doi:10.1016/j.jmb.2004.11.027 J. Mol. Biol. (2005) 345, 1083–1097

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# From an Enhanceosome to a Repressosome: Molecular Antagonism between Glucocorticoids and EGF Leads to Inhibition of Wound Healing

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Wound healing in its complexity depends on the concerted activity of many signaling pathways. Here, we analyzed how the simultaneous presence of glucocorticoids (GC), retinoic acid (RA) and epidermal growth factor (EGF) affect wound healing at the molecular, cellular and tissue levels. We found that GC inhibit wound healing by inhibiting keratinocyte migration, whereas RA does not. Furthermore, GC block EGF-mediated migration, whereas RA does not. On the molecular level, these compounds target expression of one of the earliest markers of wound healing, cytoskeletal components, keratins K6 and K16. Both GC and RA repress their transcription, whereas EGF induces it. Interestingly, the GC inhibition is mediated by a repressosome complex consisting of four monomers of the GC receptor, β-catenin and coactivator-associated-arginine-methyltransferase-1. GC are dominant, EGF cannot rescue GC-mediated inhibition. Pre-treatment of keratinocytes with GC shifts the balance towards the repressosome, allowing for dominant inhibition of K6 even in the presence of EGF or c-fos/c-jun. Although RA receptor gamma and glucocorticoid receptor bind to the same response element repressing transcription of keratins K6/K16, RA receptor interacts with the components of the EGF-enhanceosome (co-activators: glucocorticoid-receptor-interactive protein-1(GRIP-1)/steroid-receptors coactivator-1 (SRC-1)) without breaking it. Consequently, RA has a co-dominant effect with EGF: when present simultaneously, their effects balance each other. When keratinocytes are pre-treated with mitogen-activated protein kinase (MAPK) inhibitor, thus blocking EGF, the balance is shifted towards the RA repression. Similar to clinical findings, pre-treatment of keratinocytes with RA blocks GC-mediated inhibition. In summary, our results identify complex molecular mechanisms through which RA alleviates GC-mediated inhibition of wound healing.



*Keywords:* wound healing; glucocorticoids; retinoids; epidermal growth factor; keratinocytes

## Introduction

Skin integrity and its maintenance depend directly on the awareness of keratinocytes of their position in the structure of the epidermal tissue. Within moments of wounding, keratinocytes must inform each other that the barrier has been broken and they must communicate the urgent need to repair the gap. The communication skills of keratinocytes during wound healing translate into signal transduction cascades that have been studied extensively over the years. These signaling

Abbreviations used: GC, glucocorticoids; GR, glucocorticoid-receptor; GRE, glucocorticoid response element; RA, retinoic acid; RAR, retinoic acid receptor; RARE, RA response element; EGF, epidermal growth factor; GRIP-1, glucocorticoid-receptor-interactive protein-1; SRC-1, steroid receptors coactivator-1; MAPK, mitogen-activated protein kinase; TNFα, tumor necrosis factor alpha; IL-1, interleukin-1; PDGF, platelet-derived growth factor; T3, thyroid hormone; DEX, dexamethasone.

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molecules, such as epidermal growth factor (EGF), tumor necrosis factor alpha (TNFα) and interleukin-1 (IL-1), are molecular switchers that compel keratinocytes to become activated, which results in their ability to migrate and proliferate. The inhibitors of wound healing, such as glucocorticoids (GC), have also been identified.[1] However, very little is known about the molecular interplay and its outcome that occurs when several factors are present simultaneously during this process and, in particular, when such factors have opposing effects. Under certain circumstances (diabetes, external pressure and venous reflux) this innate healing program fails, leading to the development of chronic ulcers. The attempts to use growth factors/cytokines in the therapy for wound healing disorders have not been very successful.[2] Topical and systemic platelet-derived growth factor (PDGF) decreased ulcer volume and recently recombinant human PDGF has been approved by the Food and Drugs Administration (FDA) as the only growth factor for use in therapy of specific types of chronic ulcers.[3] These examples show that our knowledge of how these factors operate during wound healing, although significant, is not complete.

Growth factors and cytokines as well as hormone receptors mediate their effects through signaling cascades that regulate gene transcription. Transcriptional activity of any gene depends on the particular group of transcription factors interacting with its promoter at any given time. Activation of the promoters is rarely mediated by binding of a single transcription factor, but instead by clusters of simultaneously bound factors that interact further with co-regulators, resulting in opening of the chromatin and induction of transcription.[4] Such a cluster of transcription factors interacting with co-activators and histone-modifying enzymes to activate transcription is designated as an enhanceosome.[4–7] Conversely, a cluster of transcription factors interacting with co-repressors and histone-modifying enzymes that represses transcription is designated as a repressosome.[8]

One of the earliest markers of keratinocyte activation during wound healing is the expression of cytoskletal components that provide sufficient flexibility for cellular migration, keratins K6 and K16. We have shown that the wound healing stimulators EGF, TNFα and IL-1, target K6 by inducing simultaneous binding of AP1, C/EBP and NFκB to their respective binding sites, thus forming an enhanceosome structure.[9–12] We have also shown that both GC and retinoic acid (RA) repress transcription of keratin genes through unique molecular mechanisms that involve monomers of glucocorticoid receptor (GR), β-catenin and CARM-1, and homodimers of RA receptor (RAR).[13–17] In addition, EGF, through a mitogen-activated protein kinase (MAPK)-dependent mechanism, participates in keratinocyte shape change during their migration.[18] Therefore, we investigated how the simultaneous presence of wound healing activators and inhibitors affects the expression of K6, keratinocyte migration and epidermal wound healing. We found GC to be inhibitors of wound healing, keratinocyte migration and K6 expression, even in the presence of EGF. In contrast, we found that RA has a co-dominant effect with EGF on wound healing, keratinocyte migration and K6 expression when it is present simultaneously with EGF. We propose that the dominant inhibition by GC is based on the mutually exclusive binding of either a GC-dependent repressosome or an EGF-induced enhanceosome, whereas the RAR co-dominance derives from the ability of RAR-repressosome to swap components with the EGF-enhanceosome. We have confirmed this hypothesis by adding or eliminating the components of either of the "-somes". This shift in molecular "balance" of the components resulted in the dominance of either of the two, EGF or RA. Furthermore, by dissecting these complex mechanisms, our results revealed how RA may prevent the GC inhibitory effect, which has been described clinically. Taken together, our results identify the molecular mechanisms that lead to refined regulation of keratinocyte cytoskeletal components, thus contributing to the overall effects on keratinocyte migration and wound healing. Furthermore, they provide an example of molecular interplays that multiple signaling pathways use to regulate or maintain complex cellular processes, such as wound healing.

## Results

There is a large body of evidence that implicates keratins K6 and K16 in keratinocyte migration during wound healing.[19–22] This led us to hypothesize that GC, in part through a shut-down of keratins K6 and K16, might inhibit keratinocyte migration. In addition, we have shown that RA and thyroid hormone (T3) directly suppress the expression of a large group of keratin genes, including K6 and K16.[15,23] In contrast, GC exhibit a more specific regulatory pattern. GC target only keratins expressed in keratinocytes that are involved in wound healing: the basal cell-specific keratins K5 and K14 markers of mitotically active keratinocytes, the migration-associated keratins K6 and K16, and the "contractile" keratin K17.[14] To examine if the GC repression of wound healing-specific keratin genes is significant to the *in vivo* situation, we tested the effect of topical GC on expression of wound healing-specific K6 keratin in a wound healing organ culture model. Normal human skin was wounded by 4 mm biopsy punch and treated topically by Cormax® (a potent topical glucocorticoid cream) and maintained at an air/liquid interface: 48 hours later the samples were frozen, sectioned and stained with the K6-specific antibody. We found that, indeed, wounding induces K6 expression, shown as intense staining at the wound edge (Figure 1(A)). We observed a "wave"

