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Immunolocalization of HB-EGF in Human Skin by Streptavidin-Peroxidase (HRP) Conjugate Method

Inmunolocalización de HB-EGF en Piel Humana por el Método de Conjugado Estreptavidina-Peroxidasa (HRP)

[*]P. Castrogiovanni; [**]V. Mazzone & [**]R. Imbesi

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SUMMARY: EGF family growth factors consists of growth factors, such as transforming growth factor (TGF)-a, heparin-binding EGF-like growth factor (HB-EGF), amphiregulin (AR) and epiregulin, autocrine growth factors in normal human keratinocytes. HB-EGF is mitogen for epithelial cells and like other members of the EGF family, HB-EGF exerts its biological effects via interaction with the EGF receptor (EGFR). HB-EGF is an autocrine growth factor for human keratinocytes, and has a possible role as a paracrine growth factor for fibroblast. Our report concerning immunohistochemical localization of HB-EGF in normal skin by using the streptavidin-peroxidase (HRP) conjugate method, confirms previous data, revealing specific patterns of HB-EGF localization. Identification of HB-EGF in cells of epithelial origin suggests its autocrine and/or paracrine roles in epithelial cell maintenance. Our report especially wants to give a technical contribution, easy to manage and with evident results. A simple technique that does not require use of sophisticated equipment.

KEY WORDS: Growth factors; Keratinocytes; Streptavidin-peroxidase conjugate.

INTRODUCTION

EGF family growth factors consists of growth factors, such as transforming growth factor (TGF)-a, heparin-binding EGF-like growth factor (HB-EGF), amphiregulin (AR) and epiregulin, autocrine growth factors in normal human keratinocytes and that induce each other via a cross-induction mechanism (Shirakata *et al.*, 2003). EGF family trigger an intracellular signalling cascade upon binding to their receptors, causing changes within the target cells. It has been reported that keratinocyte migration and proliferation are predominantly mediated by autocrine EGFR activation (Stoll *et al*., 2010). EGF family growth factors efficiently regulate keratinocyte growth through auto-and cross-induction pathways (Hashimoto, 2000). The differential expression profiles seen in cross-induction and differentiation suggest that these factors have distinct, non-redundant biological functions (Shirakata *et al.*, 2000). Tissue distribution, molecular characteristics, receptor binding, receptor affinity differ among these four keratinocyte autocrine growth factors. These observations suggest that the expression of individual growth factor is quite different and these EGF-related peptides are involved in different stages of proliferation, differentiation, and development (Shirakata *et al.*, 2000).

HB-EGF is mitogen for fibroblasts, epithelial cells and smooth muscle cells, and it was initially recognized as a secreted product of cultured human macrophages (Higashiyama *et al*., 2008). In common with other EGF family members, the HB-EGF precursor is cleaved extracellularly to yield a mature protein comprising approximately 86 amino acids (Tokumaru *et al.*, 2005). Studies have shown that both the mature secreted growth factor as well as its transmembrane percursor are biologically active (Ono *et al.*, 1994). Like other members of the EGF

[*] Department of Bio-Medical Science, University of Catania, Catania, Italy.
[**]Department of Human Anatomy, Diagnostic Pathology, Forensic Medicine and Public Health "G.F. Ingrassia", University of Catania, Catania, Italy.

family, HB-EGF exerts its biological effects via interaction with the EGF receptor (EGFR) (Tokumaru *et al.*, 2005; Takazaki *et al.*, 2004). With few exceptions, EGFR distribution directly parallels sites of HB-EGF immunolocalization. The co-localization of HB-EGF with its signal-transducing receptor lends further support to potential roles of HB-EGF in processes such as skin development, wound healing, and hyperproliferative skin disorders including cancers (Downing *et al.*, 1997). Moreover human keratinocytes produce HB-EGF, which stimulates the growth of these cells in culture, and it is also an important growth factor for re-epithelialization in skin wound healing *in vivo*, in fact normal skin does not require much HB-EGF, but after injury HB-EGF plays a crucial role in wound healing by up-regulating keratinocyte migration too; the synthesis of HB-EGF at the leading epithelial edge stimulates cells, via an autocrine loop, to migrate towards the centre of the wound.

