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Applications, and Efficient Large-Scale Production, of Recombinant Human Epidermal Growth Factor

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Introduction

Human epidermal growth factor (EGF) is a small polypeptide of molecular weight 6201 Daltons, with 53 amino acid residues. Since its discovery by Cohen (1962) (an achievement subsequently honoured by the award of the 1986 Nobel Prize in Medicine), all aspects of EGF biology have attracted intense research interest. A search of the Ovid™ Biological Abstract database (1991–1999), with the search phrase 'epidermal growth factor', and restriction to the English language, yielded 10,614 hits.

In the first part of this article, we review some papers from the interval 1995–1999, which have been chosen to show actual or potential uses of EGF, mainly in various facets of human health care. In choosing papers for review in this section, space constraints have compelled us to ignore many fine research contributions which deal, for example, with aspects of the interaction of EGF with its receptor, or which detail the influence of EGF upon various signal transduction pathways within the cell. The review section concludes with the suggestion that many potential applications of EGF are thwarted by the relative unavailability of large amounts of purified, biologically active, chemically authentic EGF. In the next section, then, we review earlier efforts to produce recombinant EGF in quantity, and subsequently focus on more recent work in our laboratory, which has resulted in a fast, reliable method for the production of gram quantities of purified human EGF which is chemically identical to the natural material and exhibits full biological activity. We conclude that the availability of EGF in large quantities will be of practical use to patients and their

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Abbreviations: EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; HGH, human growth hormone; OmpA, outer membrane protein A.

Biotechnology and Genetic Engineering Reviews – Vol. 18, July 2001
0264–8725/01/18/51–71 $20.00 + $0.00 © Intercept Ltd, P.O. Box 716, Andover, Hampshire SP10 1YG, U.K.

physicians, and should also aid in the development of further applications of EGF in the field of human health care.

Actual and potential uses of EGF

WOUND HEALING

While EGF has shown much promise in wound healing applications, most of that promise has yet to be realized in the development of routine, reproducible, applications in the relief of human trauma. The review of Robson (1997) remains most pertinent today. When the use of growth factors in wound healing applications is under consideration, the factor(s) selected must, first, enhance an activity (such as epithelialization or connective tissue deposition) which is of major importance in healing the particular wound type under consideration. Next, it is necessary to check that the growth factor in question is, in fact, potentially or actually limiting for wound healing in the wound environment. Finally, adequate quantities of the growth factor must be applied, at appropriate frequencies. It is likely that limitations of EGF supply have caused physicians to err on the side of sub-optimal dosing when evaluating EGF utility in the healing of human wounds.

In a human skin explant model, the histologic structure of human epidermis is maintained for 10 d *in vitro*. In this model, the longest keratinocyte outgrowth from the explant periphery (simulating epithelial regeneration from a wound edge) was obtained with EGF among other growth factors tested (Bhora *et al.*, 1995). Rats, upon which full-thickness skin wounds were inflicted, were treated with a cream containing up to 10 μg EGF/g. With this dose, a marked effect on wound re-epithelialization (measured 7 d post-wounding) was seen, with a reduction in inflammatory infiltrate (Berlanga *et al.*, 1996). In a pig wound model, the animals received full-thickness skin wounds 8 mm in diameter. Dressings were changed, and wounds cleaned, on a daily basis. Cream, containing 10 μg EGF/g, with 1% (w/v) silver sulphadiazine, was applied at intervals of 1–3 d. By a variety of measures, wound healing (measured 8 d post-wounding) occurred faster on animals treated with the EGF-containing cream, as compared with controls where the cream lacked the growth factor. Frequent applications (daily) of cream were necessary for significant enhancement of healing (Berlanga *et al.*, 1997). In another experiment, 5 pigs suffered partial-thickness wounds on the back and creams with up to 50 μg EGF/g were applied every 24 h for 7 d. The cream with the highest EGF level significantly stimulated re-epithelialization; lower levels of EGF were ineffective. It was noted that protease activity was present in acute wounds (to 24 h post-wounding), and the implication is that this will tend to destroy applied EGF. The suggestion, then, is that EGF should be generously and frequently applied to wounds, preferably in combination with a protease inhibitor (Berlanga *et al.*, 1998a). It was shown that growth factors penetrate only slightly into the upper granulating layers of full-thickness excisional wounds in rats; applications of generous amounts of growth factors will be required before they may penetrate into deeper layers of wounds (Cross and Roberts, 1999).

As evidence suggested that frequent EGF applications were required for good wound healing, Rosenthal *et al.* (1997) developed a method to supply EGF continuously in the early stages of wound healing. A biologically active EGF fragment was

fused in-frame to the signal sequence of the human granulocyte colony-stimulating factor; the construct was transfected to fibroblasts, which, when irradiated, continued to secrete EGF. The dead cells were put in 'fibrin glue' (a matrix formed immediately when separate sprays of human thrombin and fibrinogen mix) and placed into full-thickness skin wounds of mice. Secreted EGF was detectable for 7 d in the wounds, with levels dropping from 470 pg/ml to 140 pg/ml (d 7) over time – no detectable EGF was secreted from d 14. While the EGF levels seen are possibly too low to have any marked effect on wound healing, the approach is imaginative, and open to further development so that secreted EGF levels may be enhanced.

In persons recovering from plastic surgery, excessive wound contraction may result in unsightly wound healing, and, in an animal model addressing this problem, bovine collagen matrix sponges were placed in full-thickness excisional wounds of rabbits. Usually, fibroblasts tend to invade the collagen gel causing gel contraction, resulting in unsightly wound healing. Application of EGF to the collagen gel inhibited the unwanted fibroblast action. This was shown both *in vitro* (a fibroblast-populated collagen gel) and *in vivo*. When collagen and EGF were both used, the quality of the wound healing was regarded as much better than that obtained using EGF-free collagen matrix (Inoue *et al.*, 1998).

It seems clear that topical application of EGF is valuable in the mediation of local anti-inflammatory activity, with dose-related healing activities of EGF noted after ear application of the irritants 12-*O*-tetradecanoylphorbol-13-acetate, croton oil, or arachidonic acid (Casaco *et al.*, 1999).