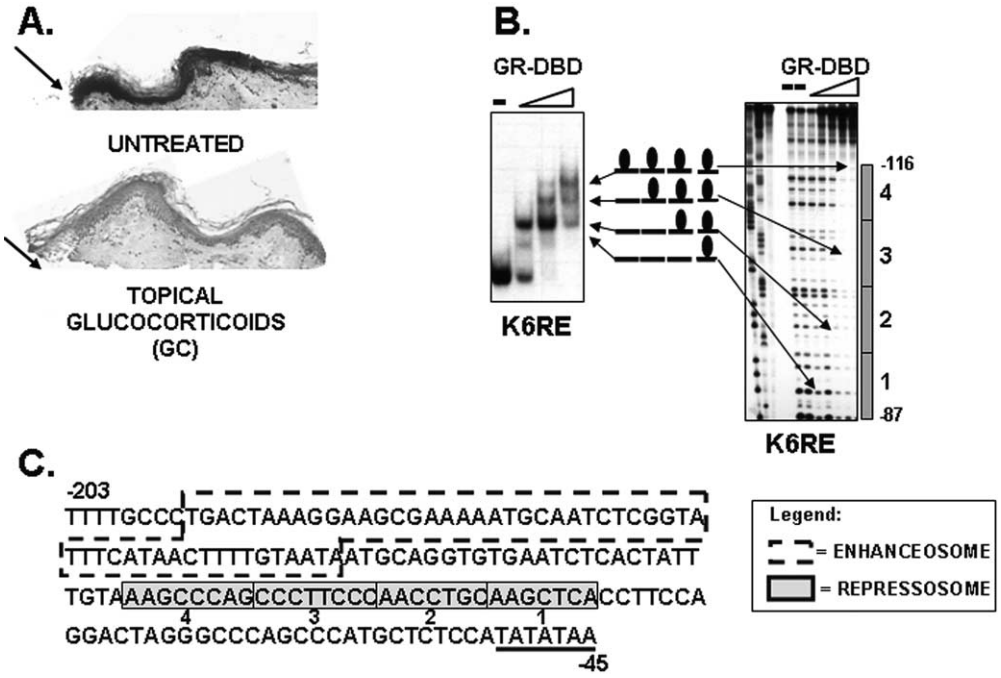


**Figure 1.** Glucocorticoids repress K6 expression. (A) Sections of wounded human skin stained with K6 specific antibody show that K6 is induced in epidermis by wounding (top), whereas topical GC treatment inhibits this induction completely (bottom). Prominent activation of K6 visible at the edge of the wound was inhibited markedly by topical GC. Arrows mark the wound edge. (B) Purified DNA-binding domain of glucocorticoid receptor (GR-DBD) binds to the K6RE in EMSA (left) and footprinting assay (right). Four monomer units of GR (arrows) bind to K6RE, which is a unique property that allows GR-mediated suppression (see also Radoja *et al*.[14]). (C) A portion of K6 promoter sequence is shown. Broken lines demarcate the enhanceosome that consists of AP1, three C/EBP and a NFkB binding sites.[10] The gray box area marks nuclear receptor (RAR and GR) binding sites and numbers below indicate GR monomer binding sites.

of K6 induction that spreads from keratinocytes at the wound edges to their neighbors. As expected, topical GC completely blocked K6 induction (Figure 1(A)), which was evident even at the protein level. This further confirms that the observed repression of wound healing keratin expression by GC is, in fact, physiologically relevant and that it occurs in wounds *in vivo*. This also suggests that GC may dominantly block the induction of K6 by EGF and other proinflammatory cytokines that participate in wound healing.

To confirm a direct interaction between the GR and the previously identified K6RE, we used electrophoretic mobility shift (EMSA) and footprinting assays. As expected, the purified recombinant DNA-binding domain of GR binds to K6RE (Figure 1(B)). The binding pattern of GR-DBD to K6RE in EMSA indicates binding of four GR monomer units, similar to previously described keratin-negative GR elements (GREs).[14] To confirm that GR binds as four monomers, we used the DNase I *in vitro* footprinting assay and 0.5 ng, 10 ng, 25 ng and 50 ng of recombinant GR-DBD. We found that GR-DBD occupies at first only one binding site at positions −87 to −92 and, as the protein concentration increases, the GR-DBD footprint grows in increments of one binding site until it occupies a total of four binding sites (Figure 1(B)). Therefore, we conclude that the repression of K6 gene expression occurs by binding of four monomers of GR to the negative K6RE.

To test if suppression of K6 and K16 genes by GC affects keratinocyte migration, we used the *in vitro* wound scratch assay. When cells grown in a tissue culture dish are "wounded" by a scratch, they migrate over the scratch to close the gap. We used primary human keratinocytes, which express high levels of K6/K16, HeLa cells as cells of epithelial origin that do not express K6/K16 and primary human dermal fibroblasts as non-epithelial cells also not expressing K6/K16. These three cell types were wounded by a scratch, incubated in the presence and in the absence of dexamethasone (DEX), and the cell migration was monitored during the next 48 hours (Figure 2). We found that DEX completely inhibits migration of keratinocytes (Figure 2(A)), whereas migration of either HeLa cells (Figure 2(B)) or dermal fibroblasts (Figure 2(C)) was not affected. This means that the GC inhibition of cell migration is specific to keratinocytes, the only cells expressing K6 and K16.

GC-mediated repression of K6 during wound healing and inhibition of keratinocyte migration (see Figures 1 and 2) occurs in the presence of other hormones, growth factors and cytokines that participate in the wound healing processes.

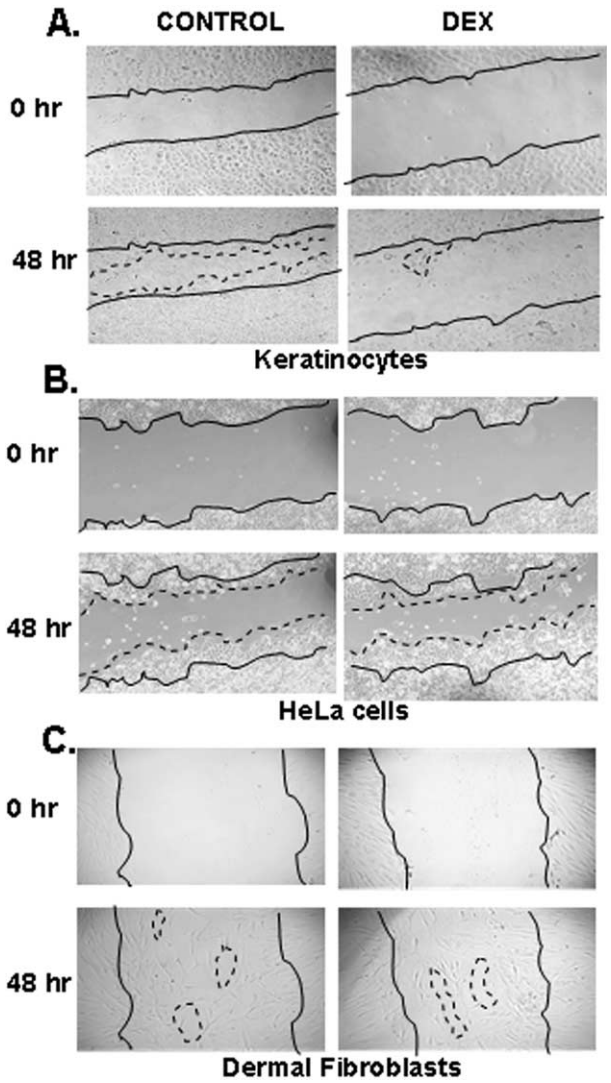


**Figure 2.** GC inhibit keratinocyte migration. Wound scratch assays are shown. All cells were pre-treated with mitomycin-C to eliminate proliferation. Dexamethasone (DEX) inhibits migration of keratinocytes (A), whereas it does not affect migration of either HeLa (B) or primary human dermal fibroblasts (C). Therefore, DEX exhibits cell type specificity, by selectively inhibiting migration of primary human keratinocytes. Continuous lines represent initial scratch and dotted lines represent the migrating front.

Therefore, our results suggest that GC-mediated repression dominates over the effects of other factors. To test this hypothesis, we have induced K6 expression in skin by adding EGF rather than by wounding, and then tested whether GC would block the EGF induction (Figure 3(A)). We found, as expected, that normal (unwounded skin) does not express K6, and that the treatment with GC alone does not affect K6 expression, whereas treatment by EGF induces strong expression of K6. Interestingly, simultaneous treatment by GC and EGF blocked the EGF-mediated induction of K6 completely. This means that the effects of GC dominate over those of EGF in regulation of K6.