Goal of our report is to give a technical contribution through a simple technique that does not require use of sophisticated equipment. Use of the streptavidin-peroxidase (HRP) conjugate method in samples of normal skin confirms data of literature, revealing specific patterns of HB-EGF localization. So the use of this method may be useful for comparative studies on specific diseases, such as skin cancer, or traumatic events, such as the healing process after injury.

MATERIAL AND METHOD

We used n. 15 bioptic specimens of normal human skin from big toe, obtained with the consent of informed engaged persons; the procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation and with the Helsinki Declaration. Specimens were processed for routine fixation in formalin 4% and inclusion in paraffin. Paraffin sections (about 5 mm thick) were deparaffinized and dehydrated by passages through bioclear and graded concentrations of ethanol, and then were washed in distilled water. Sections were incubated in 3% H2O2 solution for 5 minutes to eliminate endogenous peroxidase activity and washed in phosphate-buffered saline (PBS - pH 7.6). After incubation with normal serum for 15 minutes at room temperature, sections were incubated with polyclonal goat anti-human HB-EGF serum (primary Ac at concentration of 12.5 mg/ml) overnight at 4° C. Sections were washed in PBS, incubated with biotinylated anti-goat antibody (secondary Ac) for 45 minutes at room temperature, and then incubated with streptavidin conjugated to horseradish peroxidase (HRP) for 30 minutes at room temperature. Sections were washed with PBS and then stained with 2.5% solution of 3,3'diaminobenzidine (DAB) for 20 minutes. Sections were counterstained with Mayer's hematoxylin.

Negative control was obtained by substitution of the primary antiserum, with a preimmune serum in which anti-HB-EGF antiserum was preabsorbed with excess amount of the antigen.

RESULTS

Tissue specimens, analyzed by the streptavidin-peroxidase (HRP) conjugate method, have been observed in optical microscopy. In all samples positivity to HB-EGF has been highlighted, this further substantiated by comparison with negative controls (Fig. 1). So it's not a false

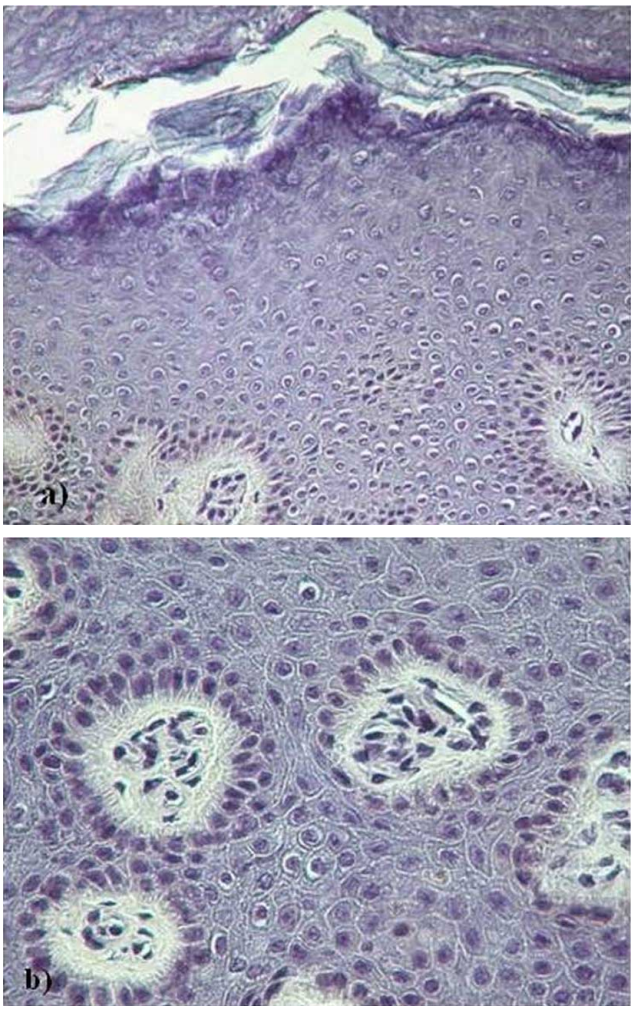

Fig. 1 Negative control with different magnification : a) 40x , b) 80x. There is no evidence of any positivity to HB-EGF, confirming the specificity of the streptavidin-peroxidase (HRP) conjugate method.