Most of the wound-healing work with EGF has used skin wound models. It appears, however, that EGF may also be valuable in assisting tendon repair. Jann *et al.* (1999) took segments of the long digital flexor tendon from chickens, and made tissue culture explants containing absorbable suture material, which was treated with EGF. In these samples, tenoblast migration was stimulated compared to EGF-free controls. The suggestion, then, is that absorbable sutures used to assist patients recovering from tendon injuries should include a slow-release form of EGF.

In summary, it seems very likely that wound healing processes which require re-epithelialization will be assisted by the frequent application of generous amounts of EGF. Further research in this area is clearly needed. In particular, EGF might enhance the wound healing facilitated by alginate dressings (which provide a moist environment conducive to granulation and re-epithelialization) (Suzuki *et al.*, 1999).

ACTIVITIES IN THE GASTROINTESTINAL TRACT

As will be reviewed, there is a wealth of evidence from animal tests which indicates that EGF will be very valuable in the treatment of various pathological conditions, or surgical disturbances, of the human gastrointestinal tract. Again, the development of regular medical protocols in this area has been slow, due in part to poor commercial availability of the considerable amounts of EGF needed for effective treatment. In a very interesting study, 75 patients with duodenal ulcers were divided into 3 groups, of which one was treated only with the anti-ulcer agent cimetidine, one with EGF at 450 μg/d, and the last group with EGF at 600 μg/d. All treatments were oral, and carboxymethylcellulose (a bioadhesive carrier matrix) at 1%, w/v, was used as an inert vehicle to deliver the drug or the EGF. After 6 weeks 76–93% of ulcers had

healed; no differences in healing efficiencies were noted between treatments (Haedo *et al.*, 1996). This means that EGF is a valuable anti-ulcer agent, and its use should probably be regarded as preferable to drug therapy.

As it is likely that EGF is susceptible to protease action in the gut (see below), a slow-release, protease-protected, form of EGF might be valuable. Pepsin inhibitor (pepstatin A) was covalently attached, *via* a spacer, to carboxymethylcellulose. When horseradish peroxidase was absorbed into this bed, it was protected from pepsin digestion. This work suggests how a slow-release EGF preparation may be formulated (Bernkop-Schnurch and Dundalek, 1996). The protection of administered EGF from proteolytic digestion in the gut may be of particular importance in patients suffering from ulcers caused by *Helicobacter pylori*, as patients with *H. pylori*-caused non-ulcer dyspepsia had 80% less EGF than controls (persons suffering from dyspepsia but without *H. pylori* involvement) in acidic gastric juice; suggesting that the pathogen may degrade EGF, presumably *via* the action of a secreted protease active at low pH (Marcinkiewicz *et al.*, 1996).

Intestinal atrophy contributes to the difficulties of patients on parenteral nutrition. While it is known that systemic EGF reverses the intestinal damage, intravenous administration of EGF is not indicated as unwanted effects on the liver, for example, may be expected. Delivery of EGF directly to the gastrointestinal tract is more desirable. Such direct delivery of EGF was achieved using a plastic infusion line; the infusate contained lactalbumin to prevent EGF adhesion to the plastic. Lactalbumin is a poor substrate for intestinal proteases and does not prevent digestion of EGF by pancreatic proteases *in vitro* (Marchbank *et al.*, 1995). In the rat, intragastric infusion of EGF at a dose of 72 μg/d increased cell proliferation in the duodenum by 26%, but did not affect the more distal regions of the intestine. When the EGF was administered with soybean trypsin inhibitor, however, a 28% rise in duodenal cell proliferation was now accompanied by a 24% increase in jejunal growth (Marchbank *et al.*, 1995). Similarly, luminal exposure to EGF (2.5 μg/d for 2 d) in starved rats prevented mucosal atrophy of the small bowel (Ulshen and Raasch, 1996). It was suggested that the use of intragastric EGF, probably with a protease inhibitor, is indicated in patients with Crohn's disease or necrotizing enterocolitis (Marchbank *et al.*, 1995).

After massive enterectomy, the remnant intestine undergoes compensatory adaptation. EGF and human growth hormone (HGH) are known to increase nutrient transport along the total length of the small intestine after this procedure. For example, when these substances were given together (HGH 0.2 mg/kg/d and EGF 1.5 μg/kg/h) to rabbits 7 d after a 70% mid-jejunoileal resection, for a 7 d period, large increases in nutrient transport in both intestinal remnants, and microvillus hypertrophy (again in both remnant intestinal portions), were noted (Iannoli *et al.*, 1997). EGF can also mitigate undesirable effects of the drug somatostatin, which is given to patients with short bowel syndrome and enterocutaneous fistulae. Somatostatin is not indicated after bowel resection – intestinal adaptation is adversely affected. In rats with an 80% small intestinal resection, however, somatostatin at 50 ng/kg/h and EGF at 1.5 μg/kg/h, administered subcutaneously *via* miniosmotic pumps, showed optimal ability to assist recovery, as judged by a combination of both histologic and molecular criteria. This work emphasized that the recovery noted when both somatostatin and EGF were administered was better than that achieved with somatostatin alone (Liu *et al.*, 1996).

Helmrath *et al.* (1998) emphasized the *role* of the submandibular gland in EGF production. When this gland was excised from mice after 50% bowel removal, adaptation was adversely affected, but the effects noted were reversed by systemic or oral EGF, suggesting that the beneficial effects of EGF may be expressed at either the luminal or basolateral surfaces of the enterocyte.

While most studies with EGF in the gastrointestinal tract have focused on intestinal applications, Vinter-Jensen *et al.* (1995) showed that EGF assists recovery in Göttingen minipigs (pigs with a genetic predisposition to small adult size, which are thus most useful as experimental animals) subjected to 3 sessions (over 3 weeks) of sclerotherapy (surgical dilation of the oesophagus, a procedure sometimes required in humans who have ingested toxic substances, or excessive amounts of alcohol). EGF was administered subcutaneously at 30 μg/kg/d, on 3–7 occasions/week, for the 3 weeks of the sclerotherapy and the 2 following weeks, with an additional weekly intravenous dose of 20–40 μg/kg. This protocol attenuated the oesophageal damage normally caused by the sclerotherapy procedure.

EGF may also assist in the mitigation of pancreatitis. In acute pancreatitis, bacterial translocation from the gastrointestinal tract to mesenteric lymph nodes and other extraintestinal organs is an important source of systemic infection. In an artificial model of pancreatitis, the biliopancreatic duct of rats was damaged with a pressure injection of the detergent taurocholate, and trypsin. The test group received 100 μg EGF/kg twice daily subcutaneously. By various measures, this treatment minimized intestinal damage, with 33% of animals showing bacterial translocation to distant sites as compared with 83% of the control group that had not been given EGF (Liu *et al.*, 1997).