To confirm that GC indeed dominate over EGF, we examined how their simultaneous presence affects keratinocyte migration in the wound scratch assay. Primary human keratinocytes were wounded by a scratch, incubated in the presence or in the absence of DEX, EGF or combined EGF+DEX (Figure 3(B)). Because both GC and RA repress expression of K6/K16, we decided to use RA as an additional control for K6/K16 repression (Figure 3(C)). Cell migration was monitored 24 hours and 48 hours after the scratch and cell migration was quantified (Figure 3(D)). After 24 hours and, more significantly, after 48 hours, the cells in the control plate were actively migrating and, in some areas, almost closed the gap. The keratinocytes incubated in the presence of DEX remained "frozen in time" i.e. they simply did not migrate at all (Figure 3(B) and (D)). As expected, EGF stimulated keratinocyte migration. We found that DEX inhibited keratinocyte migration by 80%, whereas EGF stimulated it by 50% (Figure 3(B) and (D)). Interestingly, keratinocytes incubated in the presence of both DEX and EGF did not migrate, similarly to the keratinocytes incubated with DEX alone. DEX inhibited keratinocyte migration by 80% even in the presence of EGF, thus confirming that GC block the effects of EGF. We found that RA has a milder inhibitory effect, 27%, on keratinocyte migration (Figure 3(C) and (D)). Surprisingly, we found that unlike DEX, RA did not dominate over EGF. In fact, we found that neither RA nor EGF dominate in regulating keratinocyte migration; when both were present, keratinocyte migration was very similar to that of untreated cells (Figure 3(C) and (D)). Although both GC and RA target and inhibit keratinocyte migration, only GC are dominant inhibitors in the presence of EGF. RA, on the other hand, has a co-dominant effect with EGF. This means that the dominant inhibition of keratinocyte migration is specific for GC, and is not shared with RA.

We used a wound healing organ culture model to test if GC, but not RA, are dominant inhibitors of EGF during wound healing. This model allowed us to follow the re-epithelialization from the epidermal perspective, i.e. in the presence of EGF but in the absence of circulating factors and inflammatory reactions. Normal skin was wounded by 4 mm biopsy punch and maintained at an air/liquid interface in the presence or in the absence of topical GC, RA, EGF and combinations GC+EGF and RA+EGF. Wound healing was monitored on a daily basis, for a period of seven days and wound healing was quantified on days 4 and 7 by planimetry (Figure 4(A) and (B)). In addition, organ cultures were frozen, sectioned and stained for evaluation of the healing process (Figure 4(C)). We have found, both by quantification of the healing (Figure 4(A) and (B)) and by histological evaluations (Figure 4(C)), that wounds treated with control ointment re-epithelialize fully within a week, with a fully differentiated epidermis and a defined cornified layer. Topical treatment with GC

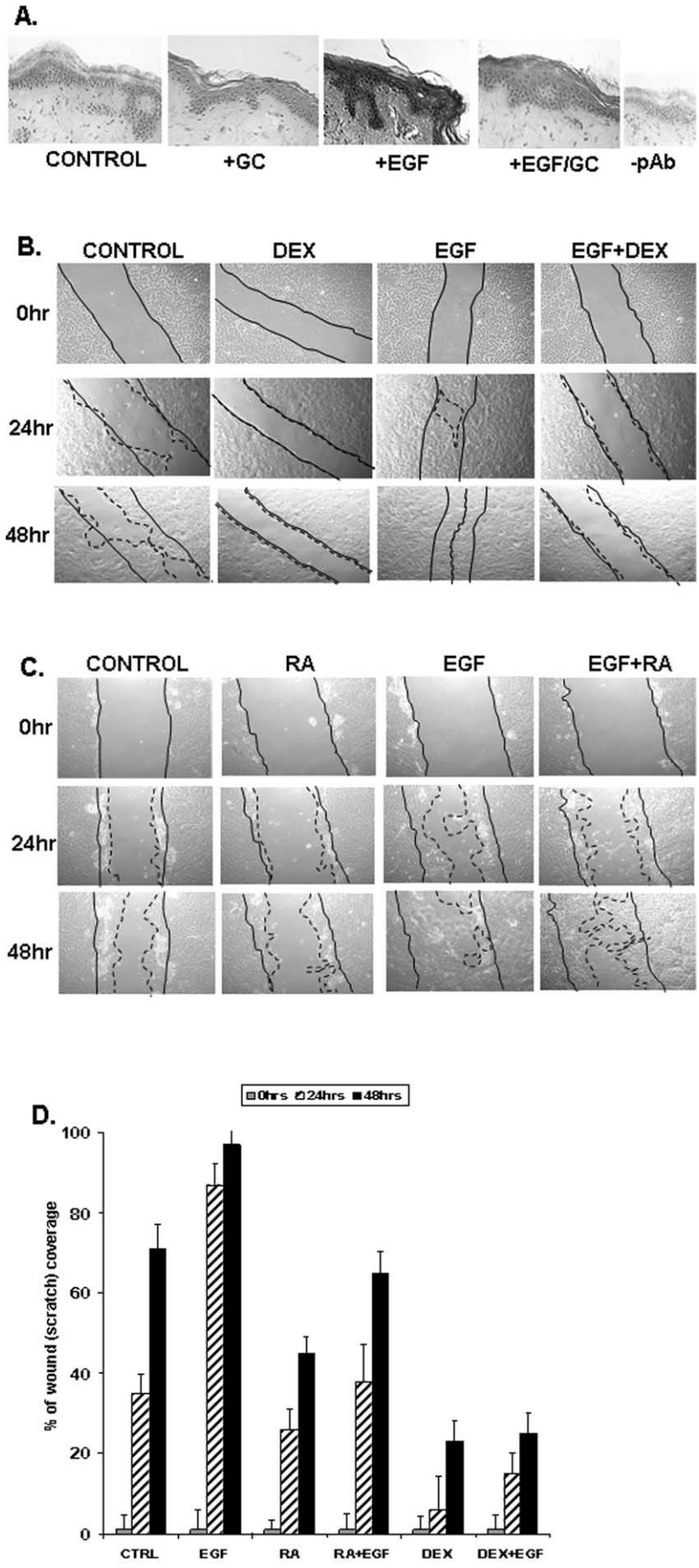


**Figure 3.** GC inhibit EGF-mediated induction of K6 in skin and block EGF-mediated keratinocyte migration, whereas RA does not. (A) Skin stained with K6-specific antibody is shown after incubation with GC, EGF and simultaneously EGF + DEX. K6 protein is not found in skin under normal condition and, therefore, GC have no effect. As expected, K6 expression is induced strongly by EGF. Interestingly, K6 is suppressed markedly when GC and EGF are present simultaneously. Therefore, GC block EGF-mediated induction of K6 in epidermis of human skin. (B) Similar effects of GC are found on keratinocyte migration in a wound scratch assay. DEX inhibits migration of primary human keratinocytes in a wound scratch assay when compared to untreated cells. Continuous lines represent initial scratch and dotted lines represent the migrating front. Inhibition is prominent even in the first 24 hours and was sustained further through 48 hours. EGF, as expected, stimulated migration and the wound was closed completely after 48 hours. Importantly, addition of DEX blocked EGF-stimulated migration completely. (C) RA inhibits keratinocyte migration slightly. However, unlike GC, it does not block EGF-stimulated migration. Continuous lines represent initial scratch and dotted lines represent the migrating front. (D) Histograms indicate the average coverage of scratch wounds widths in percentage relative to baseline wound width at the day 0, 24 hours and 48 hours after treatment with DEX, EGF, RA, DEX/EGF and RA + EGF. Quantification of the keratinocyte migration confirms that DEX inhibits keratinocyte migration even in the presence of a strong stimulator, EGF, whereas RA does not.