positivity, even though the distribution of positivity, in some cases, isn't always uniform in all samples. Positivity to HB-EGF has been detected in the epidermis and never in the underlying dermis (Fig. 2), although, by some studies, HB-EGF seems to have a paracrine role for fibroblasts. In the context of the epidermis, positivity to HB-EGF has been highlighted in the keratinocytes of the basal layer, in a well evident intracytoplasmic position (Fig. 3), sign of a synthesis of this growth factor by keratinocytes. In some cases, a diffuse staining can also detect in the layers above the basal layer, which could mean that in particular metabolic conditions, HB-EGF exerts its paracrine role to facilitate migration of keratinocytes in the upper layers and their further differentiation; in the same cases positivity to HB-EGF has been detected even in the most external layer (Fig. 4), where, however, a well-defined horny layer is not evident; the meaning of this positivity is not clear, also because in the granular layer below it, staining is not, so it may be a false positivity; futhermore at higher magnification, staining doesn't appear so specific (data not shown) as well as in the basal layer. Finally positivity to HB-EGF is evident close to skin annexes such as hair follicles (Fig. 5) which show an outer layer with a pronounced staining of cells, although localization of positivity seems to also be extracellular, validating paracrine function of HB-EGF.

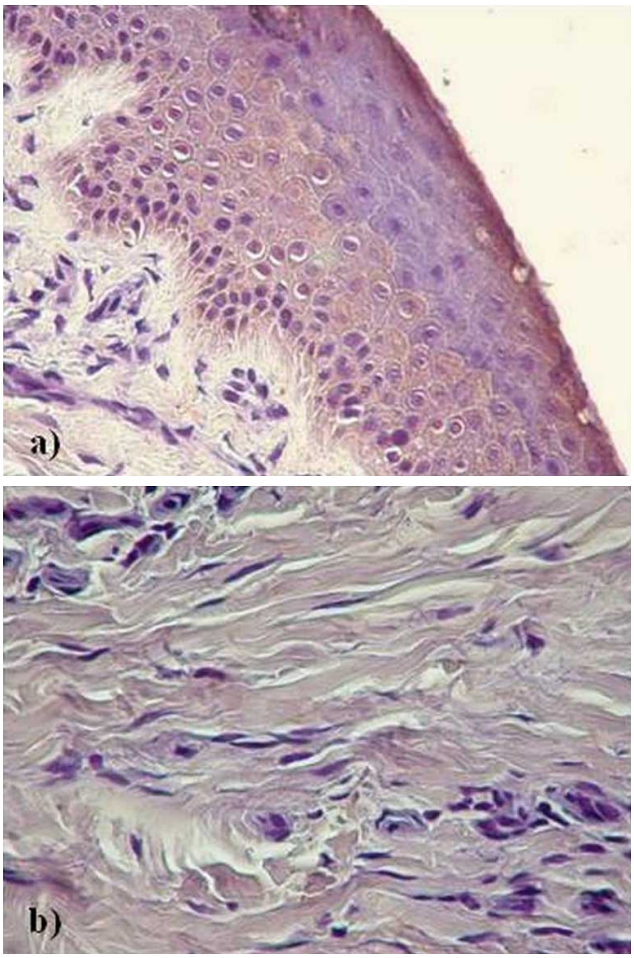

Fig. 2 By comparing the two images it is evident that positivity to HB-EGF is only in epidermis a) 80x and never in dermis b) 80x of the positive specimens analyzed.

