



Autonomous growth of BALB/MK keratinocytes transfected with a retroviral vector carrying the human epidermal growth factor gene

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Abstract

Epidermal growth factor (EGF), which promotes epidermal regeneration and wound closure, is important for the proliferation and differentiation of epidermal and epithelial tissues in animals. Exogenous EGF is a promising therapeutic agent for wound healing, but its general use is restricted by the limited availability of this protein. In this work, we show that the transfection of mouse BALB/MK keratinocytes, which are totally dependent on EGF for growth and migration, with mature cDNA for human EGF via a retroviral vector abolished the cells requirement for exogenous EGF. The transformed cells had normal morphology and a growth rate that varied according to the source of the retroviral vector used. Keratinocyte transfection with EGF cDNA provides a time- and cost-efficient means of culturing keratinocytes and yields cells that may be useful for skin grafting.

Key words: BALB/MK keratinocytes, epidermal growth factor, retroviral vector.

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Introduction

Epidermal growth factor (EGF) was first isolated from murine salivary glands (Cohen, 1962). The complete human EGF (hEGF) sequence (1,207 amino acids) includes an active EGF sequence, eight EGF-like units and a hydrophobic sequence at the carboxy terminal end characteristic of an integral membrane protein. Active, mature EGF contains only 53 amino acids, six of which are cysteines (Carpenter and Cohen, 1990).

EGF stimulates the proliferation and differentiation of epidermal and epithelial tissues in animals, and is therefore involved in wound closure and epidermal regeneration (Carpenter and Cohen, 1979; Moulin, 1995; Gibbs *et al.*, 2000). In addition, cells cultured with medium containing EGF share many of the features observed during wound regeneration (Gibbs *et al.*, 2000). EGF is synthesized by several cells involved in the regulation of wound healing, including platelets and activated macrophages. Human keratinocytes express four members of the EGF family, namely, TGF- α (transforming growth factor- α), HB-EGF (heparin-binding EGF), amphiregulin and epiregulin (Hashimoto, 2000); the expression of EGF itself in kerati-

nocytes has not yet been demonstrated. EGF receptors are expressed in cells directly involved in wound healing, including skin keratinocytes, fibroblasts, vascular endothelial cells and gastrointestinal epithelial cells (Schultz *et al.*, 1987, 1991).

Local and systemic applications of a number of growth factors have been used to treat chronic wounds (Brown *et al.*, 1986). Although EGF is an important factor in wound healing, this mediator also has important interactions with other growth factors such as PDGF (platelet-derived growth factor), IGF (insulin-like growth factor) and TGF- β (transforming growth factor- β). Additionally, EGF receptors are important for the autocrine growth of normal epidermis (Yates *et al.*, 1991). In wounded skin, the transient but dynamic elevation of EGF receptors during wound healing contributes to the migratory potential of keratinocytes (Hudson and McCawley, 1998), thereby enhancing re-epithelialization of the wound.

Topically applied growth factors can accelerate healing by inducing the migration and proliferation of target cells, and exogenous EGF is a promising therapeutic agent in this process (Brown *et al.*, 1986; Steed, 1998). Ideally, a continuous supply of EGF is required for optimal healing during the early stages of repair, but this is not always feasible. An attractive alternative to the constant application of exogenous growth factor is *ex vivo* gene transfer by retroviral vectors that would allow the continuous delivery of

EGF to the wound. In this approach, a small piece of tissue isolated from a patient is expanded into a large number of cells in culture (Green *et al.*, 1979), transfected with retroviral vectors and transplanted to the site of the lesion. Using this approach, several authors have successfully introduced a number of genes encoding proteins such as human growth hormone (Morgan *et al.*, 1987), clotting factor IX (Gerrard *et al.*, 1993) and apolipoprotein E (Fenjves *et al.*, 1994). The insulin-like growth factor-1 gene (IGF-1), which encodes another important non-autocrine growth factor for keratinocytes, has been successfully transferred to keratinocytes by retroviral vectors; the resulting cells were no longer dependent on exogenous IGF, but still required supplementation with exogenous EGF (Eming *et al.*, 1996).

