Retinyl acetate mediates autocrine proliferation and wound healing of keratinocytes through a c-neu (erbB-2)-like receptor

Abstract

Studies were initiated on retinyl acetate, a natural fatty acid retinol ester, to determine its effect on the clonal growth of normal human keratinocytes (NHK) and on the proliferation of HaCaT immortalized human keratinocytes. Both can be propagated in a chemically-defined serum-free medium. Retinyl acetate (RetAc) at physiological concentrations supports the autocrine production of an EGF-like growth factor that stimulates proliferation of both normal and HaCaT cells in serum-free media supplemented only with insulin. The possible cellular mechanism of autocrine stimulated proliferation induced by RetAc was elucidated by inhibiting clonal growth following treatment with a specific receptor tyrosine phosphokinase inhibitor, and by employing indirect immunofluorescence antibody detection microscopy to positively stain c-neu (erbB-2) cell membrane targets. C-neu antibody positively stains the cytoplasm of untreated keratinocytes, which is relocalized to focal adhesion cell surface receptors after alkaline phosphatase treatment, indicating a phosphorylation-labile receptor. By contrast, RetAc-treated keratinocytes stained positively for c-neu at cell surface focal adhesion sites that were labile to dephosphorylation by treatment with alkaline phosphatase. In addition, we investigated the effect of retinyl acetate on wound healing in an epidermal monolayer wound healing model. RetAc inhibited wound closure of damaged epidermal sheets in the wound healing zone at concentrations greater than those that stimulate keratinocyte proliferation. This result and other previous studies indicate RetAc stimulation of keratinocyte proliferation can be employed without EGF to produce a cultured stratified squamous keratinized epidermis suitable for wound healing applications.

Keywords: clonal growth, c-neu proto-oncogene, EGF, HaCaT cells, keratinocytes, retinyl acetate, wound healing

Introduction

Pleiotropic effects of retinoids on cell growth signal transduction and receptor-mediated gene regulation are well documented.1,2 The use of chemically-defined serum free medium (SFM) has identified two protein growth factors, EGF and insulin, that are required for normal human keratinocytes (NHK) proliferation in low calcium (<1mM) SFM medium.1,2 The role of retinoids in regulating proliferation of normal human keratinocytes is less well-understood. For example, all-trans retinoic acid (t-RA) stimulates proliferation in essential fatty acid-supplemented keratinocytes.3 Retinoic acid (t-RA, 10−M) treatment inhibited proliferation of HaCaT cells adapted to serum-free DME/Hams F12 medium, while retinol (ROL, 10−M) did not, but overall growth in this SFM was much curtailed.4 By contrast, a panel of retinoids including t-RA, ROL, 13-cis RA, all inhibited the clonal growth of NHK in SFM supplemented with EGF and insulin in rapidly proliferating NHK.5 The strength of inhibition was linearly correlated with their ability to suppress both ornithine decarboxylase enzyme induction by tumor promoter and papilloma formation in the mouse skin model of tumorigenesis.6 By contrast, t-RA stimulates growth-arrested adult keratinocytes in protein growth factor-deficient SFM.7 These conflicting effects appear to involve differing growth media conditions. Verani et al.8 also reported that induced alterations in membrane intracellular calcium ion fluctuations appear to underlie retinoid growth stimulation.9 Retinoid stimulation of growth-arrested NHK was also found to involve autocrine production of a heparin-binding EGF (hb-EGF), and activation of erbB receptors10 present on suprabasal cells. Moreover, autocrine stimulation via these signaling pathways appears to underlie retinoid-induced epidermal hyperplasia.10 Recently, t-RA was reported to inhibit the expression of the erbB (c-neu) receptor and other proto-oncogenes in several different epidermoid carcinoma cell lines.11 In addition, t-RA reverses the super-induction by alcohol of aryl hydrocarbon hydrylase induced by benzo(a) pyrene in SFM culture of NHK.12 Retinoids also have profound effects on epidermal keratinocyte differentiation in many different tumor cells lines.13 Retinyl acetate (RetAc) is a naturally occurring fatty acid ester of retinol, and is less toxic than t-RA. It is stored in human liver and is involved to metabolic conversion to vitamin A (retinol). Here, we explore the effect of RetAc on the clonal growth of NHK and HaCaT cells treated with different combinations of EGF and insulin. We established that serum can be dispensed with by culturing HaCaT keratinocytes in SFM supplemented with EGF and insulin. This allowed us to conduct detailed clonal assay to determine their minimal growth factor requirements. Clonal growth assay studies also examined the effect of RetAc. Unlike t-RA,7 we report that RetAc stimulates HaCaT clonal growth at physiological levels. We further explored the underlying biochemical events that reveal an autocrine signaling pathway and possible involvement of the c-neu (erbB-2) cytoplasmic receptor and a treatment-induced translocation to focal membrane areas. Finally, we examined the effect