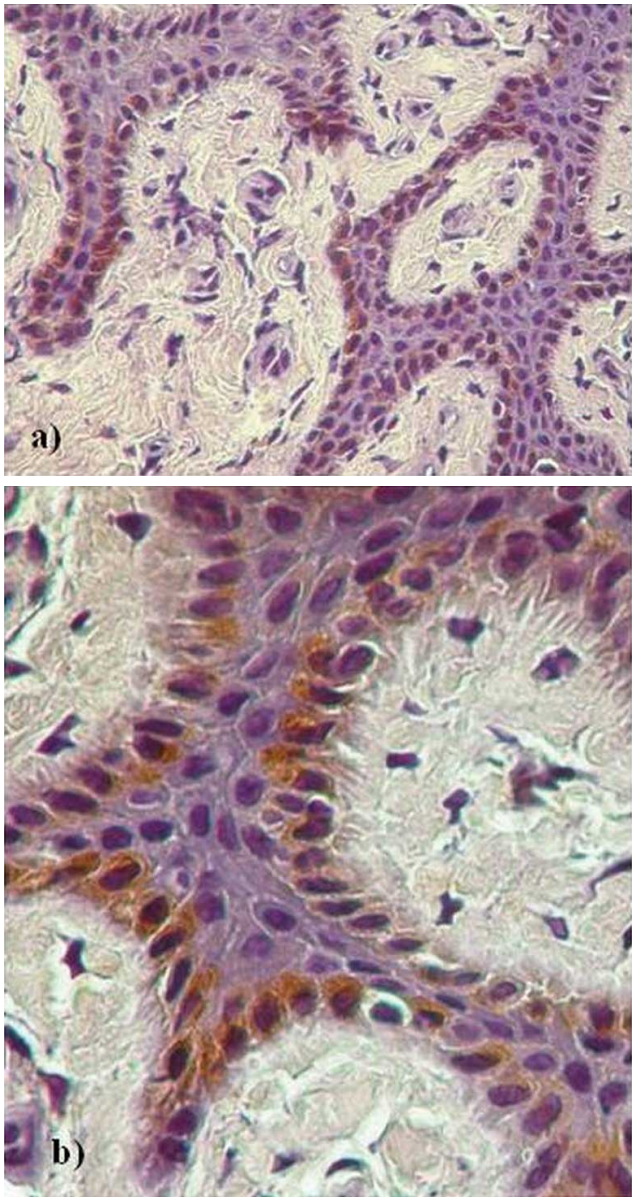

Fig. 3 HB-EGF is more evident in the basal layer of the epidermis a) 80x and at higher magnification b)130x it is possible to appreciate the intracytoplasmic localization (see text).

DISCUSSION

The growth and differentiation of keratinocytes are regulated by a variety of growth factor (Hashimoto; Piepkorn

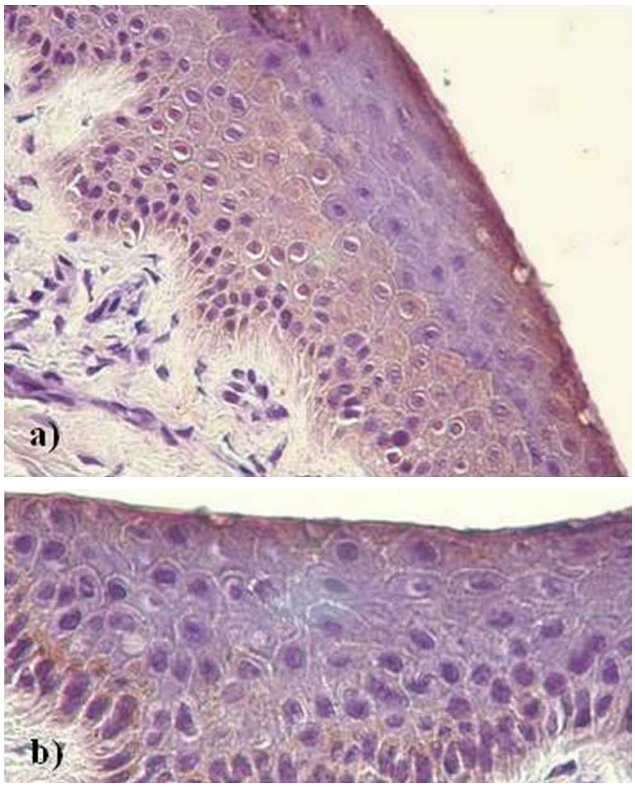

Fig. 4 Diffuse staining in layers above the basal one a) 80x, probably HB-EGF exerts its paracrine role b) 100x (see text).