In summary, it is clear that the oral administration of EGF, probably with a protease inhibitor, and possibly as a slow-release formulation, will be of considerable value to patients recovering from perturbations (surgical, ulcer-caused) of the gastrointestinal tract, and future developments in this area will be limited only by the commercial supply of EGF.

TARGETING OF BRAIN AND BLADDER TUMOURS

Treatment of brain tumours represents a major challenge to surgeons and physicians. The EGF receptor (EGFR) is over-expressed in high-grade gliomas, but is low or undetectable in normal brain. This has led to the idea that EGF, conjugated with a toxic material, would be valuable in brain tumour therapy. When a stable (5-neutron) form of boron is irradiated with low-energy thermal neutrons, some of the neutrons are captured by the nucleus, and the new isotope thus created decays, with particle emissions capable of destroying nearby tissue. Yang *et al.* (1997) took a 'fourth-generation starburst dendrimer' (a scaffolding molecule), boronated the material, linked it to EGF, and labelled the complex with [^{131}I]. When this material was administered, intratumourally, to rats with C6EGFR brain tumours expressing high levels of EGFR from a transduced gene, 22% of the injected dose was still in the tumour 24 h post-injection, while 16% remained at 48 h. With control tumours (not transduced with the EGFR-encoding gene), the figures were 5% and 1.3%. When the complex was given intravenously, it did not cross the blood–brain barrier. In an elegant approach to addressing this problem, EGF was linked to the OX26 monoclonal

antibody (which recognizes the transferrin receptor). The transferrin-binding activity of the conjugate allowed the complex to transit the blood–brain barrier after intravenous injection – the appearance of label in the brain was boosted when unlabelled EGF was given with the brain-targeted material – the unlabelled EGF saturated the (abundant) EGFR molecules in the liver (Kurihara *et al.*, 1999).

Muscle-invasive urothelial carcinoma of the urinary bladder has a poor prognosis. This tumour type also over-expresses EGFR. A complex of dextran, EGF, and (radioactive) [^{99}Tc] was prepared and injected into nude mice carrying subcutaneous xenografts of the human urothelial carcinoma cell line RT4. Mice were sacrificed 24 h post-injection. The tumour/blood radioactivity ratio ranged from 2:1 to 6:1, demonstrating uptake of the complex by the tumour. The ratios in the kidneys of the mice were similar to those in the tumours, but lower ratios were seen when other tissues were used for comparison. The suggestion, then, is that the complex should be intravesically injected at present, but that intravenous injection may later be appropriate if tumour targeting can be further improved (Bue *et al.*, 1997).

In summary, the use of EGF in the tumour-targeting area is obviously valuable, and already becoming established.

OTHER APPLICATIONS OF EGF

Minimizing the effects of ureteral obstruction

Ureteral obstruction sometimes occurs in humans. In the rat, EGF (0.1 mg/kg/d) was given systemically to 1-day-old animals with (artificial) unilateral ureteral obstruction. The treatment commenced on the day of ureteral obstruction, which was released after 5 d – EGF administration continued for a further 2 d. The EGF treatment markedly attenuated tubular and interstitial injury measured 28 d later (so 33 d after removal of the obstruction) (Chevalier *et al.*, 1999). In adult rats, subcutaneous injection of 10, 20 or 40 μg EGF/d after artificial unilateral ureteral obstruction promoted regeneration and decreased apoptosis in kidneys harvested 1–3 d after the obstructive procedure (Kennedy *et al.*, 1997). It appears, then, that systemic EGF is useful in minimizing kidney damage both during and after release of kidney obstruction, and its use in such circumstances is indicated in humans.

Assisting the regeneration of nerve tissue

The poor ability of mammalian central nervous system axons to regenerate is reflected in the limited ability of astrocytes to migrate and repopulate a site of neural injury. In an important initial study, Faber-Elman *et al.* (1996) used a scratch-wounded astrocyte monolayer *in vitro*, and confirmed that unidentified soluble materials from wounded rat optic nerves blocked astrocyte migration. Addition of EGF (and other growth factors), however, stimulated intense migration and wound closing. Application of EGF did not abrogate the inhibitory effects of the soluble materials from wounded optic nerves, but other growth factors did show this property. In a whole-animal (gerbil) model of brain ischemia, cerebroventicular infusion of EGF (24 or 120 ng/d for 7 d), starting just before or after injury, protected the cells of the hippocampus from obvious injury, and also preserved hippocampal functions, as measured by animal performance in passive avoidance tasks (Peng *et al.*, 1998).

In summary, research into the use of EGF in repairing nerve damage is clearly in its infancy, but the potential is exciting.

Use in the bioengineering of artificial organs

The growth of semi-artificial organs in culture is a major research area. Synthetic cell culture surfaces are available which are thermally responsive – after growth of a cell monolayer on these surfaces a temperature change causes the cell sheet to lift off the support, and this gentle detachment preserves structure and function in the semi-artificial organ. Synthetic culture surfaces with chemically reactive amine and carboxyl groups may be prepared. When EGF, and the extracellular matrix molecules collagen and chondroitin sulphate were attached to these groups, the immobilized EGF contributed notably to high-quality cell growth on such modified surfaces. The required polarization of cell functions was observed, and the newly grown monolayers still detached efficiently upon temperature shift (von Recum *et al.*, 1999). In another system, collagen-coated beads were mixed with hepatocytes, hepatocyte growth factor, and EGF, and then with nonparenchymal cells in a biological matrix (Matrigel 3D®). After some months, the patterns of growth showed characteristics of hepatic histological architecture, and the cells maintained their differentiation and proliferative capacities. For maximal effect, both hepatocyte growth factor and EGF were required (Michalopoulos *et al.*, 1999).