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of RetAc on wound healing using in an in vitro epidermal keratinocyte wound healing model.\textsuperscript{14}

**Materials and methods**

**Materials**

All chemicals were obtained from Sigma-Aldrich Company (St. Louis, MO). erbB (c-neu) antibodies and anti-EGF antibodies were purchased from Oncogene Sciences (Manhasset, NY).

**Cell cultures**

Normal human neonatal foreskin keratinocytes were prepared and maintained in serum free MCDB 153 medium as previously described.\textsuperscript{3} HaCaT cells, a spontaneously immortalized cell line were donated by Dr N. Fusseneg, German Cancer Institute (Berlin, Germany). They were routinely maintained by serial passage in DMEM: 10% fetal calf serum. In preliminary studies, we determined that HaCaT cells can be adapted to growth in serum-free MCDB 153 medium supplemented with EGF (5ng/ml) and insulin (5µg/ml). For clonal growth assays, HaCaT cells were washed three times with 3mL aliquots of ice-cold basal nutrient MCDB 153 medium, seeded in to sterile 35mm\textsuperscript{2} plastic disposable Petri dishes, and refed various combinations of growth factors and RetAc. The dishes were incubated at 37 °C for 10 days and the cells fixed with 50% alcohol and stained with 0.2% crystal violet and photographed previously described.\textsuperscript{4}

**Immunofluorescence -antibody staining**

The technique of indirect immunofluorescence (IIF) was performed according to previous studies.\textsuperscript{14} Briefly, we performed cytochemistry, using the c-neu antibody as follows: normal human neonatal foreskin keratinocytes were first cultured on circular glass chips. At the desired time, the chips were placed in to clean glass Petri plates and the cells fixed with drops of 3:1 acetone: water fixative and air dried. Antibody solutions were applied at the manufacturer’s recommended titer for cytochemistry applications and applied to the fixed cells for 30 minutes at room temperature. To prevent adventitious binding of c-neu antibody, the fixed cells were prewashed with a 0.1% ice-cold solution of BSA, followed by several saline washes. To detect c-neu binding a second rat anti-mouse IgG polyclonal antibody conjugated with fluorescein directed against the c-neu mouse monoclonal antibody was applied and incubated for an additional 30 minutes. The binary antibody- cell complex was rinsed several times with saline and permanent glass slides were prepared for microscopic viewing and analysis using a UV-epifluorescence Nikon phase compound microscope equipped with a Cannon microphotographic camera system.

**Wound healing studies**

HaCaT cells were plated in sterile 35mm\textsuperscript{2} plastic disposable petro dishes at 5x10\textsuperscript{3} cells/cm\textsuperscript{2} and refed SFM containing EGF (5ng/ml) and insulin (5mg/ml) every other day until the cultures reached confluence. The cells were washed 3X with ice-cold basal nutrient MCDB 153 medium and the dishes refed fresh SFM supplemented with insulin and varying concentrations of RetAc. Control dishes were refed SFM supplemented with EFG and insulin. Immediately thereafter, the control and RetAc-treated confluent epidermal keratinocyte sheets were wounded by making a 2.5mm wide scratch along the midline diameter with a fine closed tip 9cm sterile glass Pasteur pipette as previously described.\textsuperscript{15} The dishes were then placed in CO\textsubscript{2} gas humidified incubator at 37 °C for 24 hours, fixed, stained with 0.2% crystal violet stain and photographed. Percent closure of the healed wound area was measured with a 10X eyepiece micrometer ruler and 4X phase objective with a Nikon Diaphot phase contrast microscope.

