD40 (CD40+ keratinocytes), in response to CD40L transfected J558L cells and soluble recombinant CD40L. We also investigated the influence of CD40L on CD40+ keratinocytes on the expression of complement regulatory proteins MCP, DAF, and CD59.

MATERIALS AND METHODS

Keratinocyte cultures

Human keratinocytes were isolated by incubation of foreskin with thermolysin (0.50 mg per ml, Sigma, St. Louis, MO) at 4°C for 16 h and subsequent trypsinization (0.025%) for 5 min at 37°C. Trypsin (Sigma) was then neutralized by an excess 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 serum free medium (GibcoBRL) supplemented with 100 IU per ml penicillin/100 μg per ml streptomycin (GibcoBRL). The keratinocytes were plated onto 100 mm plastic Petri dishes at a density of 400,000 cells per Petri dish and incubated at 37°C in humidified, 5% CO2, tissue culture incubator. Medium was changed every 2-3 d and at 70% confluence, cultures were split after a 5 min exposure to trypsin (0.025%)/ethylene diamine tetra-acetic acid (EDTA) (1.5 mM) and recultured. For use in different experiments, cells were seeded in 6 well tissue culture plates (Costar) at a density of 100,000 cells per well in 1500 μl of medium or in 12 well plates at a density of 50,000 cells per well in 750 μl of medium. Cells in passage 2-5 were used for experiments as soon as 60-80% confluence was achieved.

Keratinocyte interaction with CD40L

CD40L transfected J558L hybridoma cells (kindly supplied by Dr. P. Lane, University of Birmingham, Birmingham, UK) and control untransfected J558L cells were cultured in IMDM supplemented with FCS. In all experiments they were irradiated with 50 Gy and washed extensively before use. For ligation studies, subconfluent keratinocyte cultures were always pre-treated with IFN-γ (10 U per ml; Pharma Biotechnology Hannover, Hannover, Germany) for 72 h at 37°C in a humidified, 5% CO2 cell culture incubator. These cells pre-treated with IFN-γ expressed high levels of CD40 as described in ‘Results’ and were referred to as CD40+ keratinocytes throughout this manuscript. CD40+ keratinocytes were then incubated with CD40L transfected or control J558L cells in a 1:1 ratio, unless indicated otherwise, and subsequently cultured in medium supplemented with IFN-γ (10 U per ml). After 72 h, supernatants were harvested, centrifuged and frozen in aliquots for the analysis of chemokines and complement components. Keratinocytes were detached with trypsin/EDTA, as described above, and used in flow cytometry experiments.

In some experiments instead of CD40L transfected cells, an 18-kD soluble recombinant CD40L (sCD40L; a gift of Dr. J-Y Bonnefoy, GlaxoWellcome Institute for Molecular Biology, Geneva, Switzerland) was used for CD40 triggering. Briefly, CD40+ keratinocytes were cultured with sCD40L in medium supplemented with IFN-γ (10 U per ml). After 72 h, supernatants were harvested, centrifuged and frozen in aliquots for the analysis of chemokines and complement proteins. sCD40L treated CD40+ keratinocytes were detached and used in flow cytometry experiments.

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Culture of CD40\(^*\) keratinocytes and CD40L transfected cells in a Transwell System

In experiments aimed at investigating the requirement of contact between CD40L transfected J558L cells and CD40\(^*\) keratinocytes for the activation of CD40\(^*\) keratinocytes a Transwell System (Costar) was used. CD40\(^*\) keratinocytes were cultured to subconfluence on the bottom of the lower wells of 24 mm diameter Transwell system. Cell number was estimated as 5-7 X 10\(^5\) keratinocytes per well. After removal of medium, 1500 \(\mu\)l fresh medium was added to the lower well. In wells in which direct contact between transfected J558L cells and keratinocytes was not desired, CD40L transfected J558L cells or control J558L cells in 500 \(\mu\)l medium supplemented with IFN-\(\gamma\) (10 U per ml) were added to the upper wells with bottoms mounted with a 0.4 \(\mu\)m pore size polycarbonate membrane, in a 1:1 ratio. In wells in which direct contact of the cells was desired, transfected or control J558L cells in 500 \(\mu\)l medium containing IFN-\(\gamma\) were added to the lower well, in a 1:1 ratio. After 72 h, supernatants were harvested, centrifuged and frozen in aliquots for the analysis of chemokines. Concentration of chemokines in supernatants in wells containing transfected or control J558L cells separated from CD40\(^*\) keratinocytes by the porous membrane were compared with those in supernatants of wells containing transfected or control cells not separated through the membrane.