Use in repair of eye damage

A body of experimental evidence suggests that EGF should be of assistance to patients undergoing eye surgery during which epithelial surfaces are damaged, but this hope has not yet been borne out in practice. When lens epithelial cells from human cataractous lenses were cultured on human anterior lens capsules, supplemented with 1 ng/ml or 10 ng/ml EGF, the epithelial cells differentiated, as did the anterior lens capsules, to show numerous microfibres. A multilayered epithelium was noted, as were ball-and-socket junction structures characteristic of healthy tissue, and nucleolar chromatin condensation. The higher EGF dose effected the most profound changes (Majima, 1998). Foreman *et al.* (1996) inflicted excisional trephine and epithelial scrape wounds on bovine and human corneo-scleral rings, and then filled the endothelial corneal cavity with an agar-collagen mix. On subsequent organ culture, re-epithelialization occurred as seen *in vivo* – a lag period was followed by migration/proliferation and reformation of intact multilayered epithelium. EGF accelerated this process. It may be advisable to use EGF in conjunction with other wound-repair agents such as hyaluronic acid. Hyaluronic acid and EGF (separately) have been shown to stimulate epithelial cell regeneration in wounded rabbit corneas (Inoue and Katakami, 1993). When used together, the stimulatory effects have been shown to be additive (Nishida *et al.*, 1991).

The application of these results to aid in human recovery after eye surgery remains unattained. Matched pairs of patients received donor corneas from the same donor, which were implanted by the same surgeon on the same day. All donor epithelium was removed mechanically at the end of surgery. Re-epithelialization was monitored in the succeeding weeks. Administration of EGF (in ophthalmic solution) at 30 μg/ml

did not speed recovery, while drops with EGF at 100 μg/ml actually retarded the healing process (Dellaert *et al.*, 1997).

The apparent disagreement between the animal tests, on the one hand, and the results with the cornea transfer patients, on the other, requires resolution. It is possible that more frequent applications of a stronger EGF solution are required before an epithelialization enhancement in human corneas post-transfer becomes apparent.

Assisting the repair of damaged ears

Large perforations of the tympanic membrane of the ear (a common human injury) usually require surgical attention. Chauvin *et al.* (1999) showed in humans, that EGF applications eliminate the need for surgery. With weekly applications of 1 μg directly to the tympanic membranes of the experimental area 100% closure was achieved by d 21 post-treatment, while only 63.6% of controls were closed by d 32. No side effects of EGF treatment were seen (canal hypertrophy was noted with other treatments). In other work relevant to the ear, basilar papillae damaged by acoustic trauma in the chicken showed hair cell regeneration in EGF-containing medium (Yamane *et al.*, 1997), suggesting that EGF should be considered for the treatment of noise-damaged human ears *in vivo*.

It is clear that EGF application is the treatment of choice for tympanic membrane damage.

Use in repair of liver injury

Carbon tetrachloride is well-known as a powerful inducer of liver injury. When rats received systemic EGF (500 or 750 μg/kg) 30 min before liver-damaging doses of carbon tetrachloride, the severe hepatic necrosis and major rises in blood liver enzymes seen in control animals were entirely prevented by the higher EGF dose (Berlanga *et al.*, 1998b). It appears that the salivary gland may act as an important source of EGF necessary for normal liver function in healthy animals, as liver regeneration after partial hepatectomy was inhibited by removal of the salivary glands in rats, while systemic injection of EGF restored normal regeneration (Jones *et al.*, 1995). As successful hepatic transplantation would be an invaluable surgical procedure, Mooney *et al.* (1996) incorporated EGF into slow release microspheres, mixed these with hepatocytes, seeded the mixture into porous sponges, and placed the sponges into the mesentery of rats. When the portal vein was shunted to the inferior vena cava, hepatocyte engraftment in the animals was twofold better than in controls with EGF-free microspheres.

Applications of this work to the treatment of people with a damaged livers (perhaps by alcoholism) are clear.

Potentiating the effects of anti-cancer drugs

Many tumours over-express EGFR. This fact, and the growth-stimulating properties of EGF, would not suggest that EGF might have an anti-cancer effect. Such an anti-cancer effect has, however, been observed. Administration of EGF enhances the sensitivities of many types of cancer cells (ovary, head and neck, cervix, colon,

pancreas, prostate, lung) to different drugs, including cisplatin, taxol, melphalan, and 5-fluorouracil, presumably because cells with EGF-enhanced growth are especially susceptible to the therapeutic agents (Kroning *et al.*, 1995).

Decreasing lung oedema

Lung liquid clearance in rats is a model of oedema in proliferative lung injury. Aerosol administration of EGF (20 μg in saline) yielded a 40% increase, compared to EGF-free aerosol, in lung active sodium transport and lung liquid clearance (Sznajder *et al.*, 1998).

Protecting against enteropathogenic bacteria

Orally administered EGF inhibits colonization of the rabbit small intestine by enteropathogenic *Escherichia coli*, and stops diarrhoea and weight loss (Buret *et al.*, 1998). The mechanism of action of EGF in this system is not known – presumably the intestinal cell receptors for the enteropathogens are occluded in some manner in cells which have been EGF-stimulated. This work has high potential applicability in the animal health area, and also, possibly, in the amelioration of diarrhoeal diseases (particularly acute episodes of 'travellers' diarrhoea') in people.

Lowering of blood pressure

There has been a single important report that intravenous injection (3–300 μg/kg) of the active 48-mer of EGF into primates resulted in the lowering of blood pressure (Keiser and Ryan, 1996) – this observation deserves further testing, particularly in hypertensive human volunteers.

Cosmetic applications of EGF

As the cosmetic industry thrives on product novelty, EGF has been used as a component of skin-care products in Hong Kong, with sales in excess of US$1 M in a few months, and with product expansion into other Asian countries planned for mid-2000. The EGF in these preparations is supposed to retard or reverse the skin aging process. The senescence of human umbilical vein cells, measured *in vitro*, was suppressed by EGF (Hasegawa and Yamonoto, 1992), and a cream containing EGF was claimed to reduce 'cutaneous senescence' in the human epidermis (Brown, 1997).

The production of recombinant EGF

The previous review section has emphasized that EGF has a range of actual and potential uses, especially in human health care, and applications are constrained only by limitations in the commercial supply of purified, active, chemically authentic EGF. Presently, the cost of commercially available recombinant EGF ranges from US$210 to US$1020 per milligram. Below, we briefly review earlier approaches to the production of recombinant EGF, and then describe recent work in our laboratory

which has resulted in the development of a fast, robust protocol for the production of gram quantities of purified EGF which is chemically identical to the natural human material, and is fully bioactive.

