REVIEW

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Formulation development of epidermal growth factor

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The information in this paper is presented to summarise various investigations in recent years to develop epidermal growth factor (EGF) delivery systems. EGF is a promising and well-characterised polypeptide that can be used in the treatment of various types of wounds and ulcers. Many approaches have been employed to deliver EGF as a stimulant for cellular activities involved in the processes of wound healing and tissue repair. Recently, emphasis is placed on the development of sustained or controlled delivery technology to optimise EGF delivery.

1. Introduction

Growth factors are molecules that promote tissue regeneration and differentiation [1]. Epidermal growth factor (EGF) is a single chain polypeptide and also potent mitogen. The importance of this polypeptide becomes rapidly recognised since its first discovery more than four decades ago. Many researchers in various fields have studied EGF not only as a therapeutic agent to cure or alleviate disease states such as wound and ulcer but also as a tool to deliver molecules of interest to the target site of cells [2–6]. Recently, the ability of EGF to accelerate cell proliferation has shown potentials to be used in tissue engineering that needs to stimulate cell growth and in cosmetics where it has decreased cutaneous senescence [7–9].
Due to the rapid progress made in the 1980s in biotechnology such as gene cloning and recombinant DNA technology, a sufficient amount of biotherapeutics become commercially available. This has made it possible to develop peptide and protein pharmaceuticals. However, the development of such biotech pharmaceuticals requires physicochemically stable formulations that can effectively deliver active components to the target site. The aim of this paper is to elaborate on EGF as a therapeutic agent and its formulation development to achieve therapeutic goals.

2. Discovery and biological activities of EGF

EGF was first discovered by Stanley Cohen when he injected crude submaxillary gland extract to new born mice during the course of his studies on nerve growth factor (NGF). The salivary gland extract unexpectedly led to an increased development in the mice such as precocious eyelid opening and tooth eruption [10]. After the isolation of the active substance responsible for these effects from murine submaxillary glands, he termed it epidermal growth factor due to its ability to stimulate the proliferation of epidermal cells in skin and cornea [11]. Amino acids analysis of mouse-derived epidermal growth factor (mEGF) showed that it is a single-chain polypeptide composed of 53 amino acid residues with the three intramolecular disulphide bonds [12]. It was in the mid-1970s that Cohen and his colleagues identified human epidermal growth factor (hEGF) from human urine [13, 14]. They revealed that hEGF processes the same biological effects that showed previously by mEGF even though the amino acid sequences of human and murine EGF are not entirely identical. In 1975, Gregory published that urogastrone, a gastric antisecretory hormone, isolated from human urine is identical to hEGF and can cause the similar biological responses in all target cells [15]. hEGF also contains 53 amino acid residues with a molecular weight of 6045 daltons. EGF has also been found in the milk, saliva, urine and plasma of various species including rats, cows and pigs [16, 17].
The early studies on the biological and physiological roles of EGF in the body were well documented [18]. EGF has demonstrated that the acceleration of proliferation and differentiation of epidermis, skin and corneal epithelial tissues, and lung and tracheal epithelia in a wide range of experimental models [18]. EGF possesses multiple biological and physiological effects like most peptide and protein drugs [19]. Some of these biological effects are: (a) inhibition of gastric acid secretion [20]; (b) enhanced cell multiplication [21]; (c) stimulation of synthesis of DNA, RNA, protein and hyaluronic acid [18, 22] and (d) stimulation of neoangiogenesis [23]. The summarisation of recent works on the biological activities of EGF may be difficult as now over 23,000 articles are found in the Medline database since 1980. However, the readers can refer to papers dealing with specific scopes of biological activities of EGF [24–29].