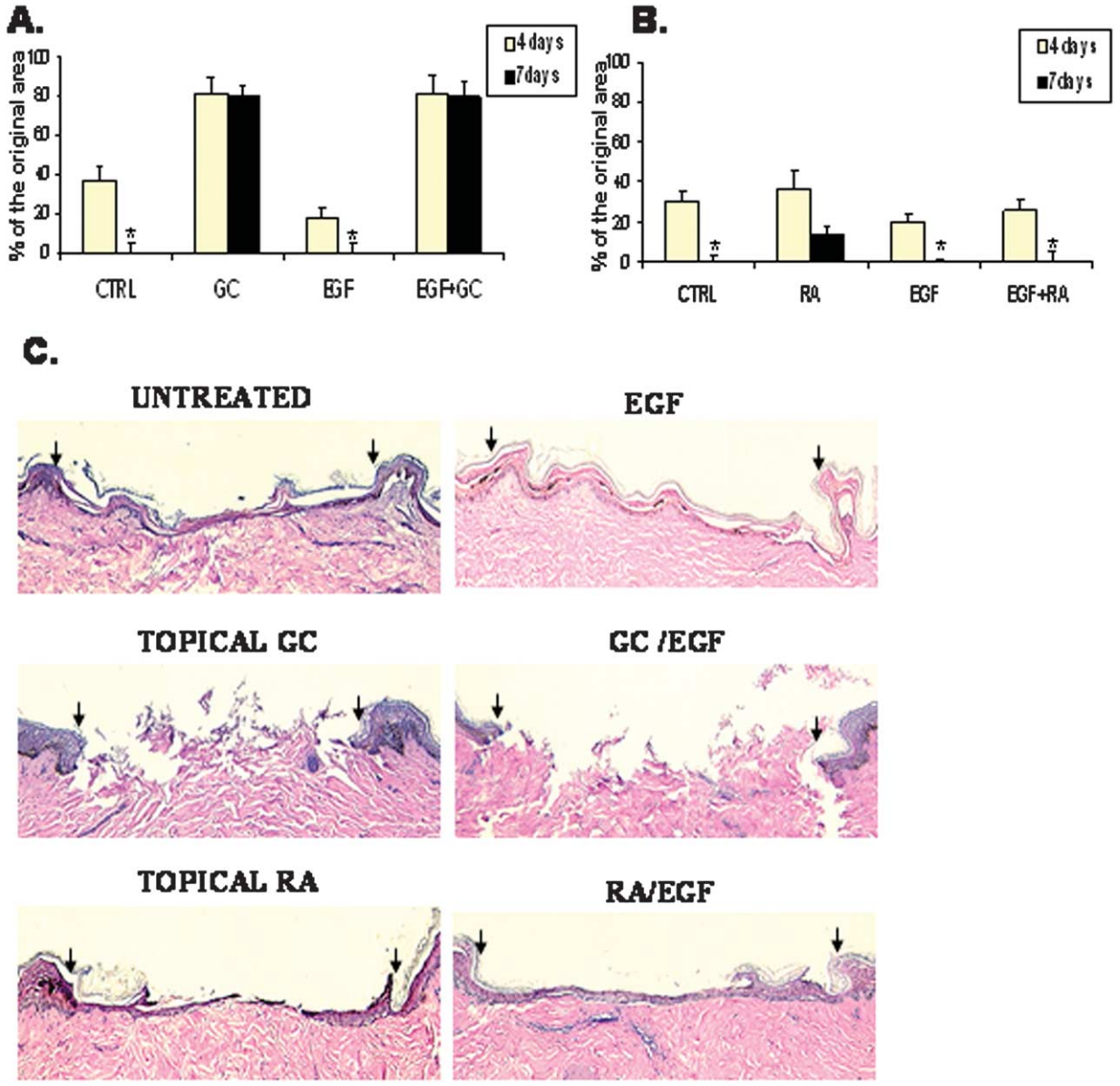


**Figure 4.** GC, but not RA, inhibit epidermal wound healing. Topical GC cause delayed wound healing in human skin organ culture wounds. (A) Quantification by planimetry of the wound size at days 4 and 7 post wounding is shown as percentage of the original area of the wound. Topical GC have similar effects on epithelialization and on keratinocyte migration *in vitro*. At day 4, there is 70% healing rate of untreated wounds and only 16% healing rate for DEX treated wounds. EGF promotes wound healing by 20%, whereas GC inhibit the EGF effect completely (healing rate 17%). At day 7, control and EGF-treated skin was healed completely, whereas GC and GC + EGF remained almost the same as at day 4, healing rates were 16.3% and 17.2%, respectively. Asterisks mark complete closure of the wound, 100% epithelialization. (B) At day 4, skin treated by topical RA shows a slight delay in healing (12%), whereas simultaneous treatment of RA + EGF shows a healing rate similar to the control. At the day 7 control, EGF and RA + EGF achieved complete closure (indicated by asterisks), whereas RA remained delayed, healing rate 86%. (C) Histology shows wound closure at day 7. Wounds treated either by topical GC or EGF + GC show no sign of healing and an absence of epithelial tongue, typical for actively healing epidermis. Wounds treated by topical RA heal slightly slower than the control (it is just about to completely close), whereas RA + EGF healed. Arrows point to the initial wound edge.

completely inhibits wound healing, i.e. the wound edges remain at the same place during the seven days, revealing no sign of keratinocyte migration or proliferation (Figure 4(A) and (C)). As expected, EGF stimulated wound healing (Figure 4(A) and (B)) and complete closure was achieved by day 6, which is visualized on histology by a thicker cornified layer (Figure 4(C)). Interestingly, when the wounded skin was treated simultaneously with GC and EGF, a complete inhibition of wound healing was observed, similar to that of GC treatment alone (Figure 4(A) and (C)). Furthermore, RA exhibited a minor inhibitory effect on wound healing (Figure 4(B)), whereas when present with EGF the two cancelled out one another, and the wound healing was very similar to that in control

