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Production of functional active human growth factors in insects used as living biofactories

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ABSTRACT

Growth factors (GFs) are naturally signalling proteins, which bind to specific receptors on the cell surface. Numerous families of GFs have already been identified and remarkable progresses have been made in understanding the pathways that these proteins use to activate/regulate the complex signalling network involved in cell proliferation or wound healing processes. The bottleneck for a wider clinical and commercial application of these factors relay on their scalable cost-efficient production as bioactive molecules. The present work describes the capacity of *Trichoplusia ni* insect larvae used as living bioreactors in combination with the baculovirus vector expression system to produce three fully functional human GFs, the human epidermal growth factor (huEGF), the human fibroblast growth factor 2 (huFGF2) and the human keratinocyte growth factor 1 (huKGF1). The expression levels obtained per g of insect biomass were of 9.1, 2.6 and 3 mg for huEGF, huFGF2 and huKGF1, respectively. Attempts to increase the productivity of the insect/baculovirus system we have used different modifications to optimize their production. Additionally, recombinant proteins were expressed fused to different tags to facilitate their purification. Interestingly, the expression of huKGF1 was significantly improved when expressed fused to the fragment crystallizable region (Fc) of the human antibody IgG. The insect-derived recombinant GFs were finally characterized in terms of biological activity in keratinocytes and fibroblasts. The present work opens the possibility of a cost-efficient and scalable production of these highly valuable molecules in a system that favours its wide use in therapeutic or cosmetic applications.

1. Introduction

The immense social and economic impact of wounds worldwide is a consequence of their high rate of occurrence of diabetes globally and their increasing frequency in the ageing population (Natarajan et al., 2000). In addition, Cushing's syndrome, poor arterial perfusion, venous hypertension, poor nutrition or sepsis are also, directly or indirectly, involved in damage of cutaneous coverage (Robson et al., 2001). Therefore, increase in the occurrence and consistent development of cost-effective and clinically efficient technologies and products (compared to traditional offerings) are driving growth in the advanced wound care market.

The healing of an adult skin wound is a complex process requiring the collaborative effort of many different tissues, cells and molecules. Injury to the skin initiates a cascade of events, which can be temporally categorized into three major groups—inflammation, tissue formation and tissue remodelling (Singer and Clark, 1999). The three phases of wound repair are not mutually exclusive but rather overlapping in time for finally lead to at least partial reconstruction of the wound area (Martin, 1997). The repair process is initiated and controlled immediately after injury by the release of various growth factors (GFs), cytokines, and low-molecular weight compounds from the serum of injured blood vessels and from degranulated platelets (Velnar et al., 2009; Greenhalgh, 1996).

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Cytokines and GFs are a group of molecules that participate, as functional messenger between cells, in the complex regulatory network, which control cellular response and are related with recombinant protein therapies (Kim et al., 2010), vaccines (Jenq et al., 2009), regenerative medicine (Werner and Grose, 2003; Barrientos et al., 2008), biosimilars (Niederwieser and Schmitz, 2011) and stem cell medicine (Tenney and Discher, 2009; Eiselleova et al., 2009). Due to their wide activity, cytokines and GFs (and their related products) are highly valuables in research, diagnostics and biopharmaceuticals sectors.

Current researches in wound healing are being focused on GFs or/and human skin substitutes (Macri and Clark, 2009) optimizing both the cellular and molecular environment, thus decreasing healing time by modifying inflammation, and accelerating the proliferative phase. Multiple studies have demonstrated a beneficial effect of many GFs, *e.g.* epidermal growth factor (EGF) and fibroblast growth factors (FGFs) families on the healing process to attract cells into the wound stimulating their proliferation and having a profound influence on extracellular matrix deposition (Pastore et al., 2008; Wang et al., 2008).

EGF is one of the first GFs to be identified (Cohen, 1962). It is a single-chain acidic polypeptide of 53 amino acid residues containing three intramolecular disulfide bonds, which are required for his proper tertiary structure (Boonstra et al., 1995; Cohen and Carpenter, 1975). It is a member of the mitogenic family of EGF-like molecules which also includes TGFα, the poxvirus growth factors, and amphiregulin (Yates et al., 1991). Its function is exerted *via* binding to the epidermal growth factor receptor (EGFR), a transmembrane protein tyrosine kinase that is expressed on many different cell types, stimulating the proliferation and the migration of cells (Carpenter and Zendegui, 1986). A series of experimental and clinical studies have demonstrated a positive effect of EGF on wound repair (Hardwicke et al., 2008).