Blocking of CD40 activation of CD40\(^*\) keratinocytes by specific antibody

CD40\(^*\) keratinocytes were cultured to subconfluence on the bottom of 6 well tissue culture plates and pre-treated for 2 h with 10 \(\mu\)g per ml of anti-CD40 monoclonal antibody 5 D12 (a kind gift of Tanox Pharma, Amsterdam, The Netherlands) in IFN-\(\gamma\) containing medium (10 U per ml). Hereafter, CD40L transfected J558L cells (or control J558L cells) were added in a 1:1 ratio to the CD40\(^*\) keratinocytes (5-7 X 10\(^5\) cells per well) in IFN-\(\gamma\) and anti-CD40 containing medium. After an incubation period of 72 h supernatants were harvested, centrifuged and frozen in aliquots for the analysis of chemokines.

Semi-quantitative determination of cell surface proteins by flow cytometry

Keratinocytes were detached with trypsin (0.025%)/EDTA (1.5 mM) for 3-5 min. For analysis of MCP, DAF, and CD59, CD40L treated CD40\(^*\) keratinocytes were used (see above). Trypsin was inactivated by fetal calf serum and detached cells were washed and resuspended in fluorescence-activated cell sorter (FACS) buffer (PBS, 2% fetal calf serum, 0.1% sodium azide). Approximately 10\(^5\) cells were incubated with specific monoclonal antibodies to CD40 (clone 5D12), MCP (clone J4-48, CLB, Amsterdam, The Netherlands), DAF (clone BRIC 110, CLB) or CD59 (1F5) or isotype control (Becton Dickinson, San Jose, CA) for 30 min at 4°C. Cells were washed two times and incubated for 30 min with fluorescein-conjugated F(ab')\(^2\) fragments of goat anti-mouse IgG (Jackson ImmunoResearch Laboratories, West Grove, PA). Hereafter, cells were washed two times with FACS buffer. Propidium iodide (1 mg per ml) (Sigma) was added to identify dead cells and 20,000 cells were analyzed immediately by FACS Calibur (Becton Dickinson). Dead cells were excluded.

The detachment of CD40\(^*\) keratinocytes with the trypsin/EDTA solution at 37°C for 3-5 min did not cause degradation of CD40 or any of the three complement regulatory proteins, as expression of these molecules on cells detached by this procedure was the same as on those detached with EDTA (2 mM) alone at 42°C for 30 min. The former procedure was selected because it did not cause loss of cell viability, whereas latter procedure caused a high degree of loss of cell viability.
ELISA for measurement of C3, factor B, IL-8, RANTES and MCP-1

For quantification of C3 and factor B, previously described sandwich ELISAs developed in our laboratory were used\(^{267}\) (described in Chapter 2). The detection limits of these ELISAs were 1 ng per ml for C3 and 100 pg per ml for factor B. Standard curves for both ELISAs were made using Human Complement Calibrator CA1 (ATAB).

The concentrations of IL-8, RANTES and MCP-1 in culture supernatants of CD40L stimulated and non-stimulated keratinocytes were estimated by ELISA as follows.