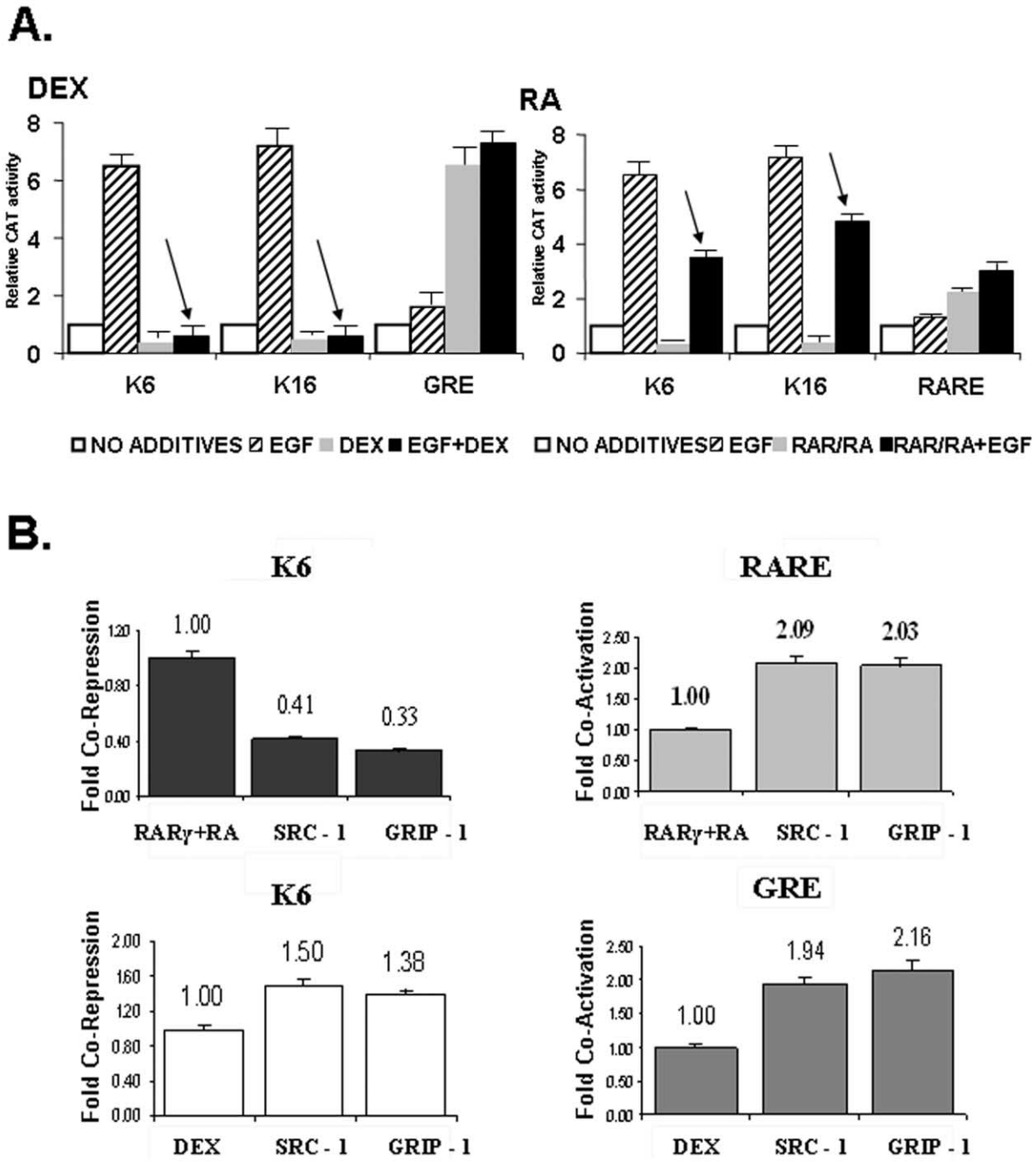


**Figure 5.** GC, but not RA, block EGF-mediated induction of K6 and K16 expression. (A) Co-transfections of primary human keratinocytes with K6/K16, GRE and RARE CAT constructs are shown. The data are presented as relative CAT activity, a measure of actual CAT activity normalized for total protein. The results show that DEX blocks EGF induction of K6/K16 expression, whereas RA does not. K6/K16 are repressed by DEX and RA. However, in the presence of EGF, only DEX completely blocks EGF-mediated K6/K16 induction, whereas RA does not. Interestingly, when present simultaneously, neither EGF nor RA has a dominant effect, but rather balance each other, having a co-dominant effect on transcription. Positive controls used in these experiments are GRE (containing a consensus glucocorticoid response element) and RARE (containing a retinoic acid response element). As expected, they were induced by their corresponding receptors, whereas none was regulated by EGF. (B) Co-activators SRC-1 and GRIP-1 become co-repressors of RAR, but not GR, in the context of K6 promoter. Basic regulation is designated as 1, and the graphs show fold co-repression/co-activation. Co-transfection of either SRC-1 or GRIP-1 enhanced repression of K6 promoter by RARγ, resulting in further repression. Therefore, in the context of K6RE, co-activators SRC-1 and GRIP-1 act as co-repressors in the presence of RA. They show no effect on GR-mediated repression of K6, even though RAR and GR receptors target the same K6 response element. GRE (containing a consensus glucocorticoid response element) and RARE (containing a retinoic acid response element) were used as a positive controls. As expected, co-activators enhanced their induction by corresponding receptors.

skin (Figure 4(B) and (C)). Therefore, we conclude that, unlike RA, GC act as a dominant inhibitor of EGF during epidermal wound healing.

To determine whether the GC dominant effect occurs at the transcriptional level, we analyzed the activity of K6 and K16 promoters in the presence and in the absence of EGF, DEX, RA, and combinations of EGF+DEX and RA+DEX in co-transfection experiments. We used the GRE (containing a consensus glucocorticoid response element) and RAR element (RARE; containing a retinoic acid response element) as positive controls. As expected, we found that the activity of K6/K16 promoters paralleled the effects we found on keratinocyte migration and wound healing: DEX repressed their activity in the presence or in the absence of EGF, whereas RA inhibited K6/K16 by itself, but was co-dominant in the presence of EGF (Figure 5(A)). Interestingly, both GR and RAR target the same response element in K6 and K16 promoters, having a similar effect on their transcriptional regulation (repression).[14,16] However, in the presence of EGF, only GR dominantly represses their expression, whereas RAR does not.

To understand how GR, but not RAR, blocks the EGF-mediated induction on the molecular level, we focused on the role of co-regulators. To determine the role of co-regulators in the context of these promoters and specific receptor configurations (homodimers of RAR and monomers of GR),[14,17] we tested the co-activators GRIP-1 and SRC-1 in co-transfection experiments. As expected, they enhanced GR-mediated and RAR-mediated activation of positive controls, GRE and RARE, respectively. Interestingly, we found that in the context of the K6 and K16 promoters, both GRIP-1 and SRC-1 act as co-repressors of RAR, but they do not affect suppression by GR (Figure 5(B) and data not shown). SRC-1 and GRIP-1 enhanced repression of K6 promoter by RAR by 2.45-fold and threefold, respectively, whereas they have very little effect on GC regulation. This means that in the context of K6/K16 response elements, the homodimers of RAR recruit co-activators that become co-repressors, whereas the GR monomers do not. We have determined that in this context GR utilizes β-catenin and arginine methyltransferase, CARM-1, as co-repressors (unpublished results). Therefore, although both receptors bind to the same promoter sequences, RAR and GR in this DNA context form different and specific regulatory complexes that have very specific implications for the promoter regulatory pattern: the GR complex inhibits EGF dominantly, whereas the RAR complex acts co-dominantly with EGF.

The K6 promoter is induced by EGF, IL-1 and TNFα through multiple response elements clustered in one region, enhanceosome, at positions −193 to −138.[9,10,11,24] Interestingly, the K6 negative nuclear receptor response element is located in the near vicinity of this cluster of positive regulatory elements, at positions −116 to −87. Therefore, we hypothesize that GR dominance over EGF is caused by the GR complex that switches an enhanceosome to a repressosome, whereas the RAR complex maintains the balance between the two, thus allowing co-dominance (see Figure 7). To test this hypothesis, we used a MAPK inhibitor (UO126) and co-transfections of c-fos and c-jun. If our hypothesis is correct, by using an inhibitor of the EGF pathway (such as UO126) we should be able to shift the balance towards repressosome formation with RAR, in which case K6 should be repressed dominantly by RA, even in the presence of EGF. Conversely, by co-transfecting the expression plasmids containing c-fos and c-jun, we should shift the balance towards the enhanceosome, in which case K6 should be induced dominantly. Indeed, this is what we found. RA strongly repressed K6 expression in the presence of EGF and UO126, whereas co-transfection of c-fos and c-jun (AP-1) in the absence of EGF induced K6 expression even in the presence of RA (Figure 6(A)). The presence of the co-activator GRIP-1 in both experiments enhanced the regulation: in the case of UO126, it enhanced the RA-mediated repression, whereas in the case of c-fos/c-jun it enhanced the activation (data not shown). This further suggests that GRIP-1 may participate as a component of both the enhanceosome and the repressosome.

To test the second part of our hypothesis, i.e. that GR, by converting an enhanceosome to a repressosome, dominantly inhibits expression of K6, we pre-treated keratinocytes with DEX for six hours before transfection. The pre-treatment activates the endogenous GR. When c-fos and c-jun expression plasmids were co-transfected with K6CAT into these keratinocytes, no regulation was found (Figure 6(B)). Please note that co-transfection of c-fos/c-jun activates K6 promoter five- to sixfold.[9,24] Furthermore, addition of DEX in the presence of c-fos/c-jun fully repressed K6 (Figure 6(B)). Lastly, when we tested RARγ activity in the presence of the AP1 complex in DEX pre-treated cells, we found that the pre-activation of endogenous GR represses K6 dominantly in the presence of both AP-1 and RARγ (Figure 6(B)). This suggests that when the GR repressosome complex occupies the K6RE, it is dominant and the enhanceosome is not able to enhance.

Clinical findings suggest that retinoids can rescue the negative effects of GC on wound healing.[25–27] This would mean that pre-treatment with RA (allowing RARγ to occupy the GR binding site) would prevent inhibitory effects of GC. That is exactly what we found in co-transfection experiments of keratinocytes pre-treated with RA six hours before transfection (Figure 6(C)). In RA-pre-treated cells, GC neither suppressed K6 expression nor blocked the AP1 activation.