EARLY WORK TO PRODUCE RECOMBINANT EGF

It was realized quite early that an efficient production system for recombinant EGF would be of value. Several studies prior to 1993 addressed the molecular biology of recombinant strain construction, but EGF yields were low (in the μg/ml range). As examples of this pioneering work, Kim *et al.* (1992) described low-level production of EGF by *Escherichia coli* in continuous culture, while Shimizu *et al.* (1991) developed a fed-batch procedure for recombinant EGF production.

In these early studies, the EGF produced remained inside the microbial cell. For several reasons, it is desirable that a recombinant product be excreted into the cell growth medium. Purification would then be simpler than for an intracellular protein as the product would not be contaminated with cytoplasmic components. In addition, the formation of inclusion bodies would be avoided and possible toxic effects of the EGF peptide product on the host cell would be reduced. Finally, excreted proteins are usually stable (and not significantly degraded) because of low levels of extracellular protease activity (Skipper *et al.*, 1985). In a promising approach, a synthetic gene for EGF was fused with the signal peptide of *E. coli* alkaline phosphatase to direct mature EGF to the *E. coli* periplasm (Oka *et al.*, 1985); yields of EGF were low. Apart from *E. coli, Bacillus brevis* has been used as the host for a recombinant EGF gene (Ebisu *et al.*, 1992). The *B. brevis* species has no external cell wall; recombinant proteins may thus be easily obtained in the culture medium. In this system, the yield (1.1 g/l) was most impressive, but the time taken (6 d) to reach this level was very long. The long growth time required for production of EGF may be expected to adversely affect the activity of the product, and would also elevate the production cost and prolong the occupancy of the fermentation equipment. In addition, long fermentation times obviously increase the risk of microbial contamination of the fermentate, which is completely unacceptable in processes designed to yield products for human therapeutic application. All these deficiencies become even more difficult to tackle in large-scale production.

The technical challenge of achieving high volumetric recombinant EGF productivity has proven to be considerable. A promising approach to the large-scale production of recombinant EGF became apparent in 1993, when an ampicillin-resistant *E. coli* JM101 strain, carrying a recombinant EGF-encoding plasmid named pETacEGF, was used to produce excreted EGF (Wong and Sutherland, 1993; Yadwad *et al.*, 1994), with EGF secretion being directed by the *E. coli* OmpA signal sequence. The OmpA protein is the major outer membrane protein of *E. coli*, and is directed to the outer membrane by a short N-terminal signal sequence, which is cleaved as the protein traverses the inner membrane of the cell, *en route* to its final destination. When the OmpA signal sequence was tagged to the gene encoding mature EGF, the EGF was also directed to the outer membrane of the cell, but did not remain there, being excreted instead into the growth medium. The levels of EGF produced by the *E. coli* JM101[pETacEGF] clone, grown under optimized conditions, was 250 mg/l/32 h. This was the best yield of EGF reported to that time. The system needed further

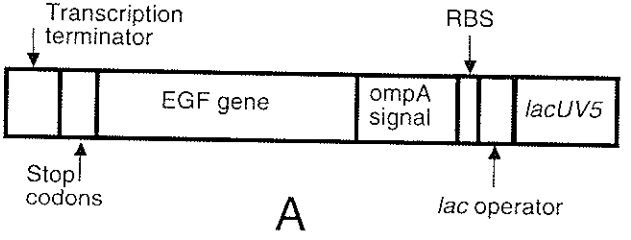
work, however. First, the yield, while good, was obtained only over 32 h in the fermentor. Again, potential problems of product quality, contamination, and cost (see above) arose. Also, the EGF produced by the *E. coli* JM101[pETacEGF] clone did not have an authentic N-terminus – 4 irrelevant amino acid residues were attached (Wong and Sutherland, 1993). While the EGF was measurable by immunoassay, the biological activity of the product was not assessed, and may well have been adversely affected by the unnatural N-terminus.

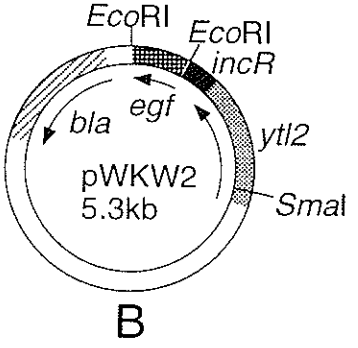
The construction of a recombinant EGF-excreting strain of *Bacillus subtilis* has been described (Lam *et al.*, 1998). The yield of EGF, in unoptimized shake-flask cultures, was 7 mg/l. While work on this system is ongoing, it was decided to focus initial attention on the development of an efficient *E. coli*-based system for recombinant EGF production, as the full range of bacterial genetic techniques are available with this organism.

THE DEVELOPMENT OF AN EFFICIENT *E. COLI* EXCRETION SYSTEM FOR HETEROLOGOUS PROTEINS

While the large-scale production of EGF was a primary research aim, it was decided to develop a DNA vector, suitable for use in *E. coli*, which would enable the cloning of a variety of genes expressing heterologous proteins, and where the recombinant strains thus created would excrete the heterologous proteins to the culture medium. The vector developed (Lam *et al.*, 1997) is a high copy-number plasmid containing (a) a cassette into which foreign DNA may be inserted, and (b) the *lacI*^{q} gene. The cassette (*Figure 3.1A*) contains, first, the *lacUV5* promoter, which was deliberately chosen to be weaker than the *tac* promoter used originally (Wong and Sutherland, 1993; Wong *et al.*, 1998a) as (over)-expression of cloned genes from the *tac* promoter sometimes results in death of recombinant cells (Lam *et al.*, 1997). The *lac* operator follows. Next, a ribosome binding site representing the *E. coli* consensus sequence is inserted, and the OmpA signal peptide sequence then commences. After the signal peptide-encoding DNA, the heterologous DNA (encoding EGF in the example of *Figure 3.1A*) is inserted in-frame. Termination codons for all 3 frames follow, and the cassette concludes with the stem–loop transcription terminator structure of the *E. coli trpA* gene. When a foreign gene is inserted into this construct, and the recombinant plasmid transformed to *E. coli*, gene expression is inhibited by the LacI repressor (expressed both from an indigeneous F' factor and from the *lacI*^{q} gene of the recombinant plasmid) unless an inducer (such as isopropyl-β-D-thiogalactopyranoside) of genes under the regulation of the *lacUV5* promoter is added to the culture. Expression of the foreign gene is then switched on. With this system, the excretion of heterologous proteins from *E. coli* can be achieved consistently and for a wide variety of proteins, without assistance from carrier proteins or membrane-lytic agents, and by using 'healthy' hosts rather than mutant strains having defective outer membranes.