In this report, we describe the successful transduction with a retroviral vector and expression of mature human EGF (hEGF) cDNA in mouse BALB/MK keratinocytes (Weissman and Aaronson, 1983) that are normally totally dependent on EGF for growth and migration.

Materials and Methods

Retroviral vectors

A vector containing hEGF cDNA was obtained from the American Tissue Culture Collection (ATCC, catalog no. 20658). The plasmid was digested with *Bam* HI to release a 1.7 kb insert that was subcloned into pBluescript SK (Stratagene) pretreated with the same enzyme. From this product, a cassette containing the hEGF sequence with the signal peptide sequence was isolated with *Eco* RI and *Sal* I, and subcloned into the retroviral vector pLXSN (Miller and Rosman, 1989) previously digested with the same enzymes. This vector was denominated as LESN.

The method for virus production has been described in detail elsewhere (Miller *et al.*, 1993). Briefly, the vectors LESN and LXSN (control) in plasmid form were used to transfect PE501 ecotropic packaging cells, and the supernatants were used to infect PA317 amphotropic packaging cells. After selection with G418, resistant colonies were isolated and expanded to generate clonal vector-producing cell lines followed by determination of the viral titer.

DNA sequencing

DNA was sequenced by the dideoxy method (Sanger *et al.*, 1977) using a T7 Sequenase kit (USB Amersham Life Science) according to the manufacturers instructions. Three primers based on the vector pBluescript SK (Stratagene) (Reverse, KS, M13-20) were used for this sequencing.

Cell culture and virus production

The amphotropic retrovirus-producing cell clone PA317/LESN was cultured in Dulbeccos modified Eagle medium (DMEM) with high glucose (4.5 g/mL), supplemented with 2 mM glutamine, 200 U of penicillin/mL,

200 mg of streptomycin/mL and 10% fetal bovine serum (FBS; Gibco) at 37 °C in a humidified atmosphere with 5% CO₂.

The murine keratinocyte cell line, BALB/MK (kindly provided by Dr. Stuart A. Aaronson), was cultured in Eagles minimum essential medium (EMEM; Biofluids Inc, Rockvill, MD) with low Ca²⁺ (0.05 mM) supplemented with 10% FBS and 5 ng of EGF/mL (Sigma) at 37 °C in a humidified atmosphere with 5% CO₂.

Cell migration assay

BALB/MK cells (5 x 10⁴ per well) were seeded on 24-well plates and PA317/LESN or PA317/LXSN (control) cells (3.5 x 10⁵ per well) were similarly seeded on 12-well plates. After 24 h, the culture medium was replaced with fresh medium. The following day, viruses were collected from PA317/LESN or PA317/LXSN cells and centrifuged at 10,000 x g for 1 min in an Eppendorf centrifuge. In the plate containing BALB/MK cells, a yellow pipet tip was used to scrape a line through the cell monolayer in each well and the wells were then washed with PBS (phosphate-buffered saline) to remove the detached cells. This was followed by the addition of 0.5 mL of virus solution (1 x 10⁵ cfu) containing 8 µg of Polybrene/mL to each well of BALB/MK cells. Cell migration was observed with an inverted microscope (Nikon). After 48 h, the cells were washed with PBS, fixed with methanol:acetone (1:1, v/v) for 4 min and stained with 0.5% Coomassie brilliant blue G in 35% ethanol and 10% glacial acetic acid for 2 min. The cells were then washed with distilled water and photographed.

Results and Discussion

Vectors and PA317 packaging cell clones

In this work, BALB/MK cells were transfected with the retroviral vector LESN. Initially, the DNA of a vector containing hEGF cDNA was sequenced and found to contain a 1.3 kb cassette with sequences for the *S. cerevisiae* signal peptide and mature hEGF between *Eco* RI and *Sal* I restriction sites (Figure 1). The amino acid sequence for hEGF contained three changes in the N-terminus compared to the original sequence reported by Bell *et al.* (1986). However, these changes do not affect the proteins biological activity (ATCC product information), and this was confirmed by the cell migration assay described below.