Taken together, our experiments confirm that K6 expression is a result of fine tuning and a balance between the enhanceosome and repressosome complexes. Additionally, both GR and RAR target and repress the same gene, K6, sharing the same DNA response element, but through different

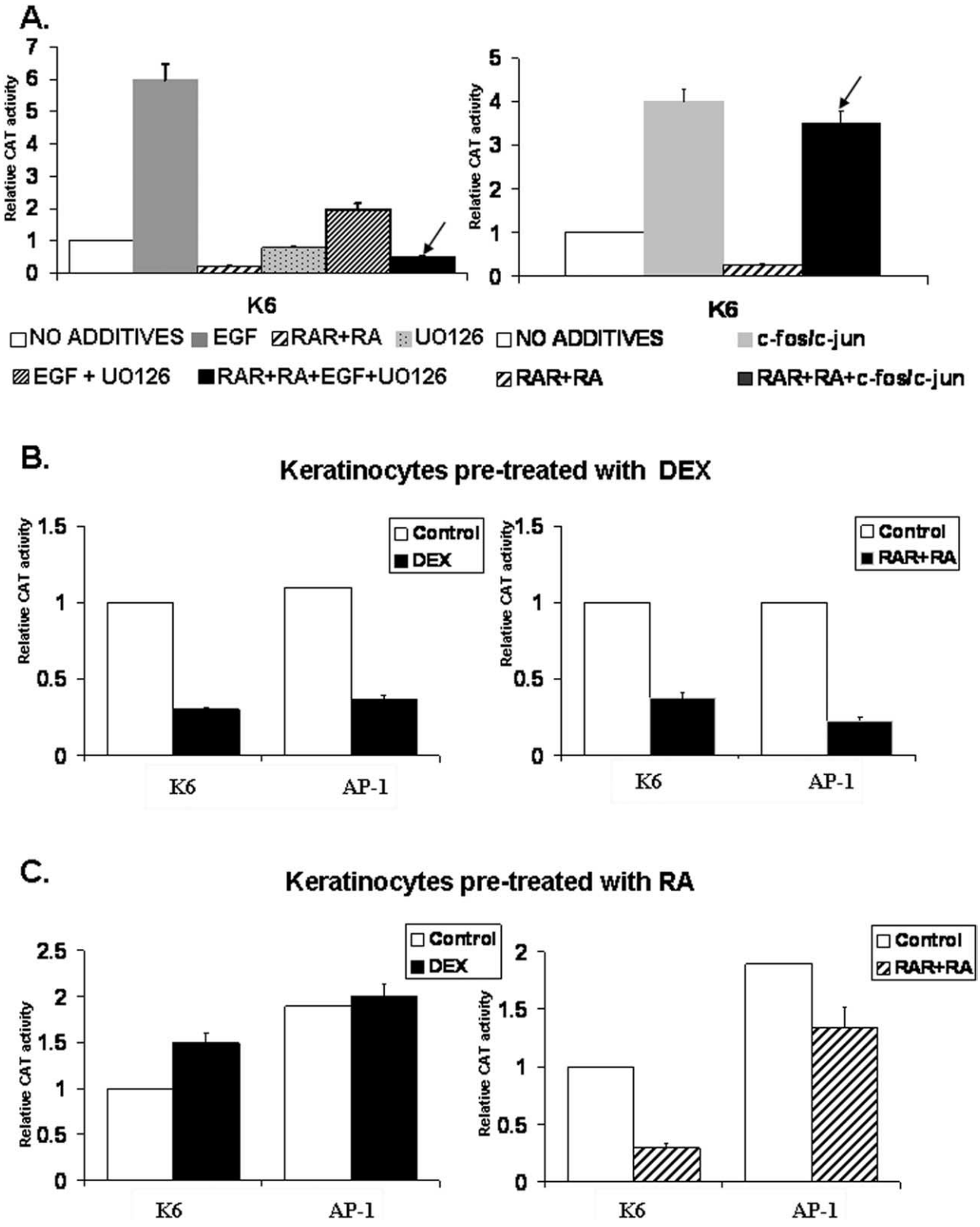


**Figure 6.** Transcriptional balance between GC, RA and EGF can be modified by a MAPK inhibitor, AP-1 overexpression or pre-activation of GR. (A) The balance between RA and EGF can be shifted either by the MAPK inhibitor or overexpression of the AP-1 complex. RA dominantly repressed K6 expression in co-transfection experiments in the presence of the MAPK inhibitor, UO126, which blocks EGF-mediated induction. This means that by blocking EGF, the balance is shifted towards K6 repression by RA. Furthermore, additional co-transfection of 1 μg of c-fos and c-jun expression plasmids shifted dominantly induced K6 expression in the presence of RA. This means that addition of fos/jun transcription factors dominantly shifts the balance towards K6 induction. (B) Pre-activated GR dominantly repress K6 even when AP-1 is overexpressed. Keratinocytes were treated by DEX six hours prior to transfection to activate endogenous GR. This GR pre-activation was sufficient to block AP-1-mediated induction of K6 even when 1 μg of c-fos and c-jun expression plasmids were co-transfected. Therefore, activated GR receptor is a dominant inhibitor of K6 even in the presence of AP-1. (C) Pre-treatment with RA can prevent the inhibitory effects of GC. Keratinocytes were treated by RA six hours prior to transfection to activate endogenous RAR. Activation of RAR was sufficient to block DEX-mediated repression of K6. Furthermore, pre-treatemnt with RA blocked the inhibitory effects of DEX on AP-1 activation.

molecular mechanisms, which leads to entirely different biological consequences.

## Discussion

The wound healing process in its complexity involves many different signals that, in concert through specific transcription factors, coordinate the process. Here, we have analyzed how GC, inhibitors of wound healing, function in the context of EGF, a wound healing stimulator, and RA, a wound healing modulator. Taken together, the results presented here reveal that GC, unlike RA, inhibit keratinocyte migration and wound healing. Furthermore, GC, but not RA, block the EGF-mediated induction of keratinocyte migration and wound healing. K6 and K16, important cytoskeletal components, are one of the first markers of epidermal wound healing. It is believed that their presence in the cytoskeleton allows for enough flexibility to support keratinocyte migration during wound healing. Using keratin K6 and K16 promoters as a paradigm of wound healing-specific genes regulated by GC, RA and EGF, we found that both GC and RA repress the expression of K6 and K16, whereas EGF induces it. Most importantly, we found that GC, but not RA, block the induction of K6 and K16 by EGF, thus confirming that GC block the EGF effects at the level of transcription. The obvious question is what is the mechanism of this repression? We believe that on the K6 gene promoter GR causes a switch from an enhanceosome to a repressosome, leading to a dominant inhibition of K6 expression. Enhanceosomes are large protein complexes comprised of several transcription factors bound to the DNA of the same promoter, co-regulators and histone-modifying enzymes.[4–6] One such complex containing AP1, C/EBP and NFκB induces K6 expression.[10] We have shown that these molecules synergize, providing several hundred-fold higher promoter activity when present simultaneously.[10] Interestingly, the response element to which nuclear receptors bind is in the close proximity of the enhanceosome cluster. If so, how does GR, but not RAR, block this enhanceosome? We note that both receptors target and bind to the same DNA response element with an identical transcriptional result: repression of K6. Yet, we show here that only GR can block the EGF effect. Our analysis of the molecular mechanisms through which GR and RAR regulate keratin gene expression offers the explanation. We believe that the recruitment of the GR–co-regulator complex and its formation causes a disassociation of the enhanceosome and leads to the formation of an inhibitory complex, a repressosome (Figure 7(A)). We have shown that when GR is in place (activated by pre-treatment of keratinocytes by DEX) neither AP-1 nor RAR can implement their effects.

In contrast to GR, RAR also binds to the same K6RE, but does not cause a repressosome formation. We found that in the context of K6RE, the co-activators, such as GRIP-1, SRC-1 and CBP, interact with RAR and become co-repressors. Hence, RAR homodimers can simultaneously bind to the K6RE with the enhanceosome structure, but instead of recruiting novel co-regulators (like GR), they share co-activators with the enhanceosome, converting them into co-repressors (Figure 7(B)). This means that EGF sends the signal "on" and RA sends the signal "off" and when both are simultaneously present the resulting signal is "no change". This means that the co-activators are dynamic structures and are "balancing" between the enhanceosome cluster and the RAR complex. When they are bound to the enhanceosome cluster, transcription is activated; when they bind to the RAR, it is not. This was confirmed in several experiments: in co-transfection experiments, in scratch experiments or wound healing experiments; neither EGF nor RA was dominant, but rather had co-dominant effect on K6 regulation, keratinocyte migration or wound healing rate. Furthermore, we have shown that it is possible to shift the balance either towards the enhanceosome (when AP-1 is in excess) or repressosome (when AP-1 is blocked by UO106).

The question remains, how are these molecular findings relevant to impaired healing. We believe that the balance of all these factors allows for the wound healing process to proceed, and changing the balance of the components of the "-somes" may lead to wound healing impairment. Importantly, we have confirmed *in vivo* that blocking the enhanceosome leads to the development of chronic wounds. NRC-1 is a well-known co-activator of AP-1.[28–30] We have shown that the inactivation of only one copy of this co-activator of AP-1 in a transgenic mouse causes an inhibition of keratinocyte migration, lack of EGF response and development of chronic wounds.[31] This means that when the balance is shifted towards the repressosome, it leads to impairment of healing.