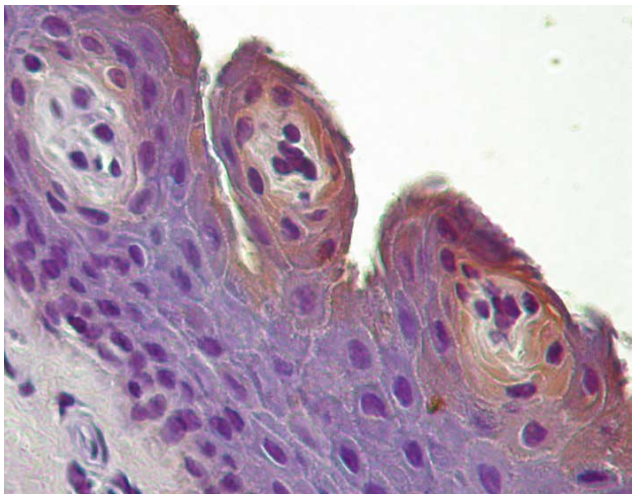

Fig. 5 Positivity to HB-EGF in keratinocytes surrounding the hair follicles. 100x (see text).

et al., 1998). *In vitro* observation suggests that EGF family members play important roles in development, epidermal morphogenesis, skin homeostasis and wound healing (Shirakata *et al*., 2005). All members of the EGF family have one or more EGF domains and a transmembrane domain. The membrane-anchored precursors of EGF family molecules are enzymatically processed externally to release the mature soluble forms, acting as autocrine growth factors (Shirakata *et al.*, 2000; Piepkorn *et al*.; Shirakata *et al*., 2005; Domaszewska-Szostek *et al*., 2009; Paini *et al*., 1997; Higashiyama *et al*., 1994). EGF family autocrine growth factors are capable of inducing other EGF family growth factors; this phenomenon is named cross-induction (Piepkorn *et al*.). Some members of the EGF family act as juxtacrine growth factors in the membrane-anchored form (Joslin *et al.*, 2010). Keratinocytes are capable of producing a large number of cytokines and growth factors, such as TGF-a (Cohen & Ealstein, 2001), HB-EGF (Higashiyama *et al.*, 1994), AR (Paini *et al*.) and epiregulin (Tokumaru *et al.*, 2000), that act as either positive or negative mediators for their growth (Shirakata *et al.*, 2000; Giltaire *et al*., 2011). Members of the EGF family are characterized by their receptor affinities; this suggest that activation of different signal transduction pathways follows receptor binding by EGF autocrine growth factors (Shirakata *et al*., 2000). Cell migration is a complex, coordinated process that allows cells to reach specific destinations during embryonic development, to maintain cellular architecture of self-renewing tissues, repair wounds, and to defend against infectious agents (Schwartz & Horwitz, 2006; Kaverina *et al*., 2002). Signals from several classes of receptors play critical roles in the regulation of the cell movement: integrins, through their ability to signal and form adhesive contacts linking the extracellular matrix (ECM) and the actin cytoskeleton (Alexi *et al.*, 2011), growth factor receptors activated through either autocrine or paracrine pathways, also regulating the actin cytoskeleton, and chemotactic receptors (Iijima *et al*., 2002). The involvement of EGF receptor signalling in various normal physiological processes requiring cell movement and deregulation of the motility response in pathological conditions such as tumour invasion is well documented (Jorissen *et al*., 2003; Jin *et al*., 2008; Kassis *et al*., 2001). There is much evidence linking EGF family members and the EGFR to physiological and pathological processes involving the skin and its epidermal annexes. Epidermal growth factor receptor (EGFR) activation causes autoinduction of multiple EGF family members in keratinocytes (Piepkorn *et al*.; Domaszewska-Szostek *et al*.; Paini *et al.*).