For quantification of IL-8, wells of 96 well flat-bottom microtiter plates were coated with 100 \(\mu\)l of 0.5 \(\mu\)g monoclonal mouse anti-human IL-8 IgG1 (Biosource, Breda, The Netherlands) per ml in carbonate buffer overnight at 4\(^{\circ}\)C. After thorough washing with Tween-80 (0.05\%) (Sigma) in PBS the wells were blocked for 1 h at room temperature with 200 \(\mu\)l of PBS containing 2\% bovine serum albumin (BSA; Sigma) and 0.05\% Tween-80. Washing was repeated and wells were incubated with 100 \(\mu\)l of sample, diluted in the same buffer that was used for blocking. Plates were incubated for 2 h at 37\(^{\circ}\)C. The wells were then washed and incubated with 100 \(\mu\)l biotinylated mouse anti-human IL-8 IgG1 (0.05 pg per ml) (Biosource) for 1 h at 37\(^{\circ}\)C. After washing, the wells were incubated for another h at 37\(^{\circ}\)C with peroxidase conjugated poly streptavidin (1:8000; Central Laboratory of the Netherlands Red Cross and Blood Transfusion Services, Amsterdam, The Netherlands). Wells were thoroughly washed and incubated with 100 \(\mu\)l 3,3',5,5' tetramethylbenzidine (Sigma) in dimethylsulfoxide (Merck, Hohenbrunn, Germany)-citrate buffer for 10 min. The reaction was stopped with 100 \(\mu\)l \(H_2SO_4\) (2 M). Optical density (OD) was measured at 450 nm. The detection limit of this ELISA was 1 pg per ml of IL-8.

For determination of RANTES, wells were coated overnight at 4\(^{\circ}\)C with 50 \(\mu\)l of 2 \(\mu\)g monoclonal mouse anti-human RANTES IgG (R&D Systems, Minneapolis, MN) per ml in PBS. After thorough washing, blocking and application of samples (100 \(\mu\)l) essentially as described above for IL-8, the wells were incubated with biotinylated polyclonal goat anti-human RANTES IgG (5 ng per ml) (R&D Systems) for 1 h at 37\(^{\circ}\)C. After further washing, the wells were incubated for 2 h at 37\(^{\circ}\)C with peroxidase conjugated poly streptavidin (1:8000). The wells were then repeatedly washed. Incubation of the wells with the peroxidase substrate, termination of the reaction, and measurement of OD was carried out as described for IL-8. The detection limit of this ELISA was 30 pg per ml.

For MCP-1 ELISA, wells were coated with 50 \(\mu\)l of 2 \(\mu\)g monoclonal mouse anti-human MCP-1 IgG1 (Pharmingen, San Diego, CA) per ml in carbonate buffer overnight at 4\(^{\circ}\)C. After thorough washing with Tween-80 (0.05\%) in PBS, the wells were blocked with 200 \(\mu\)l blocking/dilution buffer (PBS containing Tween-80 (0.05\%) and milk powder (2\%) (Nutricia, Zoetermeer, The Netherlands) for 1 h at room temperature. Washing was repeated and wells were incubated with 50 \(\mu\)l sample for 2 h at 37\(^{\circ}\)C. The wells were then washed and incubated with 50 \(\mu\)l biotinylated polyclonal rabbit anti-human MCP-1 IgG (1 \(\mu\)g per ml) (Pharmingen) for 1 h at 37\(^{\circ}\)C. Treatment of the wells with peroxidase conjugated polylstreptavidin, incubation of the wells with the peroxidase substrate, termination of the reaction and measurement of OD was carried out as described for IL-8. The detection limit of this ELISA was 7 pg per ml.