It is interesting that molecular effects of GC, RA and EGF in the context of K6 promoter correlate directly with their effects on cellular and tissue level. K6 is a marker of keratinocyte activation and is found at the leading edge of wounds. It is induced by TGFα and has been linked to EGFR activation, as well as to AP-1-induced transcription.[9,20,24,32] In mice, targeted deletion of the K6a gene resulted in a delay in re-epithelialization from the hair follicle after epidermal wounding. The lack of MK6a affects both proliferation and migration of the follicular keratinocytes *in vivo*.[32] K6 expression is also diminished at the edge of wounds in c-Jun null mutant mice, which exhibit lack of keratinocyte migration and lack of epithelial leading edge formation during wound healing.[33] However, one should keep in mind that inhibition of keratinocyte migration and epithelialization cannot be attributed solely to the suppression of K6. Such complex process occurs upon coordinated efforts of multiple cellular components, including cytoskeleton,

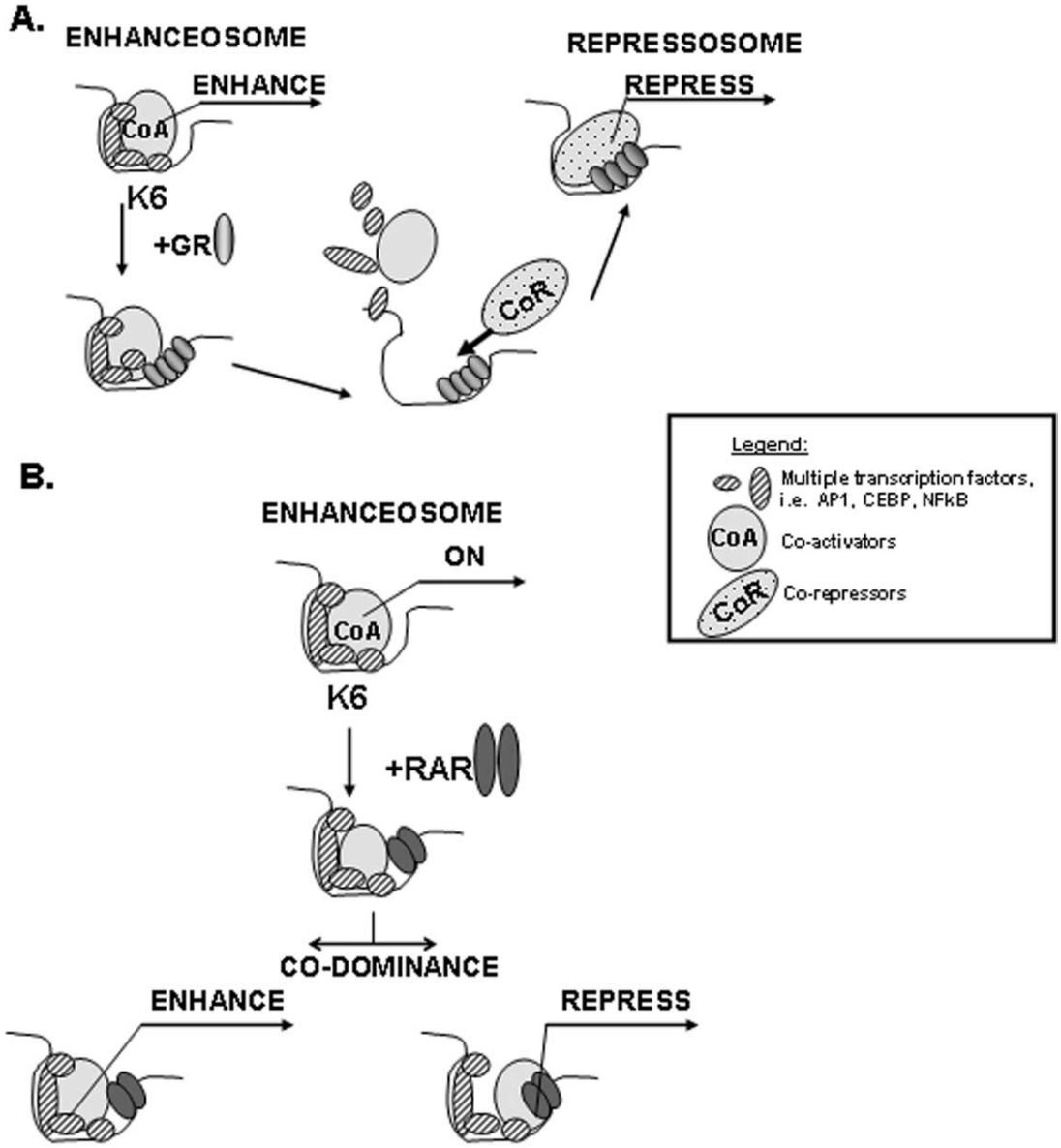


**Figure 7.** Molecular mechanism of GC dominant repression. (A) The cartoon shows our current model incorporating our previous and current findings. In this model, four monomers of GR, when bound to the K6RE, form a complex that, because of its unique monomeric conformation, blocks binding of the enhanceosome complex. This results in dominant repression. (B) RAR homodimers, because of their conformation and interactions with co-activators, may co-exist with the enhanceosome structure. The dynamic equilibrium exists between RAR-repressosome and enhanceosome. Co-activators in this context may interact with the enhanceosome structure at one point and with RAR at another, balancing between the enhanceosome and RAR complex, resulting in a balanced transcriptional regulation.

adhesion molecules and matrix deposition to name a few. The complexity of molecular interactions that occur on K6 promoter that we describe here is representative example of how GC, RA and EGF, when present simultaneously, may regulate epidermal genes that participate in the process of keratinocyte migration and epithelialization. The direct correlation of molecular mechanisms that regulate K6 expression and cellular/tissue behavior as well as clinical findings after treatment with these factors is fascinating, but is not a simple cause-and-consequence relation.

It has been shown repeatedly over the last 30 years that some of the negative effects on wound healing mediated by treatment with GC can be rescued by administration of retinoids in the early phase of wound healing.[25,26] Although most of the past studies have focused on the effects that GC and RA have on endothelial cells and fibroblasts, our results show that the keratinocyte biology and gene expression during wound healing is affected strongly by both GC and retinoids. Interestingly, retinoids can prevent experimental Cushing syndrome.[34] Although antagonistic effects of steroids and retinoids on growth factors were suggested before, this is the first time that the

molecular mechanism of such antagonism has been identified. These clinical findings can be explained by our model. It is conceivable that the RAR binding to the K6RE prevents binding of GR and blocks repressosome formation, thus rescuing the activated keratinocyte phenotype.

Another fascinating point is that both GR and RAR target and repress the same gene, K6, sharing the same response element, yet through a different molecular mechanisms, which lead to entirely different biological consequences. This further emphasizes the importance of gene expression studies in a complex environment of multiple factors that are all present simultaneously *in vivo*. Similarly, the elegant work of Gibson *et al*. showed that RA blocks anti-proliferative pro-differentiating effects of vitamin D only at specific ligand concentrations, but interestingly had different effects on involucrin.[35] Altogether, this represents an excellent example of gene and tissue specificity of hormone action.

Our findings may explain the limited success of growth factor therapy of chronic wounds. It is the combinatorial effect of complex multiple transcription factors that orchestrates the wound healing process. Furthermore, each of the transcription factors exerts its own tissue specificity by targeting specific genes and recruiting a particular set of co-regulators and histone modifying enzymes. Thus, the complexity results in a selective process of "mix and match" among regulatory complexes.[36,37]

Another point raised by the results presented here is the possibility that GC may serve as wound healing stop signals during the normal process. Very little is known about the "stop" signals, i.e. signals that "reset" the program from keratinocyte proliferation/migration (wound healing) to differentiation (normal). The question is how this specific GC regulatory mechanism becomes implemented in the epidermis and its biological significance. It is tempting to speculate, in particular because of the specificity identified in the mechanism we describe, that GC may act as a stop signal, instructing keratinocytes to stop their activating cycle and "retire" to the differentiation pathway. This would imply that GC becomes active in the late phase of wound healing. We are currently investigating this possibility.