In the experiments described here, an expression cassette containing only the mature hEGF sequence was used since the entire hEGF cDNA is > 5 kb and such a large insert tends to decrease the viral titer of packaging clones. As expected, most of the PA317/LESN clones had a viral titer > 1 x 10⁵ cfu/mL, particularly clone 9 which had a titer 10-fold greater than most of the other clones. In all of the clones shown in Table 1 the LESN vector was in-

5'- Eco RI ... Pst I ...
 ATGTTGCTGTTTTGCCATTTTCCAACAGACACAAATAACGGGTATTGTTTATAAATACT
 METLeuLeuPheCysHisPheProThrAspThrAsnAsnGlyLeuLeuPheIleAsnThr
 ACTATTGCCAGCATTGCTGCTAAAGAAGAAGGGTATCTTTGGATAAAAAGAGAGGCTGAA
 ThrIleAlaSerIleAlaAlaLysGluGluGlyValSerLeuAspLysArgGluAlaGlu
 GCTGAAGCTAACTCCGAATGTCCATTGTCCCACGACGGTACTGTTTGCACGACGGTGT
 AlaGluAlaAsnSerGluCysProLeuSerHisAspGlyTyrCysLeuHisAspGlyVal
 TGTATGTACATCGAAGCTTTGGACAAGTACGCTTGTAACTGTGTTGTTGTTACATCGGT
 CysMETTyrIleGluAlaLeuAspLysTyrAlaCysAsnCysValValGlyTyrIleGly
 GAAAGATGTCAATCAGAGACTTGAAGTGGTGGGAATTGAGATGA ... Sal I-3'
 GluArgCysGlnTyrArgAspLeuLysTrpTrpGluLeuArg---

Figure 1 - DNA sequence of hEGF subcloned into the retroviral vector. The open reading frame of hEGF is underlined and the signal peptide is in italics. Codons that differ from the original hEGF sequence (Bell *et al.*, 1986) are in bold.

tegrated into the cellular genomic DNA (not shown), as is characteristic of retroviral vectors (assayed by Southern blotting).

Effects of EGF on BALB/MK cells modified with LESN

Initially, we determined effects of exogenous EGF on the growth of BALB/MK cells by using the cell migration assay. Since EGF is one of the most important factors for stimulating cell growth, we examined the ability of a combination of EGF (10 ng/mL) and 10% FBS to promote cell growth compared to the response triggered by EGF and FBS separately. The concentration of EGF used was chosen based on a dose-response curve for this growth factor in BALB/MK cells in which concentrations > 10 ng/mL did not significantly increase the cellular response beyond that seen with 10 ng/mL.

As expected, BALB/MK cells cultured only with EMEM showed no observable migration (Figure 2A). In contrast, cells incubated with 10 ng of EGF/mL or 10% FBS showed intermediate migration when compared to those incubated with both (Figures 2B-D). The effect of