Statistical analysis

Statistical analysis was performed using a Students' t-test for data from ELISA experiments. A \(p\) value of less than 0.05 was considered significant.
RESULTS

IFN-γ at low concentrations can up-regulate the constitutive expression of CD40 on keratinocytes

Cultured human keratinocytes are known to express low but detectable amounts of CD40. In ligation studies, expression of CD40 is first up-regulated by treatment with IFN-γ, usually at a dose of 100 U per ml and for a culture period of 48 - 72 h. Keratinocytes treated with IFN-γ in doses as high as 100 U per ml become significantly stimulated. To find out the lowest concentration of IFN-γ which can cause optimal expression of CD40, the effect of IFN-γ (10-300 U per ml; 72 h) on the expression of CD40 on cultured human keratinocytes was analyzed by flow cytometry as described in 'Materials and Methods'. From 10 U per ml onwards we observed a very low dose-dependent up-regulation of the constitutive CD40 expression. A dose of 10 U per ml induced an up-regulation in CD40 expression of the keratinocytes which was more than 80% of the up-regulation induced by as high a dose of IFN-γ as 300 U per ml. (Figure 1). A dose of 10 U per ml and a time period of 72 h was therefore selected for pre-treatment of keratinocytes (CD40+ keratinocytes as described in 'Materials and Methods') in CD40-CD40L ligation studies to induce optimal CD40 expression and at the same time to prevent major stimulation of the keratinocytes.

Figure 1. The constitutive expression of CD40 on human keratinocytes is up-regulated by low concentrations of IFN-γ. Keratinocytes were cultured in the presence of increasing concentrations of IFN-γ (10-300 U per ml). After 72 h, 2 X 10⁴ cells were analyzed for the expression of CD40 by flow cytometry. Histograms for expression of CD40 are shown. Continuous line represent expression of CD40. Broken lines represent results obtained with isotype controls.
Activation of CD40+ keratinocytes by CD40L transfected J558L cells enhances chemokine production

We tested whether CD40 ligation can activate keratinocytes to produce enhanced amounts of chemokines. For this reason, CD40+ keratinocytes were co-cultured with CD40L transfected J558L cells or control J558L cells as described in 'Materials and Methods' and the concentrations of IL-8, RANTES and MCP-1 were determined in the culture supernatants. Figure 2 shows that CD40+ keratinocytes produce low levels of IL-8, RANTES and MCP-1 which was not altered upon co-culturing with increasing numbers of control non-transfected J558L cells but was markedly increased upon co-culturing with increasing numbers of CD40L transfected cells. At a CD40L transfected J558L cells : CD40+ keratinocyte ratio of 1:1 the CD40 activation caused 9-, 65- and 2.4-fold increase in production of IL-8, RANTES and MCP-1 whereas at a ratio of 5:1 of these cells CD40 activation caused 16-, 125-, and 1.4- fold increase in production of these chemokines, respectively. These results are representative of one of the two independent experiments with almost identical results. Decrease in MCP-1 production at a cell ratio of 5:1 was not a strange finding as after an optimal increase at 1:1 ratio, similar decrease in MCP-1 production with increasing ratio of transfected cells has been observed with proximal tubular epithelial cells347.

Figure 2. Chemokine production by CD40+ keratinocytes in co-cultures is dependent on the dose of CD40L transfected cells Subconfluent CD40+ keratinocyte cultures were co-cultured with CD40L transfected (CD40L+) J558L cells or control (CD40-) J558L cells in an increasing ratio with the keratinocytes (0.2, 1.0, and 5.0). Supernatants were harvested at 72 h and the concentrations of IL-8, RANTES and MCP-1 were measured by ELISA. Data represent one of two independent experiments, each in triplicate. Mean values and SD are shown.
Figure 3. Chemokine production by CD40+ keratinocytes in response to CD40L transfected cells is time-dependent. CD40+ keratinocytes were co-cultured with CD40L transfected (CD40L+) J558L cells or control (CD40L−) J558L cells in a 1:1 ratio and supernatants were harvested at 24, 48, 72 or 96 h. The concentrations of IL-8, RANTES and MCP-1 in supernatants were measured by ELISA. Data represent one of two independent experiments, each in triplicate. Mean values and SD are shown.

When cultures were followed in time, strong enhancement in release of IL-8, RANTES and MCP-1 by CD40+ keratinocytes after CD40 activation could already be detected at 24 h (Figure 3). In case of IL-8 and MCP-1, active production subsided after 24 hours whereas in case of RANTES production continued over a long period of time and was still seen at 96 hours.