Taken together, we have identified complex molecular crosstalk between a wound healing inhibitor, GC, a wound healing stimulator, EGF, and a wound healing modulator, RA. Molecular mechanisms regulating one of the participating genes, K6, suggest that pre-treatment with GC implements the inhibition, whereas both clinical and our experimental data show that pre-treatment with RA may prevent the inhibitory effects of GC. It is apparent, based on our findings on both molecular and tissue levels, that the outcome of the effects of these factors depends on the relative amounts of the ligands and their respective receptors, as well as their co-regulators. This means that the relative amounts of each of these factors are direct determinants of the wound healing process: if it will occur, how quickly it will proceed and when it will stop.

## Materials and Methods

### Skin specimens, wounding and histology

Specimens of normal human skin obtained from reduction mammoplasty (approved protocol H#9796-03) were maintained at the air/liquid interface using keratinocyte basal medium (Gibco-BRL). Specimens were divided into two groups: control and wounded. Topical glucocorticoid Cormax® (Clobetasol Propionate cream 0.05%, Oclassen Pharmaceuticals) or topical retinoid Tazorac® (Tazarotene cream 0.05%, Allergan, CA) was applied once a day using a sterile Q-tip applicator.

Samples were wounded by creating 4 mm punch biopsies through the reticular dermis. All specimens were collected and frozen sections were prepared using OCT compound (Tissue Tek). The 5 μm thick sections were stained with hematoxylin and eosin. For immunostaining with K6 antibody we used K6 monoclonal antibody (Progen) that was reconstituted in 1 ml of 1XPBS and used at 1 : 10 (v/v) dilution at 4 °C. The signal was visualized using a Vectastain ABC kit (Vector) following the commercial protocol. The sections were analyzed using a Nikon microscope and digital images were obtained using a Spot RT camera.

Wounds were quantified by planimetry. The edge of each wound was traced and the surface area for each wound was calculated for each time-point and treatment by laboratory members blinded for the experiment. Each experiment was completed for all testing conditions using the skin from the same donor. The experiments were repeated at least three times.

### Cell migration assay

All cells: HeLa, primary human keratinocytes or primary human dermal fibroblasts were grown to 80% confluency. Twenty four hours prior to the experiment, cells were transferred to basal medium; for keratinocytes KBM (Gibco-BRL) and for the others DMEM with 5% stripped serum.[14] On day 0, cells were treated with 10 μg/ml of Mitomycin C (ICN) for one hour, washed with PBS prior to scratch. Scratches were performed using sterile yellow pipette tips and photographed using a Carl Zeiss microscope and a Sony digital camera (DKC-500). Cells were incubated with either: 0.1 μl DEX (Sigma; stock in ethanol), 0.1 μl RA (Sigma, stock in DMSO), 25 ng/ml of EGF or the combinations of RA + EGF or DEX + EGF, for 24 hours and 48 hours and re-photographed in the same fields as was initially done on day 0. These are the ligand concentrations that saturate the receptor(s) identified by detailed analyses of concentration curves for each ligand (data not shown).[38] Experiments were performed in duplicates, three independent times. Cell migration was quantified as described.[39] Three laboratory members blinded for the experiment quantified the surface area that remained uncovered by the cells for each time-point and condition. A total of 30 measurements were taken for each experimental condition and expressed as a percentage of distance coverage by cells moving into the scratch wound area 24 hours and 48 hours after wounding. Three

images were analyzed per condition, per time-point, and averages and standard deviations were calculated.

### Electrophoretic mobility shift assay (EMSA) and footprinting

*Escherichia coli*-expressed DNA-binding domain portion of hGR was a gift from H. H. Samuels and has been described.[14,15] Briefly, the K6 probe was generated by labeling 1 μg of K6f primer (5′-tggagagcatgggctgggccctag) using polynucleotide kinase (Promega) and [γ-$^{32}$P]dATP (Amersham). $1.5\times10^6$ cpm of this primer were used in the primer extension reaction with K6ft (5′-cactatttg taaagcccagcccttcccaacctgcaagctcaccttccaggactagggcc cagcccatgc tctcca) as a template and Klenow endonuclease (Boehringer Mannheim). The product was gel-purified. The same probe was used in EMSA and footprinting.

For EMSA, a total of 30,000 cpm of the probe was mixed with 10 pg, 15 pg or 20 pg of GR-DBD purified receptor proteins and incubated first for 30 minutes at room temperature and then for ten minutes at 4 °C. The incubation was done in a 30 μl volume in 25 mM Tris (pH 7.8), 500 μM EDTA, 88 mM KCl, 10 mM 2-β-mercaptoethanol, 0.1 μg of aprotinin, 0.1 μg of poly(dI-dC), 0.05% (v/v) Triton X-100, and 10% (v/v) glycerol. Samples were loaded onto a 4% (w/v) polyacrylamide gel and separated by electrophoresis (20–25 mA) at 4 °C for two hours with a buffer containing 10 mM Tris, 7.5 mM acetic acid, and 40 μM EDTA (pH 7.8). Gels were dried and exposed to X-ray.

Footprinting was performed as described.[14,40] Two different reactions were performed in parallel: A/G Maxam-Gilbert sequencing (following the standard protocol)[41,42] and DNase I footprinting. We used 50,000 cpm of probe, 25 μl of the binding mix (see gel-shift protocol above) and 0.5 ng, 10 ng, 25 ng, 30 ng and 50 ng of purified receptor protein for the footprinting reaction that was incubated first for 30 minutes at room temperature and then for ten minutes at 4 °C. After 20 minutes of incubation at 4 °C, 50 μl of solution containing 10 mM $MgCl_2$ and 5 mM $CaCl_2$ was added and incubated for one minute on ice. Next, 3 μl of the 1 : 25 dilution of the DNase I (five units/ml of stock), was added and incubated exactly one minute on ice. The reaction was stopped by adding 90 μl of stop solution containing 20 mM EDTA (pH 8.0), 1% (w/v) SDS, 0.2 M NaCl, and 100 μg of yeast RNA/ml. DNA was purified by extraction with phenol followed by precipitation in ethanol. The pellet was resuspended in 1.4 μl of 9 M urea, 1% (v/v) NP-40, and, after mixing, 4.6 μl of formamide loading buffer (USB) was added. All samples were heated at 90 °C for five minutes, chilled on ice, and loaded onto the 12% polyacrylamide sequencing gel, as were the samples from the A/G Maxam-Gilbert sequencing reactions of the same DNA. Gels were subjected to 1000 V of current for one hour, dried on the gel-dryer, and exposed to the X-ray film. The footprint localization was determined by the bands that were protected by the bound protein from cleavage by DNase I, when the footprinted sample lane on the gel was compared with the sample that had no protein in the mix.

### Plasmids

Plasmids pK6CAT, pK16CAT, RARE-CAT were as described.[14,16] Plasmids containing GRE-CAT and RARγ were a gift from Pierre Chambon, expression plasmid GRIP-1 from Michael Stallcup,[43] SRC-1 from Bert O'Maley[44] and c-fos and c-jun from Edward Ziff. All plasmids were grown according to the standard protocol using a Promega kit and the commercial protocol.[16]

### Cell growth and transient transfection

Normal human keratinocytes were grown as described.[14,45] Cells were expanded through two 1 : 4 passages before transfection, which was done at 80% confluency. The Polybrene with DMSO shock method was used to transfect the DNA into the 80% confluent keratinocytes as described.[46] Cells were washed and incubated in the basal medium without EGF and bovine pituitary extract from the day before transfection until harvesting. Each transfection contained 5 μg/dish of keratin-CAT construct. The cells were then incubated with or without 0.1 μM DEX (Sigma) dissolved in ethanol, 0.1 μM retinoic acid (Sigma) dissolved in DMSO or 2.5 ng/ml EGF and harvested 48 hours later. We have constructed concentration curves for each independent ligand and the concentrations that saturate the receptor(s) were used (data not shown).[38] CAT assays were performed using FastCat (Molecular Probes) following the commercial protocol. Cell extracts used for the CAT assay were normalized by total protein determined by the BioRad protein assay. A total of 30 μg of protein was used for each reaction. CAT assay values were quantified by Fluor Imager 575 (Molecular Dynamics). The data are presented as relative CAT activity per 1 μg of protein and normalized for the basic activity of each promoter that was designated as 1. All experiments were performed in duplicate, a minimum of three times.

## Acknowledgements

Our research is supported by the National Institutes of Health grants AR45974, NR08029 DK59424, T3207190, and American Diabetes Association. We give special thanks to Evette Robinson and Heidi Bender for technical assistance.

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***Edited by J. Karn***

*(Received 16 September 2004; received in revised form 10 November 2004; accepted 12 November 2004)*