3. Designing delivery systems of EGF

3.1. Design considerations for the development of EGF formulations

Success in developing peptide and protein drugs requires optimised delivery systems since the clinical utilities of a large number of peptide and protein drugs are limited by their poor stabilities and bioavailabilities (e.g. poor pharmacokinetic properties that result in short duration of action and ineffective drug concentration at the site of action). Indeed, a large number of peptide and protein drugs are abandoned due to their extremely low bioavailabilities. Although peptide and protein drugs offer therapeutic advantages including high specificity and great potency in their biological functions, they demand a more rational approach to overcome these limitations.
Proteins and peptides undergo various physico-chemical changes during purification, formulation and storage with time. These alterations can be divided broadly into two main categories, physical and chemical instabilities. Chemical instability refers to the change in covalent bonds within polypeptides and proteins such as deamidation, oxidation, hydrolysis, racemisation, isomerisation, β-elimination and disulphide bond breakage and formation whilst

physical instability refers to the alteration of higher order structures including denaturation, adsorption, aggregation and precipitation. Physical and chemical degradations may cause the loss of biological activity and undesired side effects. In most cases, more than one pathway of physical and/or chemical alteration is responsible for the stability of peptides and proteins [30, 31]. Formulation factors can also lead to the instability of peptides and proteins. They are temperature, formulation pH, excipients containing salts and metal ions, chelating agents, shaking and shearing, non-aqueous solvents, polymorphism and pressure, and concentration, source and purity of peptides and proteins [32].

3.2. Stability and stabilisation of EGF

The most common chemical reaction found in hEGF is deamidation, resulting from the hydrolysis of the side chain amide group in glutaminyl or asparaginyl residues [33, 34]. This deamidation reaction is accelerated with increasing temperature, ionic strength, and in neutral or alkalic pH ($pH > 6.0$) [35]. The main cause of physical instability of EGF is the polymerisation of the monomer into dimer and trimer by disulphide exchange that may lead to the alteration of biological activity or immunological properties [36, 37].
Araki et al. have evaluated the stability of hEGF in various solutions [38]. hEGF was degraded in phosphate buffered saline (PBS, $pH = 7.2$) and 0.1 N acetic acid, producing unknown degradation products near the RP-HPLC peak of intact hEGF. The chemical degradation of hEGF in aqueous solution was spontaneous and temperature-dependent. At $-20\,°C$, practically no hEGF was degraded in PBS ($pH = 7.2$). Son and Kwon revealed that the deamidation of hEGF was greatly decreased in Tris-HCl solution ($pH = 7.0$) as compared to the solutions containing Na phosphate, PBS, Na borate, Na acetate and Na citrate [39]. The deamidation of hEGF in aqueous formulations could be inhibited to a larger extent by some additives such as fibronectin (0.5%), hyaluronic acid (0.2%), sucrose monocaproate or surfactants including Triton X-100 (0.02%), Tween 20 (0.01%) [39]. The deamidation did not have an influence on the mitogenic activity exhibited by hEGF and it was closely related to the degree of the peptide bond cleavage [39, 40]. The above mentioned additives and Zn ion showed the prevention of hEGF peptide bond cleavage. The addition of non-ionic surfactants such as Triton X-100 and Tween 20 (0.01–0.1%) inhibited the aggregation of hEGF in an aqueous solution. The aggregation of a polypeptide, producing higher molecular weight polymers was responsible for reduced biological activities [41].
The experiment on the degradation of hEGF in the serum suggested that hEGF was exponentially degraded by some proteases with a half life of 17 min [38]. The simulated gastric juice consisting of pepsin and hydrochloric acid ($pH = 1.6$) rapidly inactivated hEGF and the disappearance rate half time for hEGF was only 1.4 min [38]. The metabolic stability of hEGF in various gastrointestinal mucosae has been examined [42]. hEGF showed a relative stability in the colon and jejunum mucosal sites whilst it was rapidly degraded in the duodenum. Some additives such as sodium salicylate, bestatin and sodium caprate displayed the inhibition of the metabolic degradation of hEGF at a certain mucosal site like duodenum. Although Playford et al. demonstrated that EGF was labile in acidic gastric juice [43], possibilities of the substantial survival of ingested EGF in the stomach have been shown by others [44–46]. hEGF was inactivated in the skin by a number of enzymes such as protease [47]. The degradation of hEGF in the skin was decreased by adding inhibitors such as bestatin, EGTA (ethylene glycol-*bis*-(β-aminoethyl) *N,N,N′,N′*-tetraacetic acid) and TPCK (*N*-tosylphe chloromethyl ketone) [47].
The stabilisation of hEGF in pharmaceutical products can be primarily achieved by controlling physico-chemical parameters involved in the formulation process and storing under cold conditions. Also, the addition of a variety of inhibitors to prevent chemical and metabolic inactivation can be useful. However, the optimisation of formulations for local and systemic delivery of EGF might be difficult since the mechanisms of physico-chemical and metabolic instabilities of EGF (e.g. information on enzyme-sensitivity cleavage sites) have not been fully elucidated.