To prove that contact between CD40+ keratinocytes and CD40L transfected J558L cells was necessary for keratinocyte activation and to rule out the possibility that CD40L transfected J558L cells may be releasing any soluble mediator which might be causing the activation of keratinocytes to release chemokines, following experiment was performed in a Transwell system. CD40+ keratinocytes were placed in the lower well and CD40L transfected or control J558L cells in upper wells in the same medium but separated from each other through a porous polycarbonate membrane. In a parallel set CD40+ keratinocytes as well as CD40L transfected J558L cells or control J558L cells were cultured under identical conditions in lower wells without physical separation as described in ‘Materials and Methods’. After 72 h of culture neither supernatant of the wells containing transfected cells nor the supernatants of wells containing control J558L cells showed any increase in production of chemokines from CD40+ keratinocytes in wells in which both cell types were cultured without cell-cell contact (Figure 4). In supernatants harvested from CD40+ keratinocytes cultured in direct contact with CD40L transfected J558L cells, concentrations of IL-8, RANTES, and MCP-1 were greatly increased. This suggested that contact of CD40+ keratinocytes with CD40L transfected cells was essential for keratinocyte activation to produce enhanced amounts of chemokines.

After confirming that cell to cell contact was necessary for CD40L transfected J558L cells to activate CD40+ keratinocytes to produce chemokines, attempt was made to show that during cell-cell contact it was the CD40L molecule on CD40L transfected J558L cells which triggered the CD40 molecule on keratinocytes. To demonstrate this, experiments were performed
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in which interaction was selectively blocked by means of blocking antibodies directed against the CD40 molecule. CD40+ keratinocytes and CD40L expressing J558L cells were co-cultured for 72 h in the medium containing non-activating anti-CD40 antibodies or irrelevant mouse IgG1. The results showed that neutralization of CD40 on CD40+ keratinocytes with anti-CD40 abolished the induction of IL-8 and MCP-1 production, and reduced the induction of RANTES production (Figure 5). Similar concentrations of a control IgG1 showed no effect. This experiment was performed in triplicate and in all three independent experiments induction of RANTES was reduced but not completely inhibited. The specificity of CD40 activation of CD40+ keratinocytes by CD40L transfected J558L cells was confirmed by almost complete inhibition of IL-8 and MCP-1 after addition of blocking antibodies against CD40 but the reason for incomplete inhibition of production of RANTES remained unclear.

Figure 4. Physical contact between CD40L transfected cells and CD40+ keratinocytes is necessary to activate keratinocytes. CD40+ keratinocytes cultures were grown to subconfluence on the bottom of the lower wells of Transwell tissue culture plates. CD40L transfected (CD40L+) J558L cells or control (CD40L) J558L cells were added to the upper well in a 1:1 ratio (no direct contact of the transfected or control J558L cells with the CD40+ keratinocytes) or to the lower well (with direct contact of the J558L cells with the CD40+ keratinocytes). Supernatants were harvested at 72 h and the concentrations of IL-8, RANTES and MCP-1 were determined by ELISA. Data represent one of two independent experiments, each in triplicate. Mean values and SD are shown.

Figure 5. Chemokine production by keratinocytes in co-cultures of CD40+ keratinocytes and CD40L transfected cells is inhibited by antibody specific for CD40. CD40+ keratinocytes were grown to subconfluence followed by a 2 h incubation with 10 μg per ml of anti-CD40 antibodies (5D12). Hereafter, CD40L transfected (CD40L+) or control (CD40L) J558L cells were added in a 1:1 ratio to the keratinocytes and cultured. Supernatants were harvested at 72 h and the concentrations of IL-8, RANTES and MCP-1 were determined by ELISA. Data represent one of two independent experiments, each in triplicate. Mean values and SD are shown.
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Recombinant soluble CD40L (sCD40L) can also induce activation of CD40\(^+\) keratinocytes to cause enhanced production of chemokines.