**Results**

Preliminary studies established that serially- passaged cultures of HaCaT, an immortalized cell line, previously maintained in DMEM: 10% FCS can be cultured in serum-free MCDB 153 medium supplemented with insulin (5mg/ml) and EGF (5mg/ml). The question arose whether it could also be cultured in a protein growth factor-deficient SFM supplemented with RetAc medium.

Figure 1 is an image showing the morphological appearance of HaCaT cells cultured in SFM supplemented with EGF and insulin (A) compared with SFM cultures supplemented with RetAc and insulin only (B). RetAc-treated cultures appear compact resembling HaCaT cultures grown in serum-containing media containing millimole concentrations of calcium ions.

**Figure 1** Phase contrast image showing the typical culture morphology of subconfluent cultures of HaCaT keratinocytes cultured in (A) growth factor-replete SFM or in (B) growth-factor-deficient SFM supplemented with RetAc and insulin. RetAc-treated cultures appear more compact than EGF and insulin –treated cultures. Scale bars shown in white are 50 microns.

Figure 2 shows the clonal growth of HaCaT cells grown in SFM supplemented with insulin and EGF, fixed and stained one day after seeding at 5x10\textsuperscript{3} cells/cm\textsuperscript{2} (A) compared with clonal growth achieved in SFM supplemented with insulin and EGF and fixed and stained 10 days later (B) compared to enhanced clonal growth achieved after 10 days when HaCaT was grown in SFM supplemented with insulin and RetAc (C). The result show that RetAc-treated keratinocytes display enhanced clonal growth relative to EGF plus insulin supplemented cultures.

Further clonal growth studies, explored growth factor control of HaCaT cells. Induction of autocrine proliferation is validated by the fact that no growth occurs in growth factor-deficient SFM or in SFM supplemented with insulin (5µg/ml) only, while enhanced
clonal growth is observed in standard medium supplemented with 5μg/ml insulin plus 3x10^-8 M RetAc. The moderate clonal growth often observed in SFM supplemented with insulin it might be due to carry-over of EGF or incomplete removal of EGF from cell surface adherence. The above results suggest that RetAc stimulates autocrine production of EGF-like activity that subsequently binds to EGF receptors. This hypothesis was investigated using a selective inhibitor of receptor tyrosine protein phosphokinase (RTK1, PD98059) on the enhanced clonal growth of HaCaT cell stimulated to proliferate by RetAc.

These results suggest that retinoid-induced clonal growth of HaCaT keratinocytes is dependent on phosphorylation of the tyrosine residue in an activated EGF-like growth factor receptor protein required for downstream signal transduction and phosphorylation events necessary for cell proliferation. To examine this possibility, we investigated the effect of RetAc-treatment on erbB-2 (c-neu) expression using the technique of IIF microscopy detection of c-neu antibodies. Preliminary IIF studies, showed that c-neu antibody stain monopolar membrane located infocal adherence plaques, a situation that prevails in proliferating keratinocytes cultures. The effect of RetAc on c-neu expression in keratinocyte cultures propagated in SFM was also examined by IIF microscopy and detection of c-neu antibody staining as shown in Figure 4 (C). For the RetAc-treated cells the c-neu stable phosphorylation state is restricted to monopolar focal adherence areas. Figure 4 (D) shows that these membrane located c-neu phosphorylation sites are lost after AP treatment.
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Our results imply that RetAc activates proliferative skin disorders and wound healing. Moreover, the ERK signaling pathway has also been implicated in hyperproliferative epidermal skin graft, where the phosphorylation state is more stable, but can be further dephosphorylated by AP-treatment. Again, these results combined with the RTK1 loss of autocrine growth capacity in HaCaT-treated cells suggest that RetAc treatment activates c-neu-like receptors through altering the cellular location of the phosphorylation sites to target membrane associations.

Finally, our wound healing results show that physiological concentrations of RetAc do not impair the wound healing response in scratch wounded HaCaT monolayers, except at toxic concentrations. Healing under these conditions involves autocrine ligand production and activation of the erbB phosphorylation signaling pathway. These studies support our earlier disclosure, that keratinocyte cultures supplemented with insulin-like growth factor-1 (IGF-1) and RetAc in EGF-deficient SFM undergo stratified squamous keratinizing terminal differentiation suitable for preparation of an autologous cultured epidermal skin graft, and for other wound healing applications.

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Conflict of interest

The author declares that there is no conflict of interest.

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