FGFs family comprise structurally related polypeptides, consisting of 22 members in human (Ormitz and Itoh, 2001). They transduce their signals through direct interaction with heparin or heparan sulfate proteoglycans, a characteristic feature of FGFs, which stabilizes FGFs to thermal denaturation and proteolysis, and which strongly limits their diffusibility (Burgess and Maciag, 1989; Dowd et al., 1999). The complex formed can binds specifically with one of a four high-affinity transmembrane protein tyrosine kinase, FGF receptors 1–4 (FGFR1-4) with different affinity (Johnson and Williams, 1993). Most members of the FGF family have a broad mitogenic spectrum. They stimulate proliferation of various cells of mesodermal, ectodermal, and also endodermal origin. In addition to their mitogenic effects, FGFs also regulate migration and differentiation of their target cells, and some FGFs have been shown to be cytoprotective and to support cell survival under stress conditions (Itoh and Ornitz, 2011; Kinoshita et al., 2012; Werner, 1998). All of FGFs *in vivo* effects suggest a role of these growth factors in wound repair. In particular, the basic fibroblast growth factor (bFGF) or fibroblast growth factor 2 (FGF2) was shown to stimulate angiogenesis in various assay systems (Risau, 1990). Other member of the FGF family, fibroblast growth factor 7 (FGF7), also known as keratinocyte growth factor (KGF1) has been found to have profound specific stimulatory effects on keratinocyte growth at least in the adult organism (Werner, 1998; Rubin et al., 1989) or in treatment of inflammation of the mucosal lining of the gastrointestinal tract (Amgen, US Patent 5965530). Thus these are clear candidates for implementing the wound healing response.

As previously mentioned, the production of these molecules in a cost-effective manner is being a relevant matter. The Baculovirus Expression Vector System (BEVS) allows high-level of heterologous proteins production by using strong baculoviral promoters (*polh, p10*). Furthermore, BEVS allows recombinant proteins to be producing very likely to their native conformation as they can be post-translationally modified efficiently. These characteristics make BEVS an important expression system for industrial applications. The expression of various GFs in insect cells are already successfully tested (Cronin et al., 1998; Lee et al., 2006). However, a more cost-effective alternative to produce recombinant GFs would be highly desirable for a more affordable application of these recombinant products.

The use of insect larvae as living biofactories for protein production has been explored as an alternative to fermentative technologies. The recombinant protein production by the combination of recombinant baculovirus and *Trichoplusia ni* (*T. ni*) insect larva has been denominated as Improved Baculovirus Expression System t(IBES) and represents one of the best production alternatives based on baculovirus vectors. The advantages of using insect larvae for protein production include the dramatic reduction in production costs with respect to insect cell cultures, an increase of recombinant protein yields, the absence of high-tech fermentation procedures, a reduced development times and an easy production scaling-up. These advantages make the use of insect larvae as biofactories as a real alternative to standard cell-culture fermentation systems.

The use of such effective and inexpensive platform has been used to produce efficiently several recombinant antigens, including enzymes (Medin et al., 1990; Romero et al., 2011; Chazarra et al., 2010), antibodies (Reis and Blum, 1992; Gil et al., 2011; Gómez-Sebastián et al., 2012), hormones (Mathavan et al., 1995; Sumathy et al., 1996), vaccines (Gomez-Casado et al., 2011; Kuroda et al., 1989; Gil et al., 2001), cytokines (Pérez-Martín et al., 2010; Shi et al., 1996; Pham et al., 1999) and diagnostic proteins (Barderas et al., 2000; Pérez-Filgueira et al., 2006). In all these cases, proteins were processed correctly after synthesis, and their functional activities remained intact.

In the present work, we have evaluated the production of three recombinant human GFs, the human epidermal growth factor (huEGF), the human fibroblast growth factor 2 (huFGF2) and the human keratinocyte growth factor 1 (huKGF1) in *T. ni* larvae. We have used different modifications to optimize their production, the signal peptide from honeybee melittin (Mel) or the reticulum endoplasmic retention signal sequence KDEL (Lys-Asp-Glu-Leu). These sequences can facilitate an efficient translocation of recombinant proteins and their proper folding (Ruiz-Gonzalvo et al., 1996). We have also used one of the most used protein tag the polyhistidine tag (His-tag) (Terpe, 2003), that allows the purification of the tagged protein by metal affinity chromatography or a common fusion strategy in protein therapeutics, in which the fragment crystallizable region (Fc) of the human antibody IgG is used (Fc-tag) (Arnau et al., 2006). The recombinant GFs were finally characterized in terms of functionality by several biochemical assays including mitogen-activated protein kinase (MAPKs) activation and epithelial cells proliferation or migration experiments.

The results presented here show high production yields of the three human GFs analysed. These yields were around 9.1 mg (huEGF), 2.6 mg (huFGF) and 3 mg (huKGF) per gram of insect biomass, with similar functionality to their commercial counterparts in all the cases.

2. Materials and methods

2.1. Cell lines and cell culture

2.1.1. Insect cells

The insect ovarian cell line *Spodoptera frugiperda* (Sf21) was purchased from Invitrogen. Sf21 were routinely grown at 27 °C in *T. ni* Medium-Formulation Hink (TNM-FH) medium (PAN Biotech) supplemented with heat-inactivated 10% (v/v) Foetal Bovine Serum (PAN Biotech) and gentamycin sulphate 50 μg/ml (Lonza).