When CD40\(^+\) keratinocytes were cultured for 72 h and culture supernatants were harvested, IL-8, RANTES, and MCP-1 were found to be present in low concentrations. However, when CD40\(^+\) keratinocytes were cultured in the presence of increasing concentrations of sCD40L, concentrations of IL-8, RANTES, and MCP-1 in the supernatants were increased in a dose-dependent manner (Figure 6). Enhancement in production of MCP-1 was less than that of IL-8 and RANTES but was significant \((p < 0.05\) with all tested concentrations of sCD40L). These results were obtained with different keratinocyte cultures in 2 independent experiments, each in triplicate.

Activation of CD40\(^+\) keratinocytes by CD40L transfected J 558 cells or sCD40L does not enhance the production of complement components

Besides cytokines (and chemokines), keratinocytes are known to produced two complement components, C3 and factor B; production of more components is yet to be studied. Production of C3 and factor B is differentially regulated by several cytokines\(^{298,36}\). It is not known whether CD40L also acts as one of the signals for enhanced production of these components. To test this, CD40\(^+\) keratinocytes were co-cultured with increasing numbers of CD40L transfected or control J558L cells for 72 hours or both cells were co-cultured in 1:1 ratio for different time intervals and the concentrations of C3 and factor B in the supernatants were determined. In some experiments, instead of CD40L transfected or control J558L cells, increasing concentrations of sCD40L were added to CD40\(^+\) keratinocytes and cells were cultured for 72 h. The results showed that neither CD40L transfected J558L cells nor sCD40L were able to regulate the production of C3 and factor B (data not shown).

Activation of CD40\(^+\) keratinocytes by CD40L transfected J558L cells does not up-regulate the expression of complement regulatory proteins

Some cytokines (e.g. TGF-\(\beta\)), known to be present in inflammatory environment, can up-regulate the expression of MCP and CD59 on keratinocytes to protect them from their own complement\(^{37}\). Besides, expression of DAF and CD59 is down-regulated in psoriatic epidermis\(^{11}\). It is not known whether CD40L, which is transiently expressed on activated CD4\(^+\) T cells in inflammatory environment, can interact with CD40 on CD40\(^+\) keratinocytes and alter the expression of complement regulatory proteins on keratinocytes. To test this, CD40\(^+\) keratinocytes
Figure 6. Recombinant soluble CD40L can also induce CD40 activation of CD40+ keratinocytes to enhance production of chemokines. Subconfluent CD40+ keratinocytes were cultured with increasing doses of sCD40L (0.1 - 10 μg per ml). Supernatants were harvested at 72 h and the concentrations of IL-8, RANTES, and MCP-1 were measured by ELISA. Data represent one of two independent experiments, each in triplicate. Mean values and SD are shown.

In some dermatological conditions, human skin can manifest dramatic inflammation. One such condition is psoriasis in which keratinocytes undergo hyperproliferation and the epidermis is inundated with chemoattractant and proinflammatory cytokines, complement activation products (C3a, C5a, C5b-C9 etc.), and inflammatory cells (including activated T-cells). It is believed that communication between activated T-cells and keratinocytes may initiate hyperproliferation of keratinocytes. This, in turn, may produce excessive amounts of chemokines and complement. It is therefore important to elucidate the interactions involved in communication between activated T-cells and keratinocytes. One such possible interaction is the ligation of CD40 present on keratinocytes in psoriatic epidermis with CD40L transiently expressed on activated T cells. We investigated whether CD40-CD40L ligation on cultured human keratinocytes can activate keratinocytes to produce excessive amounts of chemokines and complement components and alter the expression of complement regulatory proteins in vitro, abnormalities which have been seen in psoriatic epidermis in vivo.