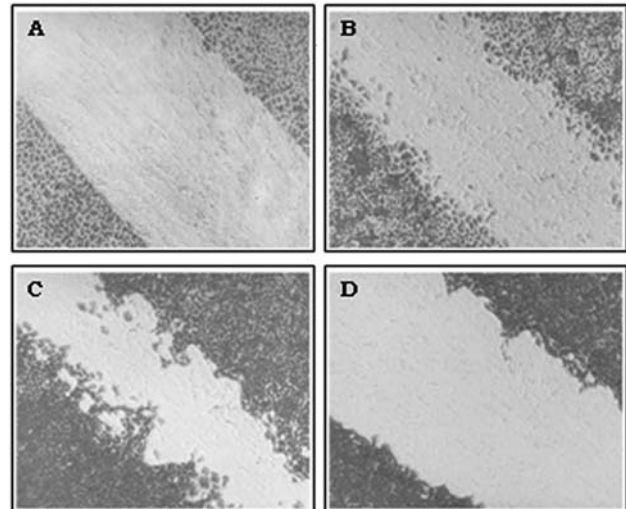


Figure 2 - Effect of EGF and/or FBS on BALB/MK cell migration. BALB/MK cells were grown as described in Methods. Except for the day of virus infection, the medium was replaced by new one without EGF and FBS (A), with 10 ng EGF/mL (B), with 10 ng EGF/mL and 10% FBS (C) or with 10% FBS (D). After incubation for 48 h, the cells were fixed and stained with Coomassie blue. Magnification: 100X.

EGF and FBS on BALB/MK cell growth differed: cells incubated with EGF were more dispersed (indicating motogenic activity) whereas those incubated with FBS formed a dense layer (indicating greater mitogenicity and less motogenicity), as previously reported for primary cultures of keratinocytes (Rheinwald and Green, 1977; Barrandon and Green, 1987).

Since cellular migration was greater in medium containing FBS and EGF, we used medium containing 10% FBS to assess the migration of BALB/MK cells modified with LESN. Of the PA317/LESN clones shown in Table 1, clones 2, 4 and 6 had more pronounced foci of cell migration (Figure 3). However, the cellular migration stimulated by the PA317/LESN clones was not totally independent of EGF since during the assay exogenous EGF was added to the medium to facilitate retroviral infection (see Materials and Methods).

To demonstrate the self-stimulated growth of transformed BALB/MK cells, the cells were selected with G418 and seeded onto new plates in the absence of exogenous EGF (Figure 4). Cells transformed with clone 6 showed greater growth than those transformed with viruses from PA317/LXSN (negative control) or PA317/LESN clone 9 (low cellular migration) (Figure 4). BALB/MK cells transformed with clone 6 reached confluence 6-7 days after infection, which was similar to the time required for non-transformed BALB/MK cells to reach confluence in the presence of exogenous EGF. In contrast, cells infected with the negative control virus (LXSN) or LESN clone 9 did not reach confluence even after two weeks in culture; indeed, most of the cells died before two weeks if EGF was not added to the culture (not shown).

Table 1 - Viral titer of PA317/LESN clones.

PA317/LESN	Titer (cfu/mL)
Clone 2	4.0 x 10 ⁵
Clone 3	1.0 x 10 ⁵
Clone 4	< 10 ⁵
Clone 5	< 10 ⁵
Clone 6	9.0 x 10 ⁵
Clone 7	3.0 x 10 ⁵
Clone 9	3.8 x 10 ⁶

cfu = colony forming units.

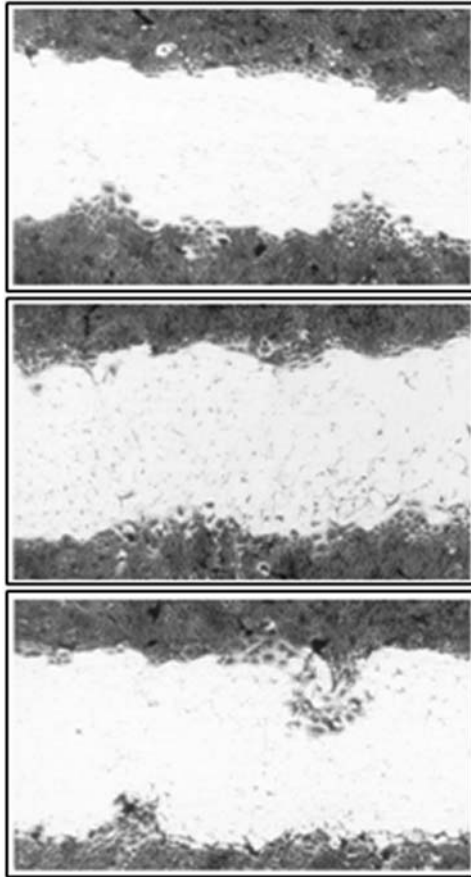


Figure 3 - Cell migration stimulated by EGF produced by BALB/MK cells transfected with LESN. The cells were grown as described in Methods. After transfection, the medium was replaced with fresh solution containing only 10% FBS. After incubation for 48 h, the cells were fixed and stained with Coomassie Blue. Panels A, B and C represent clones 2, 4 and 6 respectively. Magnification: 100X.