HB-EGF is one of the EGF family members that is initially synthesized as a 208-residue precursor (Edwards *et al.*, 2009). In common with other EGF family members, the HB-EGF precursor is cleaved extracellularly to yield a mature protein comprising approximately 86 amino acids (Tokumaru *et al*., 2005). Mature HB-EGF migrates with a molecular mass of 20-22 kDa. HB-EGF has been detected in pig uterine epithelium (Kim *et al.*, 1995), in human gastric mucosa (Dickson *et al*., 2006), in human normal skin and

skin cancers (Downing *et al*.). HB-EGF is an autocrine growth factor for human keratinocytes because it is both produced by them and stimulates their growth. Evidence for the autocrine nature of HB-EGF in human keratinocytes culture was provided by the ability of anti-HB-EGF-blocking antibody to reduce their growth. This indicates that HB-EGF may be one of the crucial factors for the autocrine growth of human keratinocytes. In addition to its distinct character as an autocrine growth factor for keratinocytes, HB-EGF has a possible role as a paracrine growth factor for fibroblast (Piepkorn *et al*.), even if in our observation we detect positivity to HB-EGF in the epidermis and never in the underlying dermis, but it may depend on particular metabolic events in which paracrine role for HB-EGF is enhanced or reduced. Our report concerning immunohistochemical localization of HB-EGF in normal skin using the streptavidin-peroxidase (HRP) conjugate method, comfirms previous data, revealing specific patterns of HB-EGF localization, in fact, we detected HB-EGF mainly in basal epithelial keratinocytes and in the outer layer of hair follicles. The intense HB-EGF staining in basal keratinocytes of normal skin confirms that HB-EGF plays a role in normal skin development and maintenance. In some cases, the layers above the basal one show a diffuse staining and this can be justified by paracrine role for HB-EGF that, in particular metabolic conditions, leads to migration of keratinocytes in the upper layers and their further differentiation. HB-EGF is mitogenic for epithelial cells, and identification of HB-EGF in cells of epithelial origin suggests its autocrine and/or paracrine roles in epithelial cell maintenance. Our report, aimed at the immunolocalization of HB-EGF in normal skin, especially wants to provide a technical contribution, easy to manage and with evident results. A simple technique that does not require the use of sophisticated equipment, for the benefit of those scientific teams that do not have large operative possibilities but are still eager to give their contribution to scientific research.

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RESUMEN: La familia factores de crecimiento EGF se compone de representantes como el factor de crecimiento transformante (TGF)-a, factor epidérmico vinculante a la heparina (HB-EGF), anfiregulina (AR) y epirregulina, factores autocrinos de crecimiento en queratinocitos humanos normales. HB-EGF es mitógeno para células epiteliales y al igual que otros miembros de la familia EGF, HB-EGF ejerce sus efectos biológicos a través de la interacción con el receptor de EGF (EGFR). HB-EGF es un factor de crecimiento autocrino de queratinocitos humanos, y tiene un posible papel como factor de crecimiento paracrino de los fibroblastos. Nuestro reporte sobre la localización inmunohistoquímica de HB-EGF en la piel normal mediante el método de conjugado estreptavidina-peroxidasa (HRP), confirma datos anteriores, revelando patrones de localización específicos para HB-EGF. La identificación de HB-EGF en las células de origen epitelial sugiere su papel autocrino y/o paracrinos en el mantenimiento de las células epiteliales. Nuestro informe quiere dar una contribución técnica, fácil de manejar y con resultados evidentes. Una técnica simple que no requiera el uso de equipo sofisticado.

PALABRAS CLAVE: Factores de crecimiento; Queratinocitos; Estreptavidina-Peroxidasa conjugada.

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Correspondence to:
Paola Castrogiovanni
Department of Bio-Medical Science
University of Catania
Comparto 10, Via S. Sofia, 87, 5123 Catania
ITALIA

Tel.:+39.0953782036
Fax:+39.0953782046

Email: pacastro@unict.it

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