Cultured keratinocytes express low amounts of CD40. Activation of keratinocytes by
CD40L does not take place in keratinocytes expressing basal levels of CD40 but does take place in IFN-γ treated keratinocytes which express high levels of CD40. Therefore in ligation studies for efficient ligation, CD40 on keratinocytes is first up-regulated by IFN-γ before treatment of keratinocytes with a source of CD40L. In previous studies, the concentrations of IFN-γ used to up-regulate CD40 on keratinocytes were 100 U per ml or more. Since this concentration of IFN-γ can directly release some inflammatory mediators, we investigated if lower concentrations can up-regulate CD40 on keratinocytes. The results showed that 10 U IFN-γ per ml can induce almost as high degree of up-regulation of CD40 as 100-300 U per ml. This low dose of IFN-γ does not induce the release of C components. IFN-γ in this dose primarily appears to act as a priming agent for keratinocytes, placing keratinocytes in a heightened state of readiness for activation through CD40 without major stimulation. Ten U of IFN-γ per ml was therefore selected for pretreatment of keratinocytes to induce optimal expression of CD40 in all subsequent experiments.

For experimental purposes, CD40 activation of CD40+ keratinocytes was achieved in two ways - using CD40L transfected J558L cells or using an agonistic recombinant soluble CD40L (sCD40L). Activation of CD40+ keratinocytes with CD40L of transfected J558L cells induced strong production of IL-8, RANTES and MCP-1. This induction was dependent on the dose of CD40L-transfected J558L cells. It was also time dependent. Experiments in which CD40+ keratinocytes and CD40 transfected J558L cells were co-cultured with and without separation from each other through a porous membrane in a Transwell system showed that contact of CD40L transfected cells with CD40+ keratinocytes was essential for up-regulation of release of chemokines. During cell-cell contact, it was the CD40L molecule on CD40L transfected J558L cells which triggered the CD40 molecule on keratinocytes. This was shown by selectively blocking CD40-CD40L ligation by antibodies against the CD40 molecule. This antibody abolished the induction of IL-8 and MCP-1 production and reduced the induction of RANTES. sCD40L could also induce CD40 activation of keratinocytes to enhance production of chemokines, further confirming the specificity of CD40-CD40L ligation for chemokine production. It also suggests that CD40+ keratinocytes can be activated through CD40 by either cell bound or secreted/shed CD40L to participate in an ongoing inflammatory response by producing chemoattractant cytokines.

The results of this study, using CD40L transfected J558L cells as well as sCD40L, showed that CD40-CD40L ligation on keratinocytes can not induce signalling involved in C3 and factor B up-regulation. This ligation also does not regulate the expression of complement regulatory proteins.

Our results if extrapolated to in vivo situation in psoriasis may suggest that the cytokine milieu surrounding the keratinocytes in vivo may represent a critical determinant of the cellular
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responses mediated through the CD40-CD40L pathway. Of particular relevance would be the tissue concentration of IFN-γ. This cytokine may provide a primary effect of up-regulating CD40 on keratinocytes for CD40 engagement in inflamed tissue and thus can facilitate CD40-CD40L ligation. This ligation may in turn result in amplified production of IL-8, RANTES and MCP-1 and possibly other chemoattractant (and proinflammatory) cytokines. IL-8 may be considered responsible for the infiltration of neutrophils and RANTES and MCP-1 for infiltration of T cells in the psoriatic lesional skin. Thus, CD40-CD40L ligation may be important not only in perpetuating and augmenting inflammatory processes in the skin but also in establishing a positive activation loop in regulation of epidermal infiltration. A pathogenic role for CD40-CD40L interaction in skin inflammatory reactions is supported by the fact that functional inhibition of T cell function by cyclosporin A which is known to prevent CD40L expression on activated T cells, is beneficial in psoriasis. It appears that production of complement and alteration of expression of complement regulatory proteins in psoriasis likely do not involve CD40-CD40L ligation.

In conclusion, CD40-CD40L ligation on keratinocytes can induce keratinocytes to produce enhanced amounts of chemokines but not complement components C3 and factor B. It also does not influence the expression of complement regulatory proteins on keratinocytes. This ligation in vivo may play a pivotal role in regulating inflammation in the epidermis.

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