3.3. EGF delivery systems

3.3.1. Topical delivery of EGF

Wound healing is a localised process. The healing process of cutaneous wounds involves a series of independent stages like inflammation, re-epithelialisation, granulation tissue formation, and matrix and collagen remodelling [48]. The role of EGF in wound healing has been addressed for many years [49–52]. Topical application is probably the most efficient way of delivering EGF to the local wound sites [53]. Vehicles delivering EGF are important since the local application of EGF in water and saline solution has not shown appreciable effects on the wound healing process [54, 55].
DiBiase and Rhodes have investigated three semisolid formulations of EGF [56, 57]. Pluronic F-127 (polyoxyethylene polyoxypropylene block copolymer) 25% gel and Carbopol 934 P (carboxypolymethylene) 0.5% gel exhibited potential for topical EGF delivery. Although the release of EGF from stearic acid-based vanishing cream was slow compared to the Pluronic and Carbopol gels, it provided an additional advantage like taking up of discharge into its oil in water vehicle structure when applied to open wound. EGF exhibited faster regeneration of the epithelium when applied in the crosslinked gelatin-hyaluronate sponge, together with an antibiotic such as silver sulphadiazine [58]. However, there was evidence that the antibiotic decreased the initial proliferating activity measured at day 5. Lanolin cream itself has demonstrated a significant enhancement of the re-epithelialisation rate, thickness of the dermis, and higher cell count in the dermis [59]. In this formulation, the effect of EGF on healing of partial-thickness wounds was marginal compared to the lanolin cream alone. Care must be taken when using the lanolin base since it induced strong inflammation reaction in the wound [59].
Corneal epithelial wounds caused by surgery, chemical burns or ulcers were also treated by the topical application of EGF [60]. Although EGF improved the epithelial wound healing in alkali-burned corneas, EGF did not have effects on preventing recurrent erosions and secondary breakdown of the corneal epithelial surface. Supply of EGF appears to be important for accelerating epithelial wound healing in corneas despite the fact that there was no evidence of dose-dependent therapeutic response [61]. Two polymeric delivery systems have been investigated for ophthalmic delivery of EGF in corneal epithelial wound healing: a Carbopol gel and a Poloxamer gel [62, 63]. Drugs can be delivered via the ocular route with ad-

vantages such as relatively fast absorption rate. A dose instilled into the precorneal site is absorbed systemically by the conjunctiva. However, most doses are rapidly cleared by drainage through the nasolachrymal duct into the nasal passages from which absorption into the systemic circulation can occur [64]. In most cases, the prolonged exposure of EGF in the healing of wound is important. Continuous EGF exposures of as few as 2 hours caused a significant increase in wound healing rate and increasing the time of exposure further increased the rate of wound healing [65]. Poloxamer has been employed to prolong residence time in the eye, leading to an avoidance of the rapid loss of the drug from the precorneal site [66]. Also, the locally applied EGF incorporated into multilamellar liposomes demonstrated long term exposure to the wound, resulting in the acceleration of wound repair [50]. Cyclodextrins were used in ophthalmic formulations of EGF due to their stabilising effect against physico-chemical degradations [67, 68].

Ideal vehicles for topical application of EGF will not only deliver EGF with capability of releasing desired amount of an active form of EGF but also stay at the target site for an extended period of time to provide sufficient exposure to the target cells.