The enzymatic degradation of waste cellulosic products, to make fuel alcohol, is a desirable biotechnological goal, and recombinant *E. coli* strains have been constructed which excrete all 3 of the required cellulosic enzymes (Lam *et al.*, 1997; Wong *et al.*, 1998a). High-level production of fish antifreeze peptides is required by the dairy industry (the peptides will be incorporated into frozen products to improve





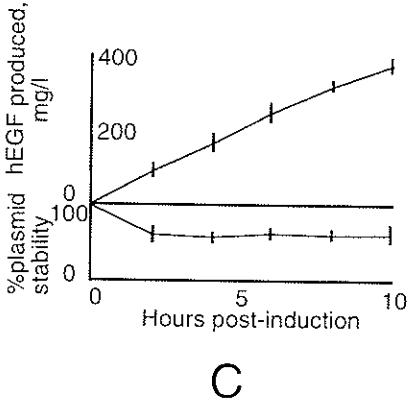


Figure 3.1. The construction of a plasmid encoding excretory EGF, and the production of EGF from a recombinant *E. coli* strain. A: The DNA cassette which allows controlled expression of recombinant proteins in *E. coli*, and also directs the recombinant products to the culture medium. The cassette contains (from right to left) (i) the *lacUV5* promoter; (ii) the *lac* operator; (iii) the consensus ribosome-binding sequence (RBS) of *E. coli*; (iv) the *ompA* signal sequence; (v) the recombinant gene (the gene encoding mature EGF in the present context); (vi) stop codons in all 3 frames; and (vii) a stem–loop terminator of transcription. B: Plasmid pWKW2, containing the EGF gene (*egf*), an ampicillin-resistance gene (*bla*), and the *ytl2-incR* stabilization system. The EGF-encoding gene is inserted into the cassette of A above, in which the *lacUV5* promoter drives transcription, and the EGF produced is N-terminally fused to the OmpA signal sequence. C: Fermentation of *E. coli* JM101[pWKW2] with the constant feeding of medium. The working volume was 1 litre, the inoculum was 10% (v/v), the initial glucose level was 2 g/l, the fermentation temperature was 32°C, and the pH was controlled at 6.8 throughout fermentation. The culture was grown to 8×10^9/ml and then induced with IPTG to a final concentration of 0.1 mM. The feeding medium was 200 g/l glucose and 20 g/l $MgSO_4.7H_2O$, and was pumped at 7.2 ml/h after the initial glucose was consumed. Both EGF production and plasmid stability during induction are shown. The tests were performed 3 times, and standard error bars are shown. From Sivakesava *et al.* (1999). hEGF: (human) EGF.

mouth feel), and the described excretion approach has found ready application in this work (Lin *et al.*, 1999; Tong *et al.*, 2000).

APPLICATION OF THE NEW *E. COLI* EXCRETION SYSTEM TO THE PRODUCTION OF EGF

Plasmids with the EGF gene

As the cassette with the *lacUV5* promoter appeared to be useful in aiding the excretion of recombinant proteins/peptides from *E. coli*, the mature EGF gene was placed in the cassette (*Figure 3.1A*). In this construct, the OmpA signal sequence is fused exactly to the N-terminus of the EGF gene. The recombinant plasmid, (termed lacUV5par8EGF) was transformed to *E. coli* and EGF production by the recombinant assessed (Sivakesava *et al.*, 1999). Production of EGF was disappointing, with only 28 mg/l produced over 10 h post-induction of batch fermentor culture. It appeared that the inclusion of the *lacI*^{q} gene on the (high copy-number) recombinant plasmid was limiting induction of the *lacUV5* promoter. When the cassette containing the EGF gene was moved to a different DNA vector, now not containing the *lacI*^{q} gene, EGF production in shake flasks was 107–140 mg/l by 20 h post-induction (Wong *et al.*, 1998b). A new problem arose, however. It appeared that synthesis of relatively high amounts of EGF was deleterious to the EGF-expressing cell, so that plasmid-free cells (essentially undetectable before induction) became obvious soon after addition of the inducer and made up 80% of bacteria 20 h post-induction. While EGF production from the minority of plasmid-containing cells was good, as detailed above, it appeared that it might be necessary to remove a large bacterial mass from the culture before EGF could be purified from the medium.

A selection of stabilizing systems has been isolated from various plasmids, and these systems have found applications in fermentation biotechnology. One such system is the *ytl2-incR* stabilization system from a large plasmid, termed pSLT, normally resident in mouse-virulent strains of *Salmonella enterica* (such as *S. typhimurium*). The *ytl2-incR* DNA (about 1 kb in size) greatly stabilizes high copy-number plasmids which carry it (Wong *et al.*, 1998b) – stability is enhanced 10^4–10^6-fold, compared to stability values shown by control plasmids. The molecular mechanism of the stabilization process appears to involve coating of supercoiled plasmid DNA by the Ytl2 protein (our unpublished results). The *ytl2-incR* system was used in an effort to stabilize the EGF-encoding plasmid.

Plasmid pWKW2 (*Figure 3.1B*) is the final construct. In this plasmid the EGF gene is fused in frame to the OmpA signal sequence. The plasmid lacks the *lacI*^{q} gene, which, as explained, inhibits efficient induction from the *lacUV5* promoter when present in high copy-number. The plasmid carries the *ytl2-incR* stabilization system. In comparative tests using constructs with or without *ytl2-incR* DNA, it was clear that inclusion of the stabilization system eliminated the appearance of plasmid-free cells post-induction in shake flasks. After addition of the inducer, bacterial growth and division ceased, but viability was maintained for at least 20 h, during which time the bacteria synthesized and excreted EGF, to give *ca.* 20 μg EGF/10^8 bacterial cells (Wong *et al.*, 1998b). While the absolute amounts of EGF produced by the 2 strains (with stabilized and non-stabilized EGF-encoding plasmids) were similar, the cell density, on EGF harvesting, of the strain with the stabilized plasmid was *ca.* 10-fold

less than that seen with the strain bearing the non-stabilized plasmid. This augured well for scale-up to fermentors.

Scale-up of EGF production

Culture and induction conditions were next optimized with respect to volumetric production of the EGF. We focused on batch and fed-batch cultivations using glucose as carbon source (Sivakesava *et al.*, 1999), and, initially, used fermentors of 2 litres volume. Fed-batch fermentations have been favoured for production of heterologous proteins by recombinant microorganisms. Such fermentations afford tight control over environmental parameters, and hence improve overall product yield compared with simple batch cultures.

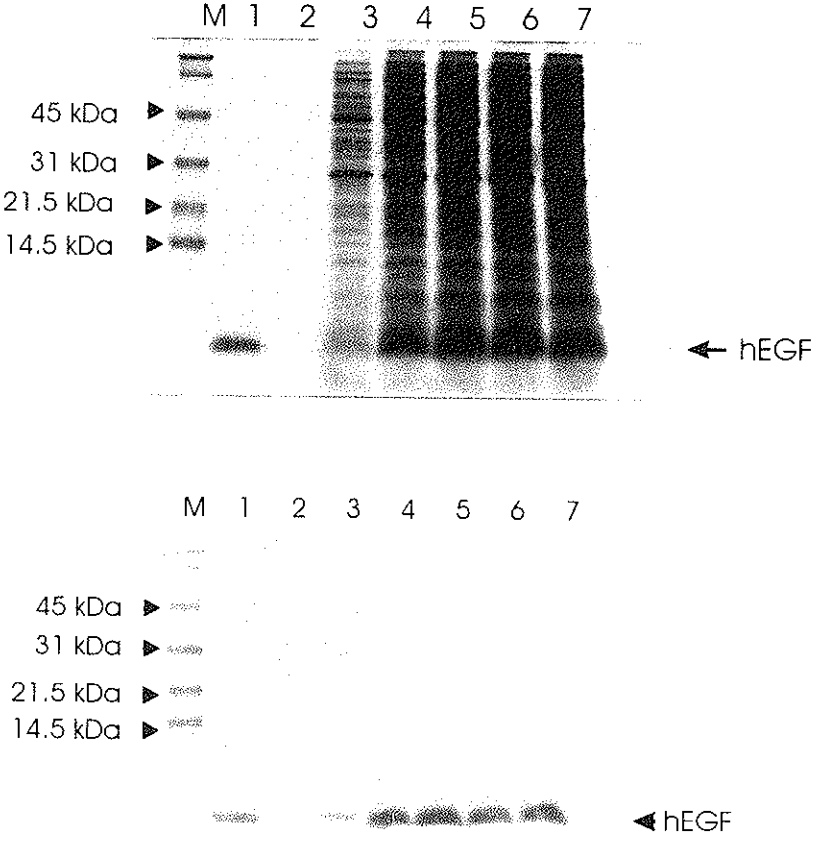


Figure 3.2. Electrophoresis and immunoanalysis of excreted EGF produced by a continuous fed-batch culture of *E. coli* JM101[pWKW2]. Top: SDS-PAGE of the samples. Lanes: M, molecular weight markers (sizes on left) (SeeBlue® pre-stained standards, Novex™); 1, 1 μg of commercial hEGF (Promega); 2, sample collected just before induction; 3–7, samples collected at 2, 4, 6, 8 and 10 h post-induction. Lanes 2–7 each contain 28 μl of culture supernatant. Bottom: Western blot analysis of the samples run on the top gel; the lane order is as in the top gel. hEGF: (human) EGF. From Sivakesava *et al.* (1999).

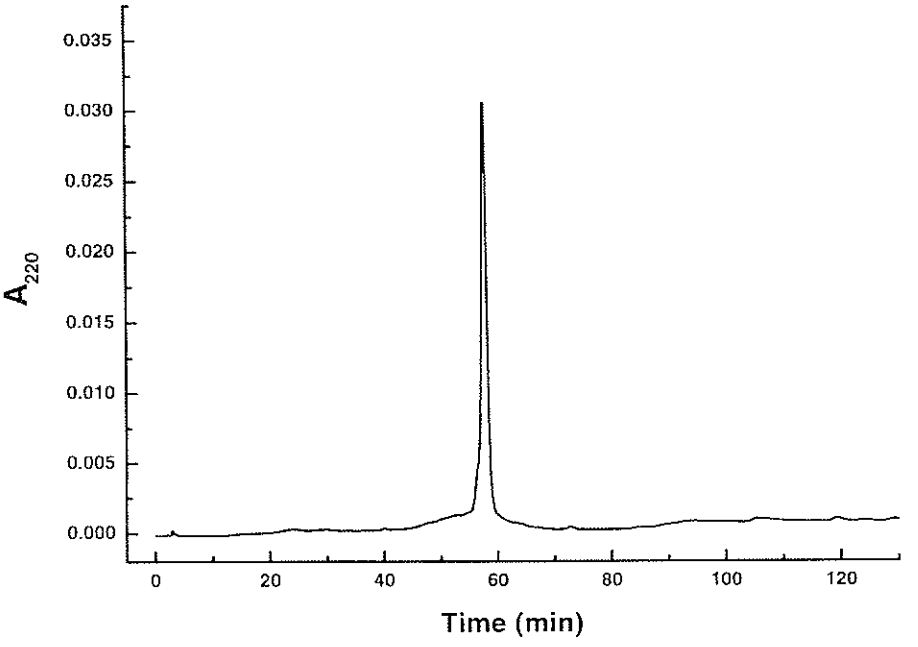


Figure 3.3. Analysis of purified EGF. A sample of purified EGF was rechromatographed on a reverse-phase HPLC column. The bound proteins were eluted with an acetonitrile gradient and the EGF assayed by radioimmunoassay. A single peak is noted. From Huang *et al.* (1999).

A simple feeding strategy for fed-batch recombinant cell fermentation was developed to maintain high excretory production of EGF. The time-course study of a fed-batch culture of an induced *E. coli* JM101[pWKW2] strain revealed a gradual increase in production of EGF in the first 10 h post-induction, to give a yield of 325 mg/l (*Figure 3.1C*). The 325 mg/l of extracellular EGF represented over 90% of the EGF produced by the cells. Under these fermentation conditions, plasmid stability was acceptable, at *ca.* 70% for the entire time of induction.

From 4 h post-induction, expression and excretion of EGF was detectable by SDS-PAGE, and soon the product became the major protein species in the culture supernatant (*Figure 3.2*), despite the presence of some intracellular proteins resulting from the lysis of cells. The EGF was detectable by immunoblot (*Figure 3.2*). The high rate of production of EGF by the *E. coli* JM101[pWKW2] clone up to the 10 h post-induction time point (32.5 mg/l/h), rendered this clone a better choice for use over other systems (Ebisu *et al.*, 1992; Yadwad *et al.*, 1994) which have also been reported to express EGF in the mg/l scale during the productive phase.

The high stability and productivity of this system facilitated scale-up of EGF production, and a protocol to purify EGF from bacterial culture supernatant was developed. The EGF in the supernatant from an induced EGF-expressing recombinant *E. coli* culture was readily purified by conventional chromatographic procedures (Huang *et al.*, 1999), and was shown to be pure by high pressure liquid chromatography (*Figure 3.3*) and SDS-PAGE (*Figure 3.4*). The N-terminus of the purified hEGF was authentic (thus: cleavage of the OmpA signal peptide was precise, and

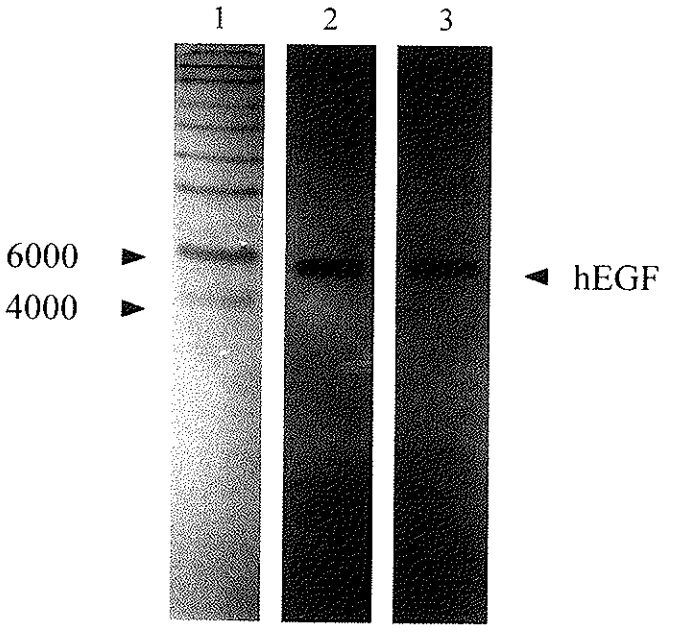


Figure 3.4. SDS-PAGE of purified EGF. Commercial pure EGF, and our purified EGF, were subjected to SDS-PAGE. Lane 1: SeeBlue® prestained markers; lane 2: commercial EGF (5 μg); lane 3: our purified EGF (5 μg). The positions of 2 molecular weight standards are indicated at the left. From Huang *et al.* (1999).

degradation from the N-terminus was absent or minimal after excretion of the protein). Commercial pure EGF, and EGF purified as described above, were assessed for bioactivity, and yielded superimposable curves (*Figure 3.5*). The recovery of EGF with this protocol was 30% of original, while the purity was 97–100%.

Since the publication of this work, the purification protocol has been simplified and refined, and gram quantities of purified, fully bioactive, chemically authentic, human EGF are reproducibly prepared in our small fermentation facility in Hong Kong. Presently, a 20-litre fermentation yields 1–2 g purified product within 2 working weeks. The fermentation/purification procedures used are readily adaptable to further scale-up.

Conclusion

Recombinant EGF has a host of actual and potential uses. The most obvious applications to date are in (a) wound healing, (b) the healing of lesions (surgical or ulcer-caused) of the gastrointestinal tract, and (c) the use (with a tagged metal isotope, either actually or potentially unstable) as a 'magic bullet' in the treatment of brain tumours. Many other possible applications are suggested by the literature; few have been adequately explored to date.

A constraint in the use, either experimentally or clinically, of EGF, lies in the fact that the material has not been routinely available in large amounts, and the small quantities that are on commercial offer are very expensive. Recent work has tagged

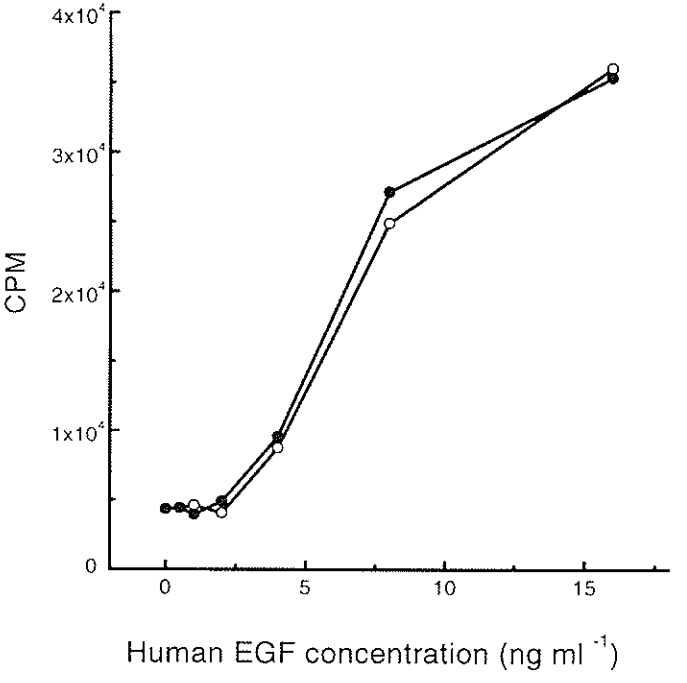


Figure 3.5. Bioassay of EGF. Commercial pure EGF (open circles), and our purified EGF (filled circles), were assessed for bioactivity, and yielded superimposable curves. The assay involves measurement of [^{3}H]-thymidine incorporation into cultured human epidermal cells exposed to EGF. From Huang *et al.* (1999).

the EGF gene, at the N-terminus, with the *E. coli* OmpA signal peptide, so that a recombinant *E. coli* strain carrying this construct may excrete large amounts of EGF into the culture medium. The EGF thus produced may be readily purified, is chemically identical to natural EGF, and is fully bioactive. Gram quantities of EGF may be easily obtained with this procedure, and this should allow increased application of EGF in the treatment of various medical conditions in humans.

Acknowledgements

Our work on human EGF production was supported by Industrial Support Fund AF/135/96 from the Hong Kong Government Industry Department, and by Competitive Earmarked Research Grants HKUST 522/94M and HKUST 639/96M from the Research Grants Council of Hong Kong.

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