For the cell migration assay, BALB/MK cells were transformed with the same volume of virus from PA317/LESN clones. Since the viral titer differed among the clones, the number of infected BALB/MK cells was also different. Consequently, cells incubated with a higher viral concentration supposedly had a greater level of EGF production and greater migration. However, PA317/LESN clone 9, which had a viral titer $> 1 \times 10^6$ cfu/mL, grew less than clone 6, which had a lower titer (Figure 4). The most likely explanation for this discrepancy is that the viral DNA incorporated into the cells suffered mutations or rearrangements that resulted in a low level of EGF gene expression. This hypothesis was supported by the finding that the viral genome in PA317/LESN clone 9 was smaller than that of the other clones, as shown by Southern blotting after digestion with *Sal* I, which cleaves in both LTR regions (not shown). In addition, northern blotting revealed a strong band of EGF RNA only in clone 6 (not shown). The latter finding supports the idea that a rearrangement of DNA in clone 9 resulted in low expression of EGF. Clone 4 of PA317/LESN had a viral titer $< 1 \times 10^5$ cfu/mL but caused

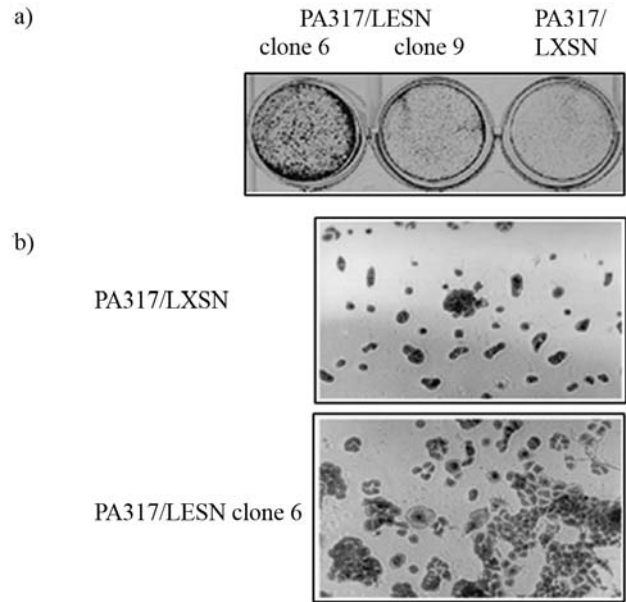


Figure 4 - Self growth of BALB/MK cells transfected with LESN. (a) BALB/MK cells transfected with LESN and selected with 500 μ g G418/mL (active compound) were seeded onto 24-well plates (5 $\times 10^4$ cells/well). After incubation for four days, the cells were fixed and stained with Coomassie Blue. (b). BALB/MK cells modified with the vector LXSN (a) or LESN (b). Magnification: 100X.

greater migration than clones 3 and 9, both of which had higher titers (Table 1 and Figure 3), probably also because of rearrangements in its genomic DNA (not shown). Together, these results indicate the need to carefully analyze genetically modified cells in order to identify the DNA rearrangement involved.

In conclusion, keratinocytes transformed by introducing the hEGF gene can be cultured without exogenous EGF. These modified cells should be useful for short-term skin grafting because they do not require a continuous supply of exogenous EGF. Since most keratinocytes have a limited life span, EGF production *in vivo* will cease when the transformed cells die, thereby minimizing the danger of keratinocytes permanently expressing this growth factor. Nevertheless, since keratinocytes that have been genetically modified by retroviral vectors are subject to insertional mutagenesis that can activate oncogenes, these modified cells require rigorous genetic analysis before their long-term use *in vivo* is approved.

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