3.3.2. *Sustained or controlled delivery of EGF*

Polymeric materials have been widely used to achieve sustained or controlled delivery of EGF to the gastrointestinal tract as well as to the open wound site. Polyvinyl alcohol (PVA) sponge containing slow-release EGF pellets was fabricated to achieve the local sustained presence of EGF [69]. Slow release of EGF, delivered from sustained release pellets in subcutaneous PVA sponge implants caused a remarkable increase in the extent and organisation of the granulation tissue at day 7, a doubling in the DNA content, and 33% increase in protein content and wet weight, as compared with placebo controls. Biodegradable polymers such as poly(DL- or L-lactic acid) (PLA) and poly(lactide-co-glycolide) (PLGA) have been extensively used for the sustained delivery of drugs [70–73]. hEGF incorporated into poly(L-lactic acid) microspheres has been delivered for the treatment of chronic gastric ulcer [74]. The microspheres were subcutaneously injected and maintained a constant EGF level in the blood for about 10 days. However, the preparation of delivery systems using PLA and PLGA causes the use of organic solvents, often resulting in the inactivation of peptide of protein drugs including EGF. In another recent study, a polymeric bilayer wound dressing containing EGF-loaded gelatin microspheres, at high-dose EGF application, showed a controlled release of EGF from the microspheres, resulting in a higher degree of reduction in the wound areas [75].

The hydrogel film of dextran dialdehyde cross-linked gelatin was able to deliver biologically active EGF in a sustained manner [76, 77]. The *in vitro* release kinetics was influenced by several formulation parameters such as the mechanical properties of the hydrogel film, temperature, storage time. The most advantageous feature of the polymeric hydrogel film over conventional wound dressings is a capability of delivering therapeutic agents into the wound site in a controlled manner. Also the proven biocompatibility of gelatine hydrogel film is expected to enhance its clinical usefulness. Polypeptide or protein release from polymeric matrices was sometimes incomplete due to non-spontaneously dissociable non-covalent protein aggregation and the surface adsorption of protein within the polymer matrix [78].

Another method to provide long-term delivery of EGF is to conjugate polyethylene glycol (PEG) to EGF [79]. The physico-chemical stability of EGF was enhanced by pegylation and a sustained release was achieved from biodegradable PLGA microspheres containing the pegylated EGF. Indeed, the PEG conjugation of therapeutic proteins has recently been introduced for increasing bioavailability and functional stability of proteins [80, 81]. A micro-sustained release system for ensuring the controlled release of microgram and smaller amounts of biologically active EGF has been reported [82]. Albumin in milligram quantities could facilitate the sustained release of picogram amounts of EGF for at least 3 weeks. This technique increased the proliferation rate of serum-starved cells.

Bioadhesives may also be useful in developing EGF delivery systems. Besides acting as platforms for sustained-release dosage forms, bioadhesive materials can offer additional advantages such as the localisation of the drug to the site of absorption or administration, the protection of the underlying cell layer, the reduction of gastric ulcers, the inhibition of proteolytic enzymes, and increase in epithelial permeability [83–85]. In an example of this approach, a bioadhesive gel was prepared in 0.2% Carbopol 940 polymer to administer EGF topically [86]. The bioadhesive gel released EGF slowly and caused a significant increase in the wound tear strength in mice compared with solution treated mice. The covalent attachment of pepstatin A, a pepsin inhibitor, to a bioadhesive carrier (sodium carboxymethyl cellulose) could allow the oral delivery of EGF in order to treat gastric ulcer [87]. The conjugation of the pepsin inhibitor also demonstrated a protective action against the enzymatic degradation of peptide drugs in the gastrointestinal tract.

4. Conclusions

EGF is emerging as a new generation therapeutic agent for the treatment of a various types of wounds and ulcers. A number of approaches have been suggested and examined for this purpose. Different wound types such as surgical incision, corneal epithelial defects or bulk loss of tissue caused by burns, trauma and diabetes will need different delivery systems. One of the most challenging tasks in the development of EGF delivery systems is to ensure its biological activity and therapeutic efficacy in the delivery systems. The ideal delivery systems of EGF for wound repair will contain an appropriate dose of EGF and will have to control the release rate of EGF since overexpressed EGF and its receptor often can cause uncontrolled cell proliferation as occurs in cancer